SH3 BINDING DOMAINS IN THE DOPAMINE D4 RECEPTOR

by

John Oldenhof

A thesis submitted in conformity with the requirements

for the Degree of Doctor of Philosophy

Graduate Department of Pharmacology

University of Toronto

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ABSTRACT

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John Oldenhof

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The dopamine D4 receptor is a G protein-coupled receptor (GPCR) that belongs to the dopamine D2-like receptor family. Functionally, the D2-like receptors are characterized by their ability to inhibit adenylyl cyclase. The dopamine D4 receptor, as well as other catecholaminergic receptors, contain several putative SH3 binding domains. Most of these sites in the D4 receptor are located in a polymorphic repeat sequence and flanking sequences in the third intracellular loop. Here we demonstrate that this region of the D4 receptor can interact with a large variety of SH3 domains of different origin. The strongest interactions were seen with the SH2-SH3 adapter proteins Grb2 and Nck. The repeat sequence itself is not essential in this interaction. The data presented indicate that the different SH3 domains in the adapter proteins interact in a cooperative fashion with two distinct sites immediately up- and downstream from the repeat sequence. Removal of all the putative SH3 binding domains in the third intracellular loop of the dopamine D4 receptor resulted in a receptor that could still bind spiperone and dopamine. Dopamine could not modulate the coupling of these mutant receptors to adenylyl cyclase and MAPK, although dopamine-modulated receptor-G protein interaction appeared normal. The receptor deletion mutants show strong constitutive internalization which may account for the deficiency in functional activation of second messengers. The dopamine D4 receptor interaction with the Nck adapter protein was confirmed using the yeast two-hybrid system. The data indicate that the D4 receptor contains SH3 binding sites and that these sites fall within a region involved in the control of receptor internalization.

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LIST OF ABBREVIATIONS

α -MEM	α -Minimum essential media
β ARK	β -adrenergic receptor kinase
5-HT	5-hydroxytryptamine (serotonin)
aa	amino acid
AC	adenylyl cyclase
AD	activation domain
APS	ammonium persulfate
ATP	adenosine triphosphate
ATP	adenosine triphosphate
BD	binding domain
BSA	bovine serum albumin
CaMK II	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CHO	Chinese hamster ovary
CIP	calf intestinal phosphatase
CNS	central nervous system
CRE	cAMP responsive elements
CREBP	cAMP responsive element-binding protein
CTP	cytosine triphosphate
ddH ₂ O	double distilled water
ddNTP	dideoxynucleotide triphosphate
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
DPM	disintegrations per minute.
dsDNA	double-stranded DNA
DTT	diethiothreitol
EC ₅₀	median effective concentration
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',-N'-tetraacetic acid
EPS	extrapyramidal side effects
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
G418	Geneticin
GnRH	gonadotropin-releasing hormone receptors
GPCR	G protein-coupled receptor
Gpp(NH)p	5'-guanylyl-imidodiphosphate
Grb2	growth-factor-receptor binding protein 2
GRK	G protein-coupled receptor kinase
GST	glutathione S-transferase
GTP	guanosine triphosphate

GTP γ S	guanosine-5'-O-(3-thiotriphosphate)
HA	hemagglutinin
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-thanesulfonic acid]
HRP	horseradish peroxidase
HS	horse serum
HS	horse serum
ib	immunoblotting
IC ₅₀	median inhibitory concentration
IGF	insulin-like growth factor
ip	immunoprecipitation
IP ₃	inositol (1,4,5)-triphosphate
JNK	Jun N-terminal kinase
JNKK	JNK kinase
K _d	equilibrium dissociation constant (saturation binding)
K _i	equilibrium dissociation constant (competitive binding)
KMES	potassium 2-(N-morpholino)ethanesulfonic acid
LB	Luria-Bertani
LPA	lysophosphatidic acid
mAChR	muscarinic acetylcholine receptor
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MEK	MAPK/ERK kinase (a MAPKK)
MEKK	MAPK/ERK kinase kinase (a MAPKKK)
MEKK	MEK kinase (a MAPKKK)
Nck	novel cytoplasmic protein
NHE	Na ⁺ /H ⁺ exchanger
PAK	p21-activated kinase
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PI	phosphatidylinositol
PI3-K	phosphatidylinositol 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PPII	polyproline type II
PTK	protein tyrosine kinase
PTX	pertussis toxin
PVDF	polyvinylidene difluoride
Pyk2	proline-rich tyrosine kinase 2
Ras	rat sarcoma virus p21 GTPase
RIA	radioimmunoassay
rpm	rotations per minute
RT	room temperature
RTK	receptor tyrosine kinase

SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shc	SH2 domain-containing, α 2-collagen-related protein
Sos	son of sevenless
TEMED	N,N,N,N-tetramethyl-ethylenediamine
TM	transmembrane
UAS	upstream activator sequence
VNTR	variable number of tandem repeats
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

Chapter 1

Introduction

1.1 Summary

The dopamine receptors belong to the large family of seven transmembrane G protein-coupled receptors (GPCR). To date five different dopamine receptor genes have been identified and cloned from mammalian species (for review see Seeman and Van Tol, 1994). The dopamine receptors are classified into two subgroups according to their structural, pharmacological and functional characteristics: the dopamine D1-like receptors (D1 and D5) which can activate adenylyl cyclase, and the D2-like receptors (D2, D3 and D4) which can inhibit adenylyl cyclase upon agonist binding.

The physiological role of the dopamine D4 receptor is still unclear. This receptor is predominantly expressed in the mesocortical and mesolimbic areas of the brain, most notably the GABA-ergic interneurons of the frontal cortex (Mrzljak *et al.*, 1996; Van Tol *et al.*, 1991). In addition, D4 receptor mRNA has been detected in the retina and the heart (Matsumoto *et al.*, 1995a; O'Malley *et al.*, 1992). The distribution profile of this receptor and its relatively high affinity for clozapine, as compared to the D2 receptor, has resulted in the implication of this site as an important target for atypical neuroleptics (Seeman *et al.*, 1997; Van Tol *et al.*, 1991). Genetic association and linkage studies suggest a role of the D4 receptor in attention deficit hyperactivity disorder, novelty seeking and Tourette's syndrome, although the latter two findings have been disputed (Barr *et al.*, 1996; Benjamin *et al.*, 1996; Ebstein *et al.*, 1996; Grice *et al.*, 1996; LaHoste *et al.*, 1996; Malhotra *et al.*, 1996). Similar studies have not supported a major role for this receptor in schizophrenia and bi-polar disorder (Lim *et al.*, 1994a; Tanaka *et al.*, 1995).

The human dopamine D4 receptor has a variable number of tandem repeats (VNTR) within the third intracellular loop domain that is not observed in other dopamine receptors (Van Tol *et al.*, 1992). This polymorphic repeat consists of between two and ten repeated units of sixteen amino acids in length. To date, 20 different receptor protein variants have been found for this polymorphism (Asghari *et al.*, 1994; Lichter *et al.*, 1993; Van Tol *et al.*, 1992). The first (α) and last (ζ) repeat units, however, are invariant, irrespective of the total number of repeat units (Asghari *et al.*, 1994; Lichter *et al.*, 1993; Van Tol *et al.*, 1992). D4 receptor genes encoding receptors with two (D4.2), four (D4.4) or seven (D4.7) repeat units are the most abundant in the human population. A similar VNTR is also present in various non-human primate species but is not seen in rodents (Livak *et al.*, 1995; Van Tol *et al.*, 1992). With respect to functional coupling to adenylyl cyclase, it has been observed that the D4.7 isoform has a two- to three-fold decreased potency for dopamine as compared to the D4.2 and D4.4 isoforms (Asghari *et al.*, 1995).

Examination of the repeat sequence and its immediate surrounding sequences revealed the presence of multiple putative Src homology 3 (SH3) binding motifs in this region. SH3 domains are recognized as modular binding domains for protein-protein interactions that are essential for full functional activity of, for example, the receptor tyrosine kinase (RTK) complex (see Pawson, 1995 for review). SH3 domains recognize proline-rich sequence motifs of about ten amino acids in length which contain a conserved PXXP amino acid sequence (Pawson, 1995; Ren *et al.*, 1993; Sparks *et al.*, 1996; Yu *et al.*, 1994). A scan of the currently identified GPCRs revealed that there were other receptors that also contained potential SH3 binding motifs, including the α - and β -adrenergic and muscarinic acetylcholine receptors. To date, the involvement of SH3 domains in seven transmembrane receptor signalling has not been fully investigated (Ren *et al.*, 1993; Xu *et al.*, 1994). The identification of such domains could direct the search for additional essential components of the GPCR complex, that may elucidate other functions of the receptors or give further insight into the

mechanisms for GPCR signal transduction.

In this study we demonstrate that the putative SH3 binding domains within the dopamine D4 receptor, the β_1 -adrenergic and the m4 muscarinic receptor can interact with SH3 domains. In particular we have investigated the interaction of the D4 receptor with the Src-homology 2 (SH2)-SH3 adapter proteins Nck and Grb2. Furthermore, we show that deletion of all the putative SH3 binding domains in the third intracellular loop of the D4 receptor results in constitutive internalization of the receptor.

1.2 Statement of Problem

After the cloning of the dopamine D4 receptor and the identification of the unique highly polymorphic repeat sequence in the third intracellular loop, a large number of studies have attempted to characterize the receptor pharmacologically and biochemically, and determine its functional role. The high affinity of the dopamine D4 receptor for clozapine, an atypical neuroleptic that shows few extrapyramidal side effects normally associated with antipsychotic medication, suggested the involvement of this receptor in this disease state. Further studies have not supported a major role of this receptor in schizophrenia and suggest that the receptor only plays a modifying role in neuroleptic therapy (Wilson *et al.*, 1998).

A large number of studies have investigated the VNTR polymorphism of the dopamine D4 receptor. The different D4 variants do not show major differences in ligand binding, G protein-coupling or the ability to inhibit adenylyl cyclase (Asghari *et al.*, 1995; Asghari *et al.*, 1994). To date no major biochemical differences between the different D4 variants has been established, nor has any role for the repeat sequence, on the cellular level, been identified. Some recent linkage and association studies, however, have demonstrated that individuals show differences in certain personality traits depending on the number of repeats that are expressed in these individuals

(Benjamin *et al.*, 1996; Ebstein *et al.*, 1996).

The apparent role of the VNTR polymorphism in a number of personality traits, the conserved nature of the repeat sequences and the requirement for the expression of at least two repeats in the human dopamine D4 sequence suggests that this sequence serves a definite role in receptor function. More than eight years after the cloning and identification of the unique repeat sequence, the function of both the dopamine D4 receptor and the repeat sequence remains to be identified.

1.3 Purpose of Study and Objectives

The purpose of this study was to establish if the repeat region serves a specific function which may help to establish a role for the dopamine D4 receptor itself. We wanted to investigate the possibility that, by means of the repeat region, the D4 receptor may be able to interact with SH3 domain containing proteins. The prevalence of PXXP motifs in the third intracellular loop and repeat region make the D4 receptor a likely candidate for SH3 binding. The demonstration of a D4-SH3 interaction would represent the first such direct interaction among the GPCRs.

The specific objectives of this body of research were to:

- 1) Determine if SH3 binding domains can directly interact with the dopamine D4 receptor and other GPCRs.
- 2) Determine if this interaction is in the third intracellular loop or restricted to the repeat region of the dopamine D4 receptor.
- 3) Determine which SH3 domains can interact with the dopamine D4 receptor.
- 4) Determine the strength of the D4-SH3 interaction.
- 5) Demonstrate the D4-SH3 interaction *in vivo*.
- 6) Determine the function of the D4-SH3 interaction.

1.4 Statement of Research Hypothesis

Based on the proline-rich nature and presence of multiple PXXP sequences in the repeat region and immediate flanking sequences that are contained in the third intracellular loop of the dopamine D4 receptor, it is hypothesized that these sequences may represent sites for protein-protein interactions mediated by modular SH3 domains. In addition, other GPCRs have multiple PXXP sequences that may also serve as SH3 binding domains, indicating that SH3 mediated protein-protein interaction is a more general phenomenon of GPCRs. The interaction with SH3 domains may represent an important mechanism by which the dopamine D4 receptor and other GPCRs participates in protein scaffolds that leads to various cellular signalling pathways and the regulation of the receptor itself.

1.5 Literature Review

The dopamine D4 receptor belongs to the superfamily of GPCRs. These receptors demonstrate extensive and complex activation and regulation of signalling pathways. In addition, research in the past decade has demonstrated that GPCRs activate and regulate many signalling mechanisms that have traditionally been associated with RTK signalling. This literature review details the functioning and regulation of GPCRs and their ability to activate a number of second messenger systems including those shared with RTKs, and where applicable, how this relates to the dopamine D4 receptor or closely related receptors. In addition the regulation of GPCRs by phosphorylation, and endocytosis is reviewed. Particular attention will be given to the nature of direct protein interactions with the GPCRs. Furthermore, protein-protein interactions mediated by modular binding domains, especially SH3 domains, will be reviewed with respect to signalling and endocytosis events.

1.5.1 The Dopamine D4 Receptor and G Protein-Coupled Receptors

1.5.1.1 The Dopamine System

Dopamine is a catecholamine that acts as a neurotransmitter in the brain and in the periphery. Dopamine acts as a modulator of cardiovascular function, hormone secretion, vascular tone, renal function and gastrointestinal motility (for comprehensive and recent reviews see Lachowicz and Sibley, 1997; Missale *et al.*, 1998; Wilson *et al.*, 1998). In the central nervous system (CNS) the dopaminergic system has been shown to be involved in motor and endocrine control, emotion, reward and cognition. These functions are modulated primarily by the following pathways (Gerfen, 1992): 1) motor control is predominantly mediated by nigrostriatal neurons (dopamine neurons project from the substantia nigra to the striatum) 2) reward, cognitive and emotional processes are mediated by the mesolimbic neurons (dopamine neurons project from the ventral tegmental area and retrorubral field to the nucleus accumbens, olfactory tubercle, septum and amygdala) and the mesocortical neurons (dopaminergic cells project from A10 and A8 groups to the prefrontal cortex, entorhinal, piriform and anterior cingulate cortices), and 3) endocrine function is mediated by the tuberoinfundibular neurons. Dysfunction of the dopamine system is believed to underlie such conditions as Parkinson's disease, schizophrenia, Tourette's syndrome and hyperprolactinemia. Dopamine antagonists are effective at blocking hallucinations and delusions associated with schizophrenia, while dopamine agonists are effective at alleviating the hypokinesia of Parkinson's disease and treating hyperprolactinemia.

The original pharmacological and functional identification of the dopamine system suggested that dopamine acted upon at least two receptor populations (Kebabian and Calne, 1979; Spano *et al.*, 1978). Upon dopamine stimulation in the neostriatum, adenylyl cyclase was activated and an increase in the level of intracellular adenosine 3',5'-cyclic monophosphate (cAMP) was observed (Kebabian and Greengard, 1971). Dopamine stimulation of receptors in the anterior pituitary gland

inhibited prolactin release, but did not stimulate adenylyl cyclase (Caron *et al.*, 1978) while in the striatum dopamine stimulation resulted in the inhibition of adenylyl cyclase preventing increases in cAMP levels (Stoof and Kebabian, 1981). These results suggested that dopamine acted upon at least two receptor populations and led to the early classification of the D1 receptor that stimulates adenylyl cyclase and increases the levels of cAMP, and the D2 receptor that inhibits adenylyl cyclase and inhibits increases in the levels of cAMP (Kebabian and Calne, 1979; Spano *et al.*, 1978). No other dopamine receptors could be distinguished pharmacologically until molecular cloning techniques revealed that there were at least five distinct subtypes of dopamine receptors (for review see Seeman and Van Tol, 1994). These have now been classified into the two subfamilies, based on their stimulatory or inhibitory effect on adenylyl cyclase, their amino acid sequence, their structural similarities, and their pharmacology, as the D1-like dopamine receptors that include the D1 (D1a) (Sunahara *et al.*, 1990) and D5 (D1b) (Sunahara *et al.*, 1991) dopamine receptors and the D2-like dopamine receptors that include the D2 (Bunzow *et al.*, 1988), D3 (Giros *et al.*, 1990; Sokoloff *et al.*, 1990) and D4 (Van Tol *et al.*, 1991) dopamine receptors.

The DNA sequences that encode the D1 and D5 receptors do not contain introns in their nucleotide coding regions (Sunahara *et al.*, 1991; Sunahara *et al.*, 1990), as seen for most GPCRs (Dohlman *et al.*, 1987). The D1 receptor gene, however, does contain an intron in the 5' non-coding region (Minowa *et al.*, 1992). The D2-like receptors do contain introns in their DNA coding sequence; six in the D2 receptor (Dal Toso *et al.*, 1989), five in the D3 receptor (Sokoloff *et al.*, 1990) and three in the D4 receptor (Van Tol *et al.*, 1991).

Based on the amino acid sequence, the expressed receptors are predicted to have a seven transmembrane (TM1-7) structure connected by intra- and extracellular loops. The transmembrane domains of these receptors display a more conserved amino acid sequence between receptors as compared to the amino acids encoding the loops. The D1 and D5 dopamine receptors show about

a 50% homology in their overall amino acid coding sequence, but 80% for the amino acids encoding the transmembrane domains. Furthermore, both the D1 and D5 receptors have a long carboxyl-terminal tail. For the D2-like receptors, the D3 receptor shows an overall homology of 52% and a transmembrane homology of 75% with that of D2. The dopamine D4 receptor has a 41% overall homology and a 56% transmembrane homology with D2 making it the least similar of all the dopamine receptors. The D2-like receptors are characterized by a relatively short carboxyl-terminal tail as compared to the D1-like receptors, and a long third intracellular loop.

The D1 and D5 receptors cannot be differentiated pharmacologically (Missale *et al.*, 1998; Sunahara *et al.*, 1991). Only relative differences in antagonist binding are seen, with (+)-butaclamol being the most discriminating ligand (Seeman and Van Tol, 1994). Dopamine binds to the D5 receptor with a 5-10 fold higher affinity as compared to that for D1 (Sunahara *et al.*, 1991). Unlike the dopamine D1-like receptors, the D2-like receptors can be distinguished pharmacologically. The D2 and D3 receptors show differences in the binding of antagonists, and even show differences in the binding of the agonist dopamine (Sokoloff *et al.*, 1990). The dopamine D4 receptor is remarkable because of its high affinity binding of the atypical neuroleptic clozapine, which shows the highest affinity for the D4 receptor, while the other D2 binding ligands show a reduced affinity for D4 as compared to D2 and D3 (Van Tol *et al.*, 1991).

The different dopamine receptors show differences in expression levels and distribution. The D1 receptor is the highest and most widely expressed of all the dopamine receptors. In the central nervous system high levels are expressed in the nucleus accumbens, olfactory tubercle, limbic system, hypothalamus and thalamus (Fremeau *et al.*, 1991; Weiner *et al.*, 1991). Compared to the D1 receptor, the D5 receptor is poorly expressed in the central nervous system. Expression of the D5 receptor has been detected in the cerebral cortex, lateral thalamus and striatum with lower levels in the substantia nigra, medial thalamus and hippocampus (Choi *et al.*, 1995; Huntley *et al.*, 1993;

Rappaport *et al.*, 1993). The dopamine D2 receptor is mainly expressed in the striatum, olfactory tubercle and the nucleus accumbens as well as the prefrontal cortex, cingulate and amygdala (Bouthenet *et al.*, 1991; Meador-Woodruff *et al.*, 1989; Weiner *et al.*, 1991). The D3 receptor is distributed in the limbic areas, olfactory tubercle and the islands of Calleja (Bouthenet *et al.*, 1991; Landwehrmeyer *et al.*, 1993). Expression of the dopamine D4 receptor is low compared to the other dopamine receptors.

Expression and distribution studies, based on mRNA levels, have shown that the highest levels of the dopamine D4 receptor are found in the heart, retina, frontal cortex, amygdala, olfactory bulb, hippocampus, mesencephalon and hypothalamus (Cohen *et al.*, 1992; O'Malley *et al.*, 1992; Van Tol *et al.*, 1991; Van Tol *et al.*, 1992). Very low levels of D4 are detected in the olfactory tubercles, and basal ganglia (Van Tol *et al.*, 1991). Recent immunohistochemical techniques, with a specific D4 antibody, have revealed that D4 receptors are expressed in the GABAergic interneurons in pyramidal and nonpyramidal neurons in the cerebral cortex and hippocampus (Mrzljak *et al.*, 1996). In addition, D4 receptors have been detected in the GABAergic neurons of both segments of the globus pallidus, the substantia nigra pars reticulata and in the reticular nucleus of the thalamus (Mrzljak *et al.*, 1996).

The dopamine D1 receptor plays an important role in locomotor activity, however the role of the D5 receptor remains mostly undefined. The D2-like receptors, and in particular the D2 receptor, have been implicated in locomotor activity, reward, learning and memory. The D1 receptor has also been shown to mediate aspects of these functions. Activation of the D2 receptor results in an increase in motor activity, while activation of the D3 receptor reduces motor activity. The physiological role of the dopamine D4 receptor is still mostly unknown. The expression of the D4 receptor in the limbic and cortical regions suggests a role of this receptor in cognition and emotion.

In functional studies, dopamine D2 receptor knockouts in mice resulted in neurological

impairment and Parkinsonian-like symptoms (Balk *et al.*, 1995). D3 activation affects locomotion, reinforcement and reward, while D3 knockouts give healthy mice with slight hyperactivity (Accili *et al.*, 1996). The dopamine D4 receptor has been suggested to be the therapeutic target in the treatment of schizophrenia based on the high affinity for the atypical neuroleptic clozapine and the elevation of D4 receptors in schizophrenics (Seeman and Van Tol, 1995; Van Tol *et al.*, 1991). Recent studies, however, indicate that the D2 receptor likely mediates most of the antipsychotic actions of neuroleptics, while the D4 receptor may play a modulating role in improving the clinical profile of these medications (Wilson *et al.*, 1998). D4 knockout mice demonstrated that this receptor is important in mediating locomotor activity and coordination (Rubinstein *et al.*, 1997), while an individual who was homozygous for a D4 null allele did not demonstrate any major psychiatric diseases (Nöthen *et al.*, 1994). Certain variants of the dopamine D4 receptor have also been associated with novelty seeking behaviour in humans (Benjamin *et al.*, 1996; Ebstein *et al.*, 1996).

1.5.1.2 Dopamine Receptors are G Protein-Coupled Receptors

Molecular cloning, sequence analysis, expression and functional studies of the dopamine receptors have revealed that these receptors belong to the large super family of GPCRs. GPCRs are membrane-spanning cell surface proteins characterized by their seven transmembrane topology (Dohlman *et al.*, 1987) and their ability to transduce signals, upon agonist stimulation by means of an exchange of GTP for bound GDP on the G alpha ($G\alpha$) subunit of heterotrimeric G proteins (for review see Ferguson *et al.*, 1998; Gutkind, 1998b; Hamm, 1998; Rohrer and Kobilka, 1998; Selbie and Hill, 1998; Vaughan, 1998). This super family has over 1000 members making up as much as 1% of the total proteins encoded in the human genome (Wess, 1998). GPCRs are stimulated by a wide range of stimuli, including neurotransmitters, peptides, hormones, phospholipids, photons, odorants, growth factors and certain taste ligands, leading to many and varied cellular responses.

GPCRs have a seven transmembrane structure: briefly, the amino-terminal tail of the receptor is extracellular while the receptor traverses the membrane seven times creating alternate intra- and extracellular loops with the receptor finally terminating intracellularly on the carboxyl-terminal end. GPCRs couple to heterotrimeric G proteins (composed of three distinct subunits α , β , and γ) on the intracellular surface of the receptor. According to the widely accepted “shuttling model”, extracellular ligand binding to the GPCR causes an overall conformational change to the receptor that promotes the exchange of GTP for GDP on the $G\alpha$ subunit (for review see Wess, 1998). The $G\alpha$ subunit then dissociates from the intimately-linked $G\beta\gamma$ complex and both $G\alpha$ and $G\beta\gamma$ from the receptor (Wess, 1998). Both the free $G\alpha$ -GTP subunit and free $G\beta\gamma$ subunit are then able to regulate a number of effectors, including adenylyl cyclase, cGMP-dependent phosphodiesterases, phospholipase C (PLC), proteins in the Ras-mitogen-activated protein kinase (MAPK) pathway, ion channels, and proteins involved in GPCR signal regulation, leading to a host of physiological responses.

Not all literature is unequivocal with respect to the widely accepted “shuttling model”. Recent evidence argues for a simultaneous receptor-G protein-effector complex (for recent reviews see Rebois *et al.*, 1998; Chidiac, 1998). In this alternative model the G protein is believed to function as an activated heterotrimer rather than as dissociated subunits. Ligand binding to the receptor is believed to result in a conformational change in the receptor that is transferred to the G protein that now allows it couple to the effector molecules as a multiprotein complex. This model may better account for the established receptor-effector coupling specificity and explain how a limited number of G proteins are able to mediate very specific intracellular signalling pathways.

The GPCRs show significant amino acid and structural homology among its members. The hydrophobic transmembrane domains display considerable amino acid sequence conservation between different GPCRs. Depending on the classification system used, the GPCRs can be divided

into three major subfamilies (Rohrer and Kobilka, 1998): 1) The rhodopsin/ β -adrenergic receptors, 2) vasoactive intestinal polypeptide (VIP)/secretin receptors and 3) the metabotropic glutamate receptors. The hallmark characteristic for the rhodopsin/ β -adrenergic receptors is the ligand binding pocket, which is believed to be formed largely by the transmembrane domains and about 20 highly conserved amino acids on the cytoplasmic side of these domains that are found only in members of this subfamily (Wess, 1998). These amino acids are important for receptor stability and for mediating the conformational changes associated with receptor activation (Baldwin, 1994; Wess, 1998). The peptide-binding-secretin GPCRs display a significantly longer amino-terminal domain containing six well conserved cysteine residues (Rohrer and Kobilka, 1998; Wess, 1998). The metabotropic glutamate receptors show no overall homology to the other GPCRs. These receptors show novel structural organization with a very large amino-terminal domains (more than 300 amino acids) believed to be important for ligand binding (Segaloff and Ascoli, 1993; Vassart and Dumont, 1992).

Much of what is known about GPCR function has been derived from studies conducted with the rhodopsin and in particular the adrenergic receptors (Sakmar, 1998). Dopamine receptors fall within the very large rhodopsin/ β -adrenergic receptor subfamily based on sequence homology and structural and functional characteristics. Furthermore, the dopamine receptors belong to the catecholamine receptor family, that include the adrenergic receptors, based on sequence homology to these receptors and the binding of this class of ligand. The high sequence homology of the β -adrenergic receptor was used to clone the first dopamine receptor in rat, the D2 receptor (Bunzow *et al.*, 1988). The dopamine D4 receptor shows a similar amino acid sequence homology to both the D2-like dopaminergic and the adrenergic receptors. The D4 receptor shares a 43% and 41% amino acid identity with the D2 and D3 receptors respectively, but also has a 41% identity with the adrenergic α_{2c} receptor (Lanau *et al.*, 1997b). Other receptors that fall within the rhodopsin/ β -adrenergic receptor family and are related to the dopamine receptors are the adenosine, serotonin and

muscarinic acetylcholine receptors.

The catecholamine receptors share many of the same functional characteristics and display a number of highly conserved amino acids found in all members of this family. The catecholamine receptors share a conserved aspartate and two serine residues in the putative transmembrane regions of TM3 and TM5 respectively (Fong and Strader, 1994; Hibert *et al.*, 1993; Mansour *et al.*, 1992; Neve *et al.*, 1991; Strader *et al.*, 1988; Woodward *et al.*, 1996). In the carboxyl-terminal tail, the dopamine receptors contain a conserved cysteine residue that is conserved in all G protein-coupled receptors and is palmitoylated in the β -adrenergic and rhodopsin receptors (O'Dowd *et al.*, 1989; Ovchinnikov *et al.*, 1988). Another similarity are two cysteine residues in extracellular loops 2 and 3 which are believed to form an intramolecular disulfide bridge to stabilize GPCRs (Civelli *et al.*, 1993). Furthermore, a phenylalanine in TM6 and an aspartate in TM2 are highly conserved in all receptors that interact with catecholamine neurotransmitters and are important in catecholamine binding and receptor activation (Missale *et al.*, 1998; Neve *et al.*, 1991).

In addition to the homology and structural similarities of the dopamine receptors to the β -adrenergic receptors, the neurotransmitter dopamine is related to the catecholamines epinephrine and norepinephrine that act on the adrenergic receptors. In the biosynthesis of the catecholamine epinephrine, dopamine and norepinephrine are intermediate products of the same biosynthetic pathway. These catecholamines act with varying efficacy on the different catecholamine receptors. The adrenergic agonist isoproterenol has been used to distinguish between the α - and β -adrenergic receptors. In general for the α -adrenergic receptors the order of potency is epinephrine > norepinephrine > dopamine > isoproterenol and for the β -adrenergic receptors the order of potency is isoproterenol > epinephrine > norepinephrine > dopamine. Epinephrine and norepinephrine, in fact, have been shown to bind the dopamine D4 receptor in CHO-K1 cells with K_d values in the nanomolar range (Lanau *et al.*, 1997b). Only five-fold higher concentrations of epinephrine over

dopamine were required to stimulate the D4 receptor. Suggestions have been made that the D4 receptor, based on its pharmacologic properties, could be classified both as a dopamine and as an adrenergic receptor (Lanau *et al.*, 1997b).

1.5.1.3 The Dopamine D4 receptor

The last dopamine receptor cloned was the dopamine D4 receptor (Van Tol *et al.*, 1991). A partial length human D4 receptor cDNA was cloned using homology screening of the human neuroblastoma cell line SKNMC with the full length dopamine D2 receptor as a probe (Van Tol *et al.*, 1991). The human dopamine D4 receptor is 387 to 515 amino acids in length varying in size dependent on the expressed isoform.

The dopamine D4 receptor is unique in that it has a highly variable polymorphism in the third intracellular loop. The identification of this D4 polymorphism was first shown in 1992 by Van Tol *et al.*, (1992). It consists of a variable number tandem repeat (VNTR) consisting of 48 base pairs in the nucleotide sequence and 16 amino acids in the coding sequence encoded in exon III. The repeat occurs two to ten times in the human population (Lichter *et al.*, 1993). A number of other additional polymorphisms outside of the third intracellular loop within the coding region of the receptor have been identified, one of which is a nucleotide repeat that occurs most often as a 12 nucleotide duplicated repeat, but less frequently as a single copy (Catalano *et al.*, 1993). It is found in the amino- terminal extracellular tail located in exon I of the gene (Catalano *et al.*, 1993). These two particular polymorphisms have been referred to as Exon I and Exon III polymorphisms.

The three most common variants of the VNTR in the dopamine D4 receptor in the human population are those with two repeats (D4.2), four repeats (D4.4) and seven repeats (D4.7). The repeat units show a moderate amount of sequence variation. Nineteen different variants have been identified and Greek letter designations have been assigned to differentiate the repeats based on their nucleotide

```

D1      MRTLNTSAMDGTLVVERDFSVWRI LTA-CFLSLLI LSTLLGNTLVCAAVI
D2      MDPLNLSWYDDDLERQNWSRPFNGSDGKADRPYNYA-TLLTLLI AVIVFGNVLVCMAYV
D3      MASLSQLSSHLNNTCGAENSTGASQARPK-AYYA-LSYCALI LAIVFGNGLVCMAYL
D4.4    MGNRSTADAGLLAGRGAAGASAGASAGLAQQAALVGGVLLI GAVLAGNSLVCSVA
D4 RAT  MGNSSATGDDGLLAGRGPESLGTGT- -GLGQAGAAALVGGVLLI GMVLAGNSLVCSVA
D5      MLPPGSGNTAYPGGFALYQQLAQNAGVGSAGAPPLGPSQVYTA-CLLTLLI IWTLLGNVLVCAAVI

D1      RFRHLRSK-TNFFVISLAVSDLLVAVLVMPWKAVAEIAGF-WPFG-SFCNIWVAFDIMCSTASILNLCVISVDQRYWAISSPFRYERKM-TPK-
D2      REKALQT-TTNYLIVSLAVADLLVATLVMPWVVYLEVVGGE-WKFSRIHCDIFVTLDVMMCTASILNLCAISIDRYTAVAMPMLYNTRY-SSKR
D3      KERALQT-TTNYLVVSLAVADLLVATLVMPWVVYLEVVGGE-WKFSRIHCDIFVTLDVMMCTASILNLCAISIDRYTAVAMPMLYNTRY-SSKR
D4.4    TERALQT-PTNSFIVSLAAADLLALLVLPFLVYSEVGGAWLLSPRLCDALMAMDVMLCTASIFNLCAISIDRYTAVAMPMLYNTRY-SSKR
D4 RAT  SERILQT-PTNYFIVSLAAADLLALLVLPFLVYSEVGGAWLLSPRLCDALMAMDVMLCTASIFNLCAISIDRYTAVAMPMLYNTRY-SSKR
D5      RSRHLRANMTNMFIVSLAVSDLFVALLVMPWKAVAEVAGY-WPFGA-FCNIWVAFDIMCSTASILNLCVISVDQRYWAISSPFRYERKM-TPK-

D1      AAFILISVAVTSLVLSIFIPVQLSWHKKAKPTSPSDGNATS LAETID- - - - -NCDSSLRTYAISSSVISFYIPVAIMIVTYTRIY
D2      RVTVMISIVVWLSFTISC-PL-LFQLNNA-DQNECII- - - - -ANPAFVYSSIVSFYVFPFIVTLLVYIKIY
D3      RVALMI TAVVWLAFVAVSC-PL-LFQFNTTGDPTVCSI- - - - -SNPDFVYSSVVSFYLPFGVTVLVYARIY
D4.4    RQLLLIGATWLLSAAVAA-PV-LCQLNDVGRDPAVCRLL- - - - -EDRDYVYSSVCSFFLPCPLMLLLYWATF
D4 RAT  -QLLLIATWLLSAAVAA-PV-VCQLNDVPGDPTVCCL- - - - -EDRDYVYSSVCSFFLPCPLMLLLYWATF
D5      MALVMVGLAWTSLISFIPVQLNWHRDQAA SWGGDLDPNNLANWTPWEEDFWEPOVNAENCSSLNRTYAISSSVISFYIPVAIMIVTYTRIY

D1      RIAQ--KQIRRIAA-LERAAVHAKNCQTTTGNGKPFV- - - - -
D2      IVL- - - - -RKRVTNKRSSRAFAHLRAPLKGNCTHPEDMKLCTVIMKSNGSFPVNNRRV- - - - -EAARRAQELEMEMLSSTSPERTRYSPIPP
D3      VVLKQRR- - - - -RKRI LT-RQNSQCNSVRPGFPQQTLSPOPAHLE- - - - -LKRYY
D4.4    RGL-QRWEVARRAKLHGRAPRRPSGPGPPSPTPAPRLPGDPCGPGCA- - - - -
D4 RAT  RGL-RRWEAARHTKLHGRAPRRPSGPGPPVSDPTGGPLFS- - - - -DCPPSPSLRTSPTVSSRPESQLSPGCLLPDAALAPPA- - - - -
D5      RIAQ--VQIRRISS-LERAAEHAQSCRSSA- - - - -

D1      - - - - -CSQPESSFKMSFKRETKVLTLSVIMGVFVCCWLPPFFIL
D2      HHQLTLDPDS- - - - -HHGLHSTDSPAKPEKNGAK-DHPKIAKIFEIQTMPNGKTRTSLK-TMSRRKLSQKQEKKATCMLAIVLGVFIICWLPPFFIT
D3      ICQDTALGQPFQERGGEKREKTRNSLSPTAPKLSLEVRRKSNGLRST- - - - -LKLGPLOPRGVPLREKKATCMVAIVLGAFAIVCWLPFFLT
D4.4    - - - - -PAPSLPPDPCGPGNCA- - - - -PDAYRAALPPQTPPQT-RRRRRAKITGRERKAMRVLPVVVGAFLLCWTPFFVV
D4 RAT  - - - - -PSSRRKRGAKITGRERKAMRVLPVVVGAFLLCWTPFFVV
D5      - - - - -ACAPDTSLRASIKKETKVLKTLTSVIMGVFVCCWLPPFFIL

D1      NCILPFC-GSGE--TQPF-C-IDSNTFDVFWFGWANSSLNPIIYA-FNADFRKAFSTLLG-CYRLCPATNNAIETVSINNNGAAMFSSHHEPR
D2      HILNIHC- - - - -DCNI PPVLYSAFTWLGYNVSAVNPIIYTTFNIEFRKAFKILHC
D3      HVLNTHC- - - - -QTCMVSPELYSAFTWLGYNVSAVNPIIYTTFNIEFRKAFKILHC
D4.4    HITQALC- - - - -PACSVPRRLVSAFTWLGYNVSAVNPIIYTTFNIEFRNVRKALRACC
D4 RAT  HITRALC- - - - -PACFVSPRLVSAFTWLGYNVSAVNPIIYTTFNIEFRSVFRKTLRLRC
D5      NCMVDFCSGHPEGGPPAGFPC-VSETTFDVFVFWFGWANSSLNPIIYA-FNADFRKAFSTLLG-CYRLCPATNNAIETVSINNNGAAMFSSHHEPR

D1      GSISKECNLVLYIPHAYGSSSEDLKKEEAAGIARPLEKLSALSVIDDYDTVSLEKI QPITNGQMPT
D2
D3
D4.4
D4 RAT
D5      AAAYIHMPNAVTPGNREVDNDEE-EGPFDRMFQIYQTSDDGDPVAESVWLDCEGEISLOITPFTPNGFH

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FIGURE 1.1 Amino acid sequence of the D1-like and D2-like dopamine receptors.
Transmembrane domains are indicated by boxed sequences. The VNTR of the dopamine D4.4 receptor is indicated by the shaded sequence.

sequence. The differences in sequences can be seen in figure 1.2. In general there is no more than a four amino acid difference between the different repeat units. The amino acid sequence of the first (α) and last (ζ) repeat sequence are invariable in the human population. The intervening repeats show variability in sequence and order. As a consequence this means that an individual may encode for a D4.7 receptor, but this may be composed of different repeat units than another individual also encoding a D4.7 receptor. The nucleotides encoding the repeat region is G/C rich

B

		Amino Acid Position															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
α	alpha	Pro	Ala	Pro	Arg	Leu	Pro	Gln	Asp	Pro	Cys	Gly	Pro	Asp	Cys	Ala	Pro
β	beta				Gly			Arg	Gly								
δ	delta	Ala			Ser												
ε	epsilon				Gly												
γ	gamma				Gly												
η	eta	Ala			Gly			Pro									
ι	iota	Ala			Gly			Arg									
κ	kappa				Cys			Arg	Gly								
λ	lambda				Gly												
μ	mu	Ala			Gly												
ν	nu				Gly			Arg	Gly								
ο	omicron				Gly			Pro					Ser	Asn			
π	pi				Gly												
θ	theta	Ala			Ser												
ρ	rho				Ser												
σ	sigma				Gly			Gly									
ξ	xi				Gly			Pro					Ser	Asn			
ψ	psi	Ala			Gly												
ζ	zeta				Gly			Pro					Ser	Asn			

FIGURE 1.2 Schematic of the variable repeat units in the dopamine D4 receptor. A) The repeat unit composition of the third intracellular loop is depicted. Individuals with between 2 and 10 repeat units have been identified. The repeat units and their order within the various identified D4 variants is depicted using Greek lettering. B) The amino acid sequence for the different variants is indicated. Some variants show differences in their nucleotide sequence, but their amino acid sequence is unaffected.

(guanosine/cytosine). The repeat units are also proline rich and all the repeat units contain at least two cysteine residues.

The VNTR polymorphism seen in humans is not present in the rodent species *Mus musculus* and *Rattus norvegicus* and has not been identified in other GPCRs (O'Malley *et al.*, 1992). The sequence flanking the repeat sequence in humans shows a high homology to that of the rat in the third intracellular loop (in the order of 95% DNA, 100% amino acid sequence). The flanking region, as seen both in humans and rodents, has a moderate homology to the sequence of the repeat region seen in humans (Makoff, 1992). The VNTR polymorphism has been identified across various primate species of both Old World and New World monkeys (Livak *et al.*, 1995; Matsumoto *et al.*, 1995b). The repeat sequence, therefore, has either been conserved in humans and lost in the rodent species, or it has been introduced in the primate species after the divergence between the rodent and primate species (for discussion see Lichter *et al.*, 1992; Makoff, 1992). The first and last repeat, although being invariable in humans, shows some variability among primate species (Livak *et al.*, 1995).

1.5.1.4 Pharmacology of the Dopamine D4 Receptor

Many of the D2 ligands show a reduced affinity for the dopamine D4 receptor, but in general show a similar binding profile to that of the D2 and D3 receptors (Van Tol *et al.*, 1991). The dopamine D4 receptor displays high affinity binding, in the same range as D2, for the antagonists spiperone and haloperidol (Van Tol *et al.*, 1991). The dopamine D4 receptor is approximately two orders of magnitude weaker in the binding of the antagonist raclopride, as compared to D2, and is not stereoselective for the antagonist sulpiride (Van Tol *et al.*, 1991). Studies on the dopamine D4 receptor revealed that the atypical neuroleptic clozapine binds the D4 receptor with a ten-fold higher affinity than the other D2-like receptor subtypes (Van Tol *et al.*, 1991). The catecholamine agonists bind D4 in the order dopamine > epinephrine > norepinephrine (Lanau *et al.*, 1997b). Epinephrine

and norepinephrine bind D4 with a similar affinity (nM) as dopamine, but require at least a 100-fold higher concentration to bind at the D2 receptor (Lanau *et al.*, 1997b).

Agonist binding to the dopamine D4 receptor is guanine nucleotide sensitive, confirming that it belongs to the GPCR superfamily (Van Tol *et al.*, 1991). The dopamine D4 receptor, like the other D2-like receptors is pertussis toxin (PTX) sensitive and couples through the G_i heterotrimeric G protein that acts to inhibit adenylyl cyclase upon stimulation (Asghari *et al.*, 1995). In contrast, the D1-like receptors couple to G_s , which stimulates adenylyl cyclase. The D4, like other GPCRs that couple to G_i , have a relatively long third intracellular loop and short carboxyl-terminal tail as opposed to the D1-like and all other G_s -coupled aminergic receptors that have a short third intracellular loop and long carboxyl-terminal tail (Civelli *et al.*, 1993).

Despite the presence of the highly variable repeat sequence in the dopamine D4 receptor, no major pharmacological or functional differences have been observed between the different variants. D4 variants with between two and ten repeats showed similar binding profiles and guanine nucleotide sensitivity (Asghari *et al.*, 1994; Jovanovic and Van Tol, in preparation). Furthermore, modulation of intracellular cyclic AMP levels by the different receptor variants showed a two-fold reduction in potency of dopamine acting on the D4.7 variant compared to D4.2 and D4.4 (Asghari *et al.*, 1995), while D4.10 displayed a 2-fold increase in potency for dopamine (Jovanovic and Van Tol, in press). In addition, removal of all the repeats resulted in a receptor that showed normal binding and coupling and could still inhibit adenylyl cyclase normally (Asghari *et al.*, 1995; Asghari *et al.*, 1994). These results indicate that the repeat only has minor effects on binding and G protein-coupling.

A number of linkage and association studies have attempted to determine if the number of repeats in the dopamine D4 receptor is related to various behaviours and disorders including novelty seeking, depression, attention deficit hyperactivity disorder, schizophrenia and Tourette's syndrome. The results of these studies are inconsistent. In 1996 it was shown that novelty seeking behaviour was

related to the number of repeats in the D4 receptor and that up to 15% of this trait was determined by the D4 polymorphism (Benjamin *et al.*, 1996; Ebstein *et al.*, 1996). Follow up studies have both confirmed and rejected this claim (Malhotra *et al.*, 1996; Pogue-Geile *et al.*, 1998). Similar studies have also shown an association for Parkinson's (Ricketts *et al.*, 1998), attention deficit hyperactivity disorder (LaHoste *et al.*, 1996), chronic alcoholism (George *et al.*, 1993) and major depression (Manki *et al.*, 1996), but these findings, with the exception of attention deficit hyperactivity disorder, have also been disputed (Malhotra *et al.*, 1996; Pogue-Geile *et al.*, 1998; Tanaka *et al.*, 1995). Genetic studies between this VNTR and schizophrenia have been largely negative, suggesting that the dopamine D4 is not a major locus contributing to this disease (Hong *et al.*, 1998; Shaikh *et al.*, 1994; Tanaka *et al.*, 1995; Wilson *et al.*, 1998). It remains unclear if and what possible function the VNTR may play in dopamine D4 function.

1.5.1.5 GPCR and Dopamine D4 Receptor Structure

Theoretical structural analysis of the amino acid sequence of the dopamine D4 receptor, like all GPCRs, suggests that the receptor has a seven transmembrane topology. The amino-terminal tail is found on the extracellular surface of the cell, while the carboxyl-terminal tail is located intracellularly. The receptor traverses the membrane seven times thus creating alternating intracellular and extracellular loops. The transmembrane domains are characterized by their hydrophobic amino acids and a relatively conserved amino acid sequences between different GPCRs (Probst *et al.*, 1992). Each transmembrane domain is composed of about 20-25 residues that form α -helices (Farahbakhsh *et al.*, 1995; Strader *et al.*, 1994). The intracellular and extracellular loops are characterized by their hydrophilic amino acids and their relatively low conservation of amino acid sequences between different GPCRs.

Three-dimensional models based on the structural information derived from experiments with

bacteriorhodopsin have supported the seven transmembrane topology of GPCRs and the dopamine D4 receptor. The results from high resolution electron cryomicroscopy on the non-GPCR, seven transmembrane bacteriorhodopsin protein has been used for many GPCR models despite the lack of sequence homology (Hibert *et al.*, 1993; Kyle *et al.*, 1994). No high resolution structure is available

FIGURE1.3 Three dimensional view of a typical GPCR. The seven transmembrane domains are connected by loops that alternate between the extracellular (EL) and intracellular surface (IL). The amino-terminal tail is extracellular and the carboxyl-terminal tail is intracellular. Glycosylation sites are situated on the amino-terminal tail, while phosphorylation sites are located on the intracellular loops and the carboxyl-terminal tail. The transmembrane domains and extracellular loops participate in ligand binding, while the intracellular loops are involved in G protein-coupling, and contain binding sites for kinases, including the G protein-coupled receptor kinases (GRKs).

for the GPCRs mainly because of the difficulty in generating crystals from the integral membrane proteins which are suitable for X-ray crystallography (for review see Wess, 1997; Wess, 1998). Low resolution imaging of the transmembrane core of the rhodopsin receptor, however, has supported the seven transmembrane model (Schertler and Hargrave, 1995; Schertler *et al.*, 1993; Unger and Schertler, 1995) and has been used in general models of GPCR structure (Baldwin, 1993; Baldwin, 1994). These studies involved the electron cryomicroscopic analysis of 2-dimensional rhodopsin crystals and indicated that the seven transmembrane helices are arranged in a tightly packed helical ring-like structure. Baldwin used the low resolution imaging results and compared the sequence of over 200 different GPCRs to generate a general structural model for GPCRs. According to this model TM4, TM6 and TM7 are nearly perpendicular to the plane of the membrane, while TM1, TM2, TM3 and TM5 are tilted about 25° (Baldwin, 1993; Baldwin, 1994; Baldwin *et al.*, 1997). These and mutagenesis studies indicate that the transmembrane domains of GPCRs are arranged in a clockwise fashion if viewed from inside of the cell (Baldwin, 1993; Liu *et al.*, 1995b; Mizobe *et al.*, 1996; Schertler *et al.*, 1993).

Unlike the arrangement of the transmembrane domains, little is known about the extracellular receptor surface (Wess, 1998). Recent biophysical studies have given some insight on the intracellular receptor surface that is believed to be involved in G protein-coupling. Results from nuclear magnetic resonance and circular dichroism experiments with biologically active peptides derived from β -adrenergic and rhodopsin receptors indicate that the carboxyl-terminal portion of the third intracellular loop and amino portion of the carboxyl-terminal tail are α -helically arranged (Jung *et al.*, 1995; Jung *et al.*, 1996; Yeagle *et al.*, 1996). Little is known about the intracellular structure of the dopamine D4 receptor, and in particular the third intracellular loop region containing the VNTR polymorphism. The third intracellular loop region of the D4, and especially the repeat region, is extremely proline rich. Prolines tend to induce a short left-handed polyproline type II helical (PPII)

conformation (Adzhubei and Steinberg, 1993). How this affects the overall structure of the third intracellular loop is not known. Detailed structural knowledge of this region will help to determine the function for the repeat and aid in determining the mechanisms of protein interactions.

GPCRs require a number of post-translational modifications for proper functioning of the receptor. Some of these modifications have also been shown to be important in dopamine receptor signalling and regulation. The GPCRs are extensively phosphorylated by both second messenger-dependent kinases and G protein-coupled receptor kinases (GRK). Many GPCRs contain recognition sites for these kinases which are situated mostly in the third intracellular loop and/or carboxyl-terminal tail. Phosphorylation of GPCRs is an important step in receptor desensitization and may serve to prepare some GPCRs for specific protein interactions. The dopamine receptors also contain potential glycosylation sites in the amino-terminal tail and/or second extracellular loop (Missale *et al.*, 1998). The dopamine D4 receptor only contains one potential glycosylation site (Missale *et al.*, 1998). Palmitoylation of a highly conserved cysteine residue is important for anchoring the carboxyl-terminal tail to the plasma membrane for GPCRs including the β -adrenergic and rhodopsin receptors (O'Dowd *et al.*, 1989; Ovchinnikov *et al.*, 1988).

The seven transmembrane topology of the GPCRs is believed to be stabilized by two cysteine residues found in the second and third extracellular loops which are believed to form an intramolecular disulfide bridge (Dohlman *et al.*, 1990; Fraser, 1989). These residues are also conserved in the dopamine D4 receptor and possibly play a similar function.

1.5.1.6 Ligand Binding to the GPCRs and the Dopamine D4 Receptor

From site directed mutagenesis studies and protein modeling with the dopamine D2 receptor, as well as with the related β_2 - and α_2 -adrenergic receptors, it is believed that agonist binding occurs and is determined mainly by contact points within the hydrophobic transmembrane domains of the

receptor (Hibert *et al.*, 1992; Hibert *et al.*, 1993; Strader *et al.*, 1989; Strader *et al.*, 1988; Trumpp-Kallmeyer *et al.*, 1992). In particular a conserved aspartate residue in TM3 is probably involved in binding the amine group of the catecholamine side chain (Hibert *et al.*, 1993; Strader *et al.*, 1988), while two serine residues in TM5 are important hydrogen bond donors to bind the hydroxyl groups of the catechol moiety for the β_2 , α_2 , and D2 receptors (Cox *et al.*, 1992; Mansour *et al.*, 1992; Tomic *et al.*, 1993). A phenylalanine in TM6 and an aspartate in TM2 are also highly conserved among the catecholamine receptors, which affects agonist binding and receptor activation (Hibert *et al.*, 1993; Neve *et al.*, 1991; Sidhu *et al.*, 1992; Tomic *et al.*, 1993). The interaction between the aspartate in TM2 and the agonist is thought to be allosterically modulated by either Na^+ or H^+ for the dopamine D2 receptor and the α_2 -adrenergic receptor (Hibert *et al.*, 1992; Horstman *et al.*, 1990; Neve *et al.*, 1991) and may account for differences seen in binding to the dopamine D4 receptor in the absence and presence of NaCl (Asghari *et al.*, 1994; Van Tol *et al.*, 1992).

More specific ligand binding properties of D2-like receptors were determined in chimeric studies that expressed dopamine D3/D1 transmembrane chimeras (Alberts *et al.*, 1998). These studies showed that the superficial amino acid residues of TM2, TM4, and TM6, the more deeply situated residues in TM3 and all the residues of TM7 are important for ligand binding to the D3 receptor (Alberts *et al.*, 1998). Work done on the dopamine D2 receptor, using a substituted-cysteine accessibility method, has identified an important cluster of aromatic residues in TM6 that contribute to the binding site pocket (Javitch *et al.*, 1998). Four of the identified accessible aromatic residues are completely conserved in related GPCRs and have been shown to be important for ligand binding and receptor activation (Javitch *et al.*, 1998). The arrangement of these residues is consistent with the α -helical structure predicted for the transmembranes (Javitch *et al.*, 1998). The relatively low homology of D4 to D2 and D3 may mean that other amino acid residues are important for binding in the dopamine D4 receptor, however, these studies have not been done for the D4 receptor.

Ligand binding is believed to result in subtle conformational changes in the GPCR. For the rhodopsin receptor, activation is believed to result in a relatively small outward movement of TM3 (Farahbakhsh *et al.*, 1993; Farahbakhsh *et al.*, 1995) which exposes part of the receptor on the intracellular surface to allow G protein-coupling (Wess, 1997). Agonist and antagonist activation of the yeast α -factor pheromone receptor resulted in distinct proteolytic fragments of the third intracellular loop when cleaved with trypsin, indicating differences in the accessibility of the trypsin cleavage sites in the receptor and probable differences in receptor conformation (Bukusoglu and Jenness, 1996). These conformational changes are also believed to occur among the catecholamine receptors. Ligand-dependent structural changes were observed in the β_2 -adrenergic receptor when observed using a cysteine-specific fluorescence marker molecule (Gether *et al.*, 1995).

1.5.1.7 G protein-Coupling of the GPCRs and the Dopamine D4 Receptor

All GPCRs are believed to be able to signal through heterotrimeric G proteins by means of a common molecular mechanism (Wess, 1998). The guanine nucleotide binding heterotrimeric G protein is made up of three distinct subunits; $G\alpha$, $G\beta$ and $G\gamma$. At least twenty different $G\alpha$ subunits, six $G\beta$ subunits and twelve $G\gamma$ subunits have been identified (Clapham and Neer, 1997; Gutkind, 1998b; Hamm, 1998). The heterotrimeric G proteins are traditionally named after the α subunit which can be grouped into four families based on amino acid similarity and function, G_s , $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ (Conklin and Bourne, 1993; Neer, 1995). G proteins are made up of various complements of the α , β and γ subunits which affect both the G protein's ability to couple to specific receptors (ie. the GPCR can only couple effectively to a distinct subset of the many structurally similar heterotrimeric G proteins expressed in the cell) and to modulate specific signalling pathways (Wess, 1998). Both the function and specificity of coupling to the GPCR appears to be mostly mediated by the $G\alpha$ subunit. However, a large body of evidence indicates that the $G\beta\gamma$ subunit also contributes to G protein

specificity (for review see Kalbrenner *et al.*, 1996).

The widely accepted model of receptor activation and G protein-coupling is that upon ligand binding the receptor undergoes a conformational change which promotes the heterotrimeric G protein ($G\alpha\beta\gamma$) to couple to the activated receptor, and this interaction with the activated receptor promotes a GTP-for-GDP exchange on the $G\alpha$ subunit (Bourne, 1997; Dohlman *et al.*, 1991; Neer, 1995; Wess, 1997; Wess, 1998). The activated $G\alpha$ ($G\alpha$ -GTP) subunit and the $G\beta\gamma$ subunit dissociate from the receptor and from each other. Both the free $G\alpha$ -GTP subunit and the $G\beta\gamma$ subunit are now able to act upon a diverse array of downstream effectors. For instance, $G\alpha_s$ and $G\alpha_i$ modulate cAMP levels by either activating or inhibiting adenylyl cyclase, respectively. Pertussis toxin catalyses ADP-ribosylation specifically on the $G\alpha_i/G\alpha_o$ subunit family and thus prevents $G\alpha_i/G\alpha_o$ interaction with the receptor and signalling (Ui and Katada, 1990). PTX sensitivity of GPCRs is taken as evidence of a G_i/G_o -mediated signal transduction mechanism. The free $G\beta\gamma$ subunit has been shown to activate signalling pathways and participate in receptor regulation.

The different G proteins, mediated mostly by the $G\alpha$ subunit, activate distinct second messenger systems. Members of G_s stimulate various adenylyl cyclases, while members of G_i can inhibit adenylyl cyclases, thus increasing or decreasing the levels of cAMP respectively. $G\alpha_q$ (a member of $G\alpha_i$) stimulates cGMP phosphodiesterase. All the members of the $G\alpha_{q/11}$ family activate different forms of PLC β which mediates the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP₂) and generates the second messengers inositol (1,4,5)-triphosphate (IP₃) and diacylglycerol. The functions of $G_{12/13}$ are still not clearly understood. The $G\beta\gamma$ subunit can also bind to a number of effectors and in many cases mediate either opposing or synergistic effects to those of the $G\alpha$ subunit. For example type I adenylyl cyclase is stimulated by $G\alpha_s$ but inhibited by $G\beta\gamma$, while type II adenylyl cyclase is stimulated by both $G\alpha_s$ and $G\beta\gamma$ (Taussig *et al.*, 1993). Recent evidence shows that the $G\beta\gamma$ is important for GPCR desensitization and GPCR-mediated activation of the MAPK pathway leading

to mitogenic responses in the cell.

Unlike the structure of the GPCRs, high resolution structures for at least two heterotrimeric G proteins, $G\alpha_1\beta_1\gamma_2$ (Wall *et al.*, 1995) and $G\alpha_\beta\gamma_1$ (Lambright *et al.*, 1996), have been resolved by X-ray crystallography, and this has given considerable insight into the G protein activation of downstream effectors (Ford *et al.*, 1998). The $G\alpha$ subunit is composed of a domain involved in GTP hydrolysis, which is structurally identical to that found in the superfamily of GTPases, and a domain that buries the GTP into the core of the protein (Coleman *et al.*, 1994; Noel *et al.*, 1993). The $G\alpha$ and $G\beta$ subunits interact at two distinct interfaces (Lambright *et al.*, 1996; Wall *et al.*, 1995). The $G\beta$ and $G\gamma$ subunits make extensive contacts with each other and can only dissociate under denaturing conditions (Hamm, 1998; Lambright *et al.*, 1996; Wall *et al.*, 1995). There appears to be only moderate direct interaction between the $G\alpha$ and $G\gamma$ subunits (Lambright *et al.*, 1996; Wall *et al.*, 1995). Unlike the $G\alpha$ and $G\beta$ subunits, the $G\gamma$ subunits show large differences in structure among the different forms. The conformational structure of the free $G\beta\gamma$ complex is the same as that found bound within the heterotrimeric G protein (Lambright *et al.*, 1996; Wall *et al.*, 1995) suggesting that the $G\alpha$ subunit acts, by its binding, as a negative regulator of $G\beta\gamma$ activity (Wess, 1998). Lipid modifications of the $G\alpha$ and $G\beta$ subunits are important for the attachment of the G protein to the cytoplasmic side of the plasma membrane (Wedegaertner *et al.*, 1995).

Upon GTP binding to $G\alpha$ in the heterotrimeric/activated receptor complex, a conformation switch of three flexible regions in $G\alpha$ occurs, lowering the affinity of the $G\alpha$ subunit for the $G\beta\gamma$ subunit and leading to the dissociation of $G\alpha$ and $G\beta\gamma$ (Lambright *et al.*, 1994; Lambright *et al.*, 1996; Wall *et al.*, 1995). Cleavage of GTP to GDP due to the intrinsic GTPase activity of the free $G\alpha$ subunit favors the reassociation of $G\alpha$ with the free $G\beta\gamma$ subunit, which can now once again bind to an activated receptor (Clapham and Neer, 1997; Conklin and Bourne, 1993).

The coupling of G proteins to the receptor is determined both by the G protein and the

receptor. A specific GPCR can only bind to a limited set of G proteins, and a specific G protein can only bind to a limited number of GPCRs. In general the binding of GPCRs to different G proteins is not absolute, but rather is the result of relative differences in affinity and selectivity. For example the $G_{i/o}$ -coupled m4 muscarinic and α_2 -adrenergic receptors are also capable of binding G_s as seen when G_i is inactivated (Eason *et al.*, 1992; Migeon and Nathanson, 1994). The phosphorylation of the β_2 -adrenergic receptor switches the receptor from a G_s to a G_i -coupled receptor (Daaka *et al.*, 1997). In addition, specific coupling also depends on the expression, restricted localization and density of the receptor and G proteins.

The low resolution electron diffraction structure of the rhodopsin receptor in combination with mutagenesis and biochemical studies of different GPCRs have given insight into the requirements for G protein-coupling to the GPCRs. These studies have shed light on the conformational changes of the receptor, the intracellular loops that are involved in G protein binding, and the specific amino acids critical for G protein selectivity and affinity. Although the GPCRs show differences in amino acid sequences, the GPCRs and G proteins are thought to share a common mechanism of activation because of the conserved structural nature of these proteins (Wess, 1998).

It is thought that ligand binding to the receptor causes a conformation change in the relative orientations of TM3 and TM6 resulting in a small clockwise rotation, of TM6 relative to TM3, as observed from within the cell as seen in the rhodopsin and β_2 -adrenergic receptor (Farahbakhsh *et al.*, 1995; Farrens *et al.*, 1996; Gether *et al.*, 1997). This activation is also believed to result in a small outward movement of the cytoplasmic side of TM3 and a structural change in the second intercellular loop (Farahbakhsh *et al.*, 1993; Farahbakhsh *et al.*, 1995). The movement of the transmembrane domains is transferred into the intracellular loop domains, thus altering the receptors intracellular surface and presenting previously hidden amino acid residues important for G coupling (Farahbakhsh *et al.*, 1993; Farahbakhsh *et al.*, 1995). The GPCRs are thought to trigger GDP release on the $G\alpha$

subunit of the heterotrimeric G protein by an allosteric mechanism, since the nucleotide is buried deep inside the $G\alpha$ subunit and is unlikely to be in direct contact with the receptor (Bourne, 1997; Lambright *et al.*, 1996; Wall *et al.*, 1995; Wess, 1998)

The identification of the receptor domains involved in G protein-coupling has been derived from early studies with hybrid muscarinic and adrenergic subtypes and more recently with many other GPCRs (Dohlman *et al.*, 1991; Hedin *et al.*, 1993; Wess, 1998). Hydrophobic residues in the second intracellular loop and in the extreme amino- and carboxyl-terminal regions of the third intracellular loop are the most important in G protein-coupling (Dohlman *et al.*, 1991; Hedin *et al.*, 1993; Savarese and Fraser, 1992; Wess, 1997; Wess, 1998). Short peptides corresponding to the amino- and carboxyl-terminal regions of the third intracellular loop and the second intracellular loop of the rhodopsin, muscarinic and β -adrenergic receptors were shown to either mimic or inhibit G protein-coupling (König *et al.*, 1989; Münch *et al.*, 1991; Okamoto and Nishimoto, 1992) which confirm mutagenesis studies which indicate that these regions are important for coupling (Blin *et al.*, 1995; Wong *et al.*, 1990; Wong and Ross, 1994). All these sites act in a cooperative fashion to determine both G protein selectivity and affinity of the GPCR (Blin *et al.*, 1995; Wong *et al.*, 1990; Wong and Ross, 1994). Some selectivity for G protein-coupling may also be derived from the carboxyl-terminal tail, although some GPCRs lack this region (ie. Gonadotrophin) (Wess, 1998). The amino acids in the first intracellular loop do not appear to be in direct contact with the G protein, however, the structural conformation this loop imposes on the receptor may affect coupling (Wess, 1998). It also appears that the relative contribution of the different domains involved in G protein-coupling varies considerably even among members of closely related GPCRs (Wess, 1998). These studies indicate that coupling is mostly determined by the intracellular surface of the receptor, in particular, the second and third intracellular loop, while the carboxyl-terminal tail and first intracellular loop also modulate receptor coupling.

A highly conserved amino acid triplet sequence among the rhodopsin/ β -adrenergic receptors is the Asp/Glu-Arg-Tyr (DRY) sequence in the amino-terminal portion of the second intracellular loop. The Arg residues occurs in 99% of all rhodopsin like receptor sequences (Baldwin *et al.*, 1997) including the dopamine D4 receptor. Mutation of the Arg residue abolishes or drastically reduces G protein-coupling in many receptor subtypes including the rhodopsin, m1 muscarinic and α_{1B} -adrenergic receptors (Franke *et al.*, 1992; Scheer *et al.*, 1996; Zhu *et al.*, 1994). Mutation of the preceding aspartate residue abolished G_s binding to the β_2 -adrenergic receptor (Fraser *et al.*, 1988) but not to the G_q -coupled α_{1B} -adrenergic receptor (Scheer *et al.*, 1997). A conserved leucine centrally located in the second intracellular loop of the dopamine D4 receptor and other GPCRs upon mutation results in strongly impaired G protein-coupling in the β_2 -adrenergic receptor and m1 and m3 muscarinic receptors (Moro *et al.*, 1993a). Replacement of the second intracellular loop of the α_1 -adrenergic receptor with that of a closely related, but functionally distinct GPCR has been shown to alter the receptor's G protein-coupling to adenylyl cyclase, thus indicating this region is involved in regulating G protein selectivity as seen for the α_2 -adrenergic receptor (Näsman *et al.*, 1997). Replacement of the β_2 -adrenergic and dopamine D2 receptor with the second intracellular loop of the thrombin receptor conferred G_q coupling to both of these receptors (Verrall *et al.*, 1997). A recent study using the m5 muscarinic receptor indicates that the second intracellular loop is also important for enabling G protein activation (Burstein *et al.*, 1998). These studies show that G protein-coupling selectivity is in large part determined by the second intracellular loop.

The third intracellular loop is also involved in determining G protein selectivity. Replacement of the third intracellular loop of functionally distinct receptors leads to reversal of the G protein binding profile as originally seen in the muscarinic (Wess *et al.*, 1990a; Wess *et al.*, 1990b) and adrenergic receptors (Cotecchia *et al.*, 1992; Liggett *et al.*, 1991; Wong *et al.*, 1990; Wong and Ross, 1994) and other GPCRs (see Wess, 1998). The newly created chimeric receptor does not always

display the same selectivity and/or affinity of the original wild type receptor (Cotecchia *et al.*, 1992; Wess *et al.*, 1990b). The second and third intracellular loop act in a cooperative fashion to determine maximal coupling affinity and selectivity of the receptor. The third intracellular loop may be anywhere between 15 and 240 amino acid residues in length, but only 8-15 amino acids at the amino- and carboxyl-terminal ends of the loop have been shown to be involved in G protein-coupling (Strader *et al.*, 1994). Point mutation studies indicate that the replacement of the conserved arginine residue at the TM3/second intracellular loop junction as well as the replacement of a couple of lysine residues in the carboxyl-terminal region of the third intracellular loop abolishes G protein-coupling (see Wess, 1997).

Only the most membrane proximal 8-16 amino acid residues of the carboxyl-terminal tail are believed to be required for efficient G protein-coupling (Wess, 1998). In some cases, as seen with the β -adrenergic receptor, deletion of part of the carboxyl-terminal tail leads to improved G protein-coupling efficiency (Parker and Ross, 1991), increased basal activity (Parker and Ross, 1991), or increased promiscuity for different G protein-coupling (Schneider *et al.*, 1994). Complete removal of the carboxyl-terminal tail from GPCRs results in the inactivation of the receptor (Sano *et al.*, 1997; Segaloff and Ascoli, 1993). Although, this region is believed to play an important role in G protein selectivity, replacement of the carboxyl-terminal tail from related, but functionally distinct receptors, as in the case of the α_{2A} and β_2 -adrenergic receptor, is not sufficient to alter G protein binding (Liggett *et al.*, 1991). The carboxyl-terminal tail is believed to play a role in the proper formation of the receptor/G protein complex (Wess, 1998).

The coupling of the heterotrimeric G protein to the receptor is largely determined by the $G\alpha$ subunit subtype incorporated into the G protein (Wess, 1998). In turn, G protein-coupling of the $G\alpha$ subunit is largely determined by the carboxyl-terminal tail and certain centrally located amino acid residues of the $G\alpha$ subunit that are believed to be in direct contact with the receptor (Bourne, 1997).

A six amino acid residue stretch in the amino-terminal region of $G\alpha$ has recently been shown to contribute to the discrimination between $G\alpha_q$ and $G\alpha_i$ coupling of the m2 muscarinic and dopamine D2 receptor (Kostenis *et al.*, 1998). A direct interaction between the last five amino acids of $G\alpha$ and four amino acids at the third intracellular loop/TM6 junction of the receptor is believed to contribute to G_i selectivity in the m2 muscarinic receptor (Liu *et al.*, 1995a). The $G\beta\gamma$ subunit can also modulate the selectivity of the G protein for the receptor by increasing the affinity and selectivity of coupling (Clapham and Neer, 1997). Both the $G\beta$ and $G\gamma$ subunits are believed to make direct contacts to the receptor. For the rhodopsin and β_1 -adrenergic receptors, this interaction is with the carboxyl-terminal tail (Heithier *et al.*, 1992; Phillips and Cerione, 1992). The different $G\beta$ subunits are structurally quite similar, but the different $G\gamma$ subunits are structurally quite diverse which is believed to contribute to differences in G protein-coupling selectivity (Clapham and Neer, 1997).

Agonist binding of the dopamine D4 receptor is guanine nucleotide and PTX-sensitive indicating that the receptor is a heterotrimeric $G_{i/o}$ coupled receptor (Asghari *et al.*, 1995; Senogles, 1994; Van Tol *et al.*, 1991) like the other D2-like receptors. Recently the rat D4 receptor has been shown to functionally couple, in a mesencephalic neuronal cell line (MN9D), to the PTX-sensitive cone transducin G protein ($G\alpha_{t2}$) normally coupled to opsin-like receptors (Yamaguchi *et al.*, 1997). This is the first specific G protein shown to couple to the D4 receptor. Like the other rhodopsin/adrenergic receptors, chimeric studies using the D1 and D2 receptors indicate that the second and third intracellular loops are involved in G protein-coupling (Kozell *et al.*, 1994). Most studies, including those with the adrenergic receptors, have indicated that only the amino- and carboxyl-terminal regions of the third intracellular loop participate in G protein-coupling (see above). For the dopamine D2 receptor, however, differential G protein-coupling occurs to $G\alpha_{t2}$ as a result of a splice variant that produces a D2 receptor with a 29 amino acid insert (D2L) or without (D2S) in the central portion of the third intracellular loop (Montmayeur *et al.*, 1993). Similar studies have not

been completed with the different D4 variants.

The VNTR polymorphism produces only small differences in binding and in adenylyl cyclase inhibition as seen with the D4.2, D4.4 and D4.7 variants, suggesting that the repeat region is not likely to play a major role in G protein-coupling (Asghari *et al.*, 1995; Asghari *et al.*, 1994). Removal of the repeat region did not prevent adenylyl cyclase inhibition and still exhibited G protein-coupling confirming the above result (Asghari *et al.*, 1995). These results also support the findings that only the most amino- and carboxyl-terminal amino acid residues in the third intracellular loop are important for G protein-coupling. More detailed studies on the coupling properties and amino acid residues involved in D4 receptor coupling to G proteins have not been completed. G protein-coupling of the D4 receptor likely requires the same regions as shown to be important for the other GPCRs, and not the repeat region of the receptor.

1.5.1.8 Second messenger pathways activated by the G_i-Coupled Dopamine D4 receptor

The dopamine D4 receptor has been shown to activate a number of second messenger systems believed to be mediated through the PTX-sensitive G α_i protein and G $\beta\gamma$ heterotrimeric G protein subunits. The dopamine D4 receptor, like the D2 receptor, can inhibit adenylyl cyclase and thus inhibit intracellular cAMP accumulation in retina (Cox *et al.*, 1992) and a variety of cell lines in a PTX-sensitive manner (Asghari *et al.*, 1995; Chio *et al.*, 1994; Tang *et al.*, 1994). Dopamine D4.4 activation has also been shown to potentiate the G $_s$ -stimulated activity of a form of adenylyl cyclase (ACII) by means of the G $\beta\gamma$ subunit released from the pertussis toxin-sensitive heterotrimeric G protein (Watts and Neve, 1997). Unlike the D2 receptor, the dopamine D4 receptor is not able to modulate intracellular calcium levels by the stimulation of PI hydrolysis (Missale *et al.*, 1998), but can reduce calcium levels by the negative modulation of calcium currents as seen in GH $_4$ C $_1$ cells (Seabrook *et al.*, 1994). The D4 receptor has been shown to potentiate ATP-stimulated arachidonic

acid release in CHO-K1 cells in a PTX-sensitive manner and requires protein kinase C, thus implicating the G_i proteins (Chio *et al.*, 1994). The exact mechanism of arachidonic acid potentiation is not known for either the D2 or D4 receptor. The D4 receptor also affects the activity of the amiloride-sensitive Na⁺/H⁺ exchangers responsible for regulating intracellular pH and cell volume (Chio *et al.*, 1994). In CHO cells the activation of the dopamine D4 receptor leads to an increase in the extracellular acidification rates in a PTX-sensitive manner (Chio *et al.*, 1994).

Recent work has also demonstrated the involvement of the dopamine D4 receptor in signal transduction pathways that lead to mitogenesis and/or differentiation. D4 stimulation in the mesencephalic cell line MN9D causes increases in neurite number and length (Swarzenski *et al.*, 1994). The mechanism of D4-mediated mitogenesis has not been determined, but is likely mediated by the Gβγ activation of the MAPK pathway as seen with other GPCRs (Gutkind, 1998b). Although the Gβγ has been shown to modulate a number of second messenger effectors (Clapham and Neer, 1997), these have not been delineated for the dopamine D4 receptor. Problems with receptor expression and technical difficulties have hindered experiments with the dopamine D4 receptor, which have resulted in few studies that have investigated the biochemical and pharmacological properties of the dopamine D4.

1.5.1.9 Signal Regulation of GPCRs and the Dopamine D4 Receptor

Signal regulation of GPCRs occurs at the level of the ligand, the receptor itself, the G proteins and at the level of downstream effectors. GPCR signals can be attenuated by the removal of the ligand from the extracellular fluid, by GPCR desensitization by phosphorylation, GPCR endocytosis and/or GPCR downregulation by either lysosomal break down or reduction in protein synthesis (Ferguson *et al.*, 1996a). Some GPCRs, such as the β₂-adrenergic receptor, can be desensitized by the action of GRKs (G protein-coupled receptor kinases) which phosphorylate the receptor, making it the target

for arrestin binding (Attramadal *et al.*, 1992; Freedman *et al.*, 1995; Lohse *et al.*, 1992). Receptor internalization or endocytosis cause depletion of the receptor from the cell surface. Internalized receptors are either “resensitized” and recycled to the membrane or degraded (Ferguson *et al.*, 1996a; Ferguson *et al.*, 1998). Desensitization by downregulation of the number of GPCRs occurs during long term exposure to agonists. Protein synthesis and degradation may both be affected, reducing the population of receptors at the cell surface.

1.5.1.10 Phosphorylation of GPCRs and the Dopamine D4 Receptor

The phosphorylation of the GPCRs has been extensively studied in the β_2 -adrenergic receptor and has been shown to be a general phenomenon, with few exceptions, for many other GPCRs as well (Tobin, 1997). The characterization of the phosphorylation of the dopamine receptors and in particular the dopamine D4 receptor is limited. Phosphorylation, mediated by receptor-specific and/or second messenger-dependent serine/threonine kinases, has been identified to be of two types: homologous and heterologous.

Receptor phosphorylation in response to agonist binding to that receptor is classified as homologous phosphorylation (Ferguson *et al.*, 1998). This process is mediated mainly by GPCR specific kinases (GRKs), such as the β -adrenergic receptor kinase (β ARK1), but these receptors are also subject to phosphorylation by the non-receptor-specific, second messenger-dependent kinases protein kinase A (PKA) and protein kinase C (PKC), as is the case for the β_2 -adrenergic receptor (Ferguson *et al.*, 1996a). Phosphorylation is rapid (seconds) and requires receptor occupancy. The conformational change induced by agonist binding is believed to expose the amino acid residues recognized and phosphorylated by the receptor-specific kinase (Premont *et al.*, 1995).

Receptor stimulation resulting in the phosphorylation of another receptor subtype is classified as heterologous phosphorylation. This type of phosphorylation is mediated only by the second

messenger-regulated protein kinases. For the phosphorylation of the β_2 -adrenergic receptor, PKA and PKC mediate this process, which are activated in response to increased levels of intracellular cAMP, diacylglycerol or IP₃ (Berridge, 1987; Clarke *et al.*, 1989; Hausdorff *et al.*, 1989). Heterologous phosphorylation displays a slower time course (minutes) and is independent of receptor occupancy.

The contribution of the two mechanisms to receptor phosphorylation is dependent on the level of receptor occupancy. The agonist-dependent phosphorylation by GRKs is more prevalent in the presence of high receptor occupancy as seen at the synapse (Ferguson *et al.*, 1996a). Second messenger-mediated phosphorylation by PKA and PKC is more prominent with low receptor occupancy (Hausdorff *et al.*, 1989; Lohse *et al.*, 1990a).

Phosphorylation of GPCRs occurs through the direct action of kinases on the receptor at specific phosphorylation recognition sites. The β ARK phosphorylation sites in the β_2 -adrenergic receptor are situated in the carboxyl-terminal tail, while the PKA and PKC sites are found both in the third cytoplasmic loop and carboxyl-terminal tail (Berridge, 1987; Hausdorff *et al.*, 1989). The consensus sequences for the second messenger-dependent kinases are more clearly defined than those for the different GRKs. In general, phosphorylation sites are situated in the third intracellular loop or the carboxyl-terminal tail for most GPCRs (Lachowicz and Sibley, 1997).

To date six members of the GRK family (GRK1-6) have been identified which show between 57-96% homology to each other (Ferguson *et al.*, 1998; Premont *et al.*, 1995). The receptor-specific kinase that phosphorylates the rhodopsin receptor (GRK1) was found to be structurally related to those that phosphorylate the β -adrenergic receptors (Lorenz *et al.*, 1991). β ARK1 and β ARK2 (GRK2 and GRK3 respectively) were identified as β_2 -adrenergic receptor-specific kinases, but since then have been shown to phosphorylate GPCRs of many types including the A1 and A2 adenosine receptors, the m2 and m3 muscarinic receptor and the δ opioid receptor (Krupnick and Benovic, 1998). The GRK4 subfamily includes the newly cloned and less well characterized GRK4, GRK5 and GRK6

kinases (Premont *et al.*, 1995). Considering the number of GPCRs, it is certain that the GRKs can target more than one receptor subtype and it also appears that a receptor subtype may be the substrate for more than one type of GRK (Ferguson *et al.*, 1996a; Krupnick and Benovic, 1998).

The different GRKs are targeted to the plasma membrane by distinct mechanisms (Ferguson *et al.*, 1996a). The β ARKs undergo translocation from the cytoplasm to the plasma membrane in response to receptor activation (Boekhoff *et al.*, 1994; Pitcher *et al.*, 1992). Free G $\beta\gamma$ has been implicated in the recruitment of both β ARK1 and β ARK2 to the membrane, where the complex then binds and phosphorylates the activated β_2 -adrenergic receptor (Boekhoff *et al.*, 1994; Clapham and Neer, 1997; Inglese *et al.*, 1993; Pitcher *et al.*, 1992). PKC activation of β ARK1 has been shown to enhance its kinase activity, likely by increasing the association of the protein with the membrane (Chuang *et al.*, 1995; Winstel *et al.*, 1996). β ARK1 interacts directly with the free G $\beta\gamma$ dimer, that is anchored to the plasma membrane through the G γ subunit which is isoprenylated (by a geranylgeranyl isoprenoid) on the carboxyl-terminal (Mumby *et al.*, 1990). The interaction between G $\beta\gamma$ and β ARK is mediated by a pleckstrin homology (PH) binding domain of β ARK1 (Inglese *et al.*, 1995). This interaction with G $\beta\gamma$ targets the β ARKs to the plasma membrane and facilitates the interaction with the receptor, thus increasing its intrinsic kinase activity (Daaka *et al.*, 1997; Stoffel III *et al.*, 1997). In addition, β ARK concurrently binds to the lipid PIP₂, which helps target it to the G $\beta\gamma$ subunit, for full kinase activity (DebBurman *et al.*, 1995; Pitcher *et al.*, 1995). GRK1, 4 and 6 undergo post-translational modification and are either farnesylated (GRK1) or palmitoylated (GRK4 and 6) on the carboxyl-terminal amino acid residues, which then aids in their association with the membrane (Inglese *et al.*, 1992; Premont *et al.*, 1995; Premont *et al.*, 1994; Stoffel *et al.*, 1994). Association of GRK5 to the membrane is thought to occur through an electrostatic charge, mediated by 46 highly basic carboxyl-terminal amino acids and membrane phospholipids (Premont *et al.*, 1994).

For most GPCRs, including the β_2 -adrenergic receptor, serine and threonine residues (serines 396, 401, 407 and threonine 384 of β_2) in the carboxyl-terminal tail are phosphorylated by GRKs (Fredericks *et al.*, 1996; Premont *et al.*, 1995). The exact phosphorylation sites, however, have not yet been identified for most GPCRs (Ferguson *et al.*, 1996a). Receptors, such as the G_i -coupled m2 muscarinic acetylcholine receptor and the α_2 -adrenergic receptor, have a short carboxyl-terminal tail that contains no serine or threonine residues. These receptors are phosphorylated on their serine and threonine residues in their much longer third intracellular loop (Krupnick and Benovic, 1998; Pals-Rylaarsdam *et al.*, 1997). Both of the G_i -coupled dopamine D2 and D4 receptors contain no serine or threonine residues in their short carboxyl-terminal tail; however, both receptors do contain multiple serines and threonines in the third intracellular loop which could act as potential GRK phosphorylation sites. The consensus sequences or structures that target GRKs to specific GPCR regions have not been clearly identified. The GRKs have been shown to phosphorylate multiple sites in the receptor in *in vitro* assays, however, only the initial phosphorylation of the receptor is believed to be of physiological significance in receptor desensitization (Oghuro *et al.*, 1993).

Recently, an important function for PKA phosphorylation of the β_2 -adrenergic receptor was demonstrated (Daaka *et al.*, 1997). Heterologous phosphorylation of the β_2 receptor switched the coupling specificity from G_s to G_i allowing the receptor to activate G_i -mediated signalling events (Daaka *et al.*, 1997). This result demonstrates that the coupling specificity of the receptor is not strictly dependent on its amino acid sequence, but can be modified by post-translational modifications of the receptor, adding another level of control and complexity in signal regulation.

Few reports have investigated the phosphorylation of the dopamine receptors. Technical difficulties exist in isolating pure and sufficient amounts of receptor for most GPCRs subtypes, including the dopamine receptors, unlike the rhodopsin and β -adrenergic receptors that are highly enriched in the plasma membrane fraction (Premont *et al.*, 1995). The D1 receptor has been shown

to be phosphorylated by both β ARK1 and β ARK2 and GRK5 in whole cell phosphorylation assays (Tiberi *et al.*, 1996). The dopamine D2 receptor is phosphorylated (Ng *et al.*, 1994), likely by a GRK dependent mechanism (Itokawa *et al.*, 1996). To date, no studies have looked at the phosphorylation status of the dopamine D4 receptor, the residues involved or the mechanism of phosphorylation.

1.5.1.11 Desensitization of GPCRs and the Dopamine D4 Receptor

Receptor desensitization is the processes that turns off or dampens the agonist-generated signal. This process involves phosphorylation of the receptor and interaction of the receptor with so called arrestin proteins. Sequestration or endocytosis, by internalization of the receptor, is believed to resensitize the phosphorylated receptor (Ferguson *et al.*, 1998). Desensitization may be classified as homologous or heterologous, dependent on the type of phosphorylation the receptor undergoes. Phosphorylation of the receptor contributes to desensitization, but the GPCR requires the binding of an “arresting” protein (arrestin) for its full inactivation (Ferguson *et al.*, 1998).

In the case of the β_2 -adrenergic receptor, β ARK1 phosphorylates serine residues in the carboxyl-terminal portion of the receptor and the receptor then becomes a substrate for the β -arrestin protein (Benovic *et al.*, 1991; Fredericks *et al.*, 1996; Lohse *et al.*, 1990b). β -Arrestin binding to the receptor results in the uncoupling of the receptor from the G protein, by binding to the receptor region implicated in G protein-coupling, and effectively “turns off” the receptor (Ferguson *et al.*, 1996a). β -arrestin belongs to a family of cytosolic proteins known as the arrestins. The arrestins comprise a family of six members, based on sequence homology and tissue distribution (Ferguson *et al.*, 1996a). The arrestins fall into four categories: visual arrestin, cone arrestin, β -arrestins (β -arrestin1 and β -arrestin2), and the uncharacterized D- and E-arrestins (Attramadal *et al.*, 1992; Craft *et al.*, 1994; Lohse *et al.*, 1990b; Yamaki *et al.*, 1987). β -arrestins have been shown to regulate a spectrum of GPCRs. Receptor desensitization and uncoupling are rapid processes (seconds to minutes) that occur

after receptor activation (Grady *et al.*, 1997).

Arrestins preferentially bind to GRK-phosphorylated receptors, as compared to second messenger-dependent phosphorylated or non-phosphorylated receptors (Lohse *et al.*, 1992; Lohse *et al.*, 1990b). The binding requirements of the arrestins for GPCRs have not been fully delineated. Neither the third intracellular loop nor the carboxyl-terminal tail are absolutely required for arrestin binding (Ferguson *et al.*, 1996b). The proposed model for arrestin binding involves several recognition domains (Gurevich *et al.*, 1995). Arrestin binds weakly to the non-activated, non-GRK phosphorylated receptor. Arrestin can independently bind either to a GRK-phosphorylated receptor through a phosphorylation recognition domain or to a ligand-activated receptor through an activation recognition domain, found in the first and third intracellular loops respectively (Krupnick and Benovic, 1998). Molecular characterization of the interaction between arrestin and the G_i-coupled receptors, such as the m2 muscarinic acetylcholine and α_2 -adrenergic receptors using GST-fusion constructs, demonstrate that arrestin binds to the third intracellular loop (Wu *et al.*, 1997). Simultaneous activation and GRK-phosphorylation of the receptor results in the strongest arrestin binding, allowing arrestin to bind to both recognition sites, which then induces a conformational change in the arrestin molecule that allows additional contact sites between arrestin and the receptor (Gurevich *et al.*, 1995). Full arrestin binding causes uncoupling of the receptor from the G protein by steric hindrance (Attramadal *et al.*, 1992; Freedman *et al.*, 1995; Inglese *et al.*, 1993; Lohse *et al.*, 1992; Lohse *et al.*, 1990b). The inactivated receptor no longer responds to agonist binding. Receptor endocytosis and “resensitization” appear to be necessary for the receptor to regain its function.

1.5.1.12 GPCR and Dopamine D4 Receptor Endocytosis

Certain activated GPCRs, after homologous desensitization and arrestin binding, are further regulated by endocytosis (also known as internalization or sequestration). The primary role of

sequestration might be to mediate receptor dephosphorylation and resensitization (Ferguson *et al.*, 1996a; Sibley *et al.*, 1986; Yu *et al.*, 1993). The receptor/arrestin complex is sequestered into endosomal vesicles by the mechanistic action of the GTPase dynamin as seen with the β_2 -adrenergic receptor (Goodman *et al.*, 1996; Zhang *et al.*, 1996), effectively reducing the number of receptors on the cell membrane. Putative conformational changes, possibly by lower pH, leads to the reversal of arrestin binding and receptor dephosphorylation by a protein phosphatase. The inactive and dephosphorylated receptor is presumed to be recirculated to the plasma membrane where once again it can respond to ligand binding (Ferguson *et al.*, 1996a).

Although phosphorylation is not a prerequisite for endocytosis, for the G_i -coupled receptors, including the m2 muscarinic receptor, it enhances sequestration (Moro *et al.*, 1993b). The highly conserved DRYXXV/IXXPL sequence, found to be important for G protein-coupling (Franke *et al.*, 1992; Scheer *et al.*, 1996; Zhu *et al.*, 1994), affects GPCR internalization, since mutation of the leucine residue interferes with endocytosis of the m1 muscarinic acetylcholine and the gonadotropin-releasing hormone receptors (GnRH) (Moro *et al.*, 1994). Arrestin acts as an adaptor protein, to enhance sequestration of GRK-phosphorylated receptors, by recruiting other cellular proteins required for the mobilization of receptors to endocytotic organelles (Ferguson *et al.*, 1996a). A similar process may also be involved in m2 muscarinic acetylcholine and dopamine D2 receptor endocytosis (Ferguson *et al.*, 1996a; Itokawa *et al.*, 1996; Tsuga *et al.*, 1994). Arrestin has been shown to interact with clathrin, a major protein component of the clathrin-based endocytotic mechanism (Goodman *et al.*, 1996). In this way arrestin binds the activated and phosphorylated GPCR and targets the receptor to the clathrin-coated pits by its direct interaction with clathrin. Although, arrestin-mediated endocytosis can account for m2 muscarinic internalization, it has been shown that receptor desensitization and arrestin binding are not required for internalization of this G_i -coupled receptor (Pals-Rylaarsdam *et al.*, 1997). Mutation of a serine/threonine cluster, in the third intracellular loop

of m2, to alanines leads to a receptor that is phosphorylated on another serine and threonine cluster in the third intracellular loop, but does not desensitize or bind arrestin, yet does internalize (Pals-Rylaarsdam *et al.*, 1997). Certain GPCRs, therefore, possess the ability to internalize using an arrestin and clathrin-independent mechanism (Pals-Rylaarsdam *et al.*, 1997), possibly by binding to a novel adaptor protein. The dopamine D2 receptor is phosphorylated and sequestered in response to agonist binding (Itokawa *et al.*, 1996; Ng *et al.*, 1994). If D2 endocytosis follows the same mechanism employed by other GPCRs, this is then likely mediated by GRK phosphorylation (of a serine or threonine residue in the third intracellular loop (Itokawa *et al.*, 1996)) and arrestin binding. GRK2 overexpression leads to increased endocytosis of both the D2 and D3 G_i-coupled receptors (Ferguson *et al.*, 1998). A recent report, however, demonstrates that the D1 and D2 receptors are sequestered by two distinct pathways into distinct endocytotic pools (Vickery and von Zastrow, 1999). D2, but not D1, can be internalized in a dynamin-independent mechanism (Vickery and von Zastrow, 1999) which raises the possibility that the D4 receptor may also be subject to a dynamin-independent mechanism of endocytosis. This remains to be elucidated.

Recent reports suggest that the GTPase dynamin is essential for receptor-mediated endocytosis (Sweitzer and Hinshaw, 1998). Arrestin-bound receptors are targeted to clathrin-coated pits. Dynamin, a mechanoprotein, is believed to pinch off the vesicle from the plasma membrane (Sweitzer and Hinshaw, 1998). Dynamin binds to adapter proteins involved in signal transduction, including Grb2 and PLC γ , and also with amphiphysin, an integral membrane protein required for dynamin-mediated endocytosis.

It is not known whether the dopamine D4 receptor undergoes GRK and arrestin-mediated endocytosis as described for the β_2 -adrenergic and m2 muscarinic acetylcholine receptors. The G_i-coupled dopamine D2 receptor has been shown to undergo sequestration (Itokawa *et al.*, 1996; Vickery and von Zastrow, 1999) that appears to be mediated by both a dynamin dependent and a

FIGURE 1.4 The mechanism of GPCR endocytosis for the β_2 -adrenergic receptor. The proteins and steps involved in receptor internalization, resensitization and recycling are illustrated. After receptor activation, GRK (G protein-coupled receptor kinase) is recruited to the receptor, by $G\beta\gamma$, and phosphorylates the receptor. Arrestin (Arr) binds to the phosphorylated receptor. Dynamin binds arrestin and the receptor complex is recruited to clathrin-coated pits and internalized. The receptor is resensitized and dephosphorylated by protein phosphatase 2A (PrP2A) (Fowles *et al.*, 1989; Palczewski *et al.*, 1989) and recycled to the membrane.

dynamin independent mechanism. It remains to be determined if the D4 receptor is subject to this regulation and what mechanisms are involved.

1.5.1.13 GPCR and Dopamine D4 Dimerization

Recent work has shown that certain GPCRs, including some members of the dopamine receptor family, undergo the process of oligomerization. Dimerization is a process that occurs among members of the tyrosine kinase receptor family, including the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors, and is thought to enhance agonist binding and signalling events (Hébert and Bouvier, 1998; Sorokin *et al.*, 1994; Yarden and Schlessinger, 1987a; Yarden and Schlessinger, 1987b). Two receptors of the same subtype interact with each other to form a homodimer. For GPCRs evidence for dimerization has come from cross-linking studies, immunoprecipitation experiments, chimeric studies and peptide interference studies (for review see Hébert and Bouvier, 1998). Visualization of some GPCRs separated on SDS-PAGE gel reveals molecular bands that correspond to the receptor as both monomers and dimers. In addition functional studies point to dimerization as being an important process in receptor activation and internalization (Cvejic and Devi, 1997; Hébert *et al.*, 1996). Peptides interfering with β_2 -adrenergic dimerization were also shown to reduce the ability of the β_2 to stimulate cAMP levels (Hébert *et al.*, 1996). The third intracellular loop, TM6 and TM7 have been shown to be important for the dimerization of GPCRs as derived from deletion mutant, chimera and peptide studies on the muscarinic, dopamine D2 and β_2 -adrenergic receptors (Hébert *et al.*, 1996; Maggio *et al.*, 1996; Ng *et al.*, 1996). For the δ opioid receptor interconversion from the dimeric to the monomeric form of the receptor is agonist dependent and required for receptor internalization (Cvejic and Devi, 1997). The dopamine D2 receptors have been shown to form homodimers (Ng *et al.*, 1996) and evidence from binding experiments suggests that the D4 may also form homodimers (Hidaka *et al.*, 1995), but this has not

been confirmed. Additional research needs to be done in order to establish the physiological relevance of this type of receptor interaction and the importance in signal transduction for GPCRs and the dopamine D4 receptor (Hébert and Bouvier, 1998).

1.5.1.14 GPCR and Dopamine D4 Receptor Activation of the MAPK Signalling Pathway

The function of GPCRs, in the past, has been mostly attributed to differentiated cell processes, including adenylyl cyclase, PLC and ion channel regulation. This view, however, has undergone a dramatic shift in the last several years. Functions usually associated with the RTK growth factor receptors in proliferating and differentiating cells, now also appear to be regulated by GPCRs (Luttrell *et al.*, 1997b). Several lines of evidence indicate that stimulation of GPCRs can lead to differentiation or proliferation of cells and that GPCRs have oncogenic potential (Gilman, 1987; Gutkind, 1998b; Johnson and Dhanasekaran, 1989). RTKs, such as the EGF, fibroblast growth factor (FGF), PDGF and the insulin-like growth factor (IGF) receptors, stimulate the Ras-MAPK pathway that leads to regulation in the nucleus. GPCRs are able to activate these same pathway at several entry points or activate different pathways that leads to mitogenesis (Luttrell *et al.*, 1997b). It now appears that the two major families of signalling receptors, GPCRs and RTKs, converge on this same signalling mechanism.

The RTKs, besides the GPCRs, represent the other major family of transmembrane signalling receptors that mediate extracellular signals into intracellular events (for review see Cano and Mahadevan, 1995; Cohen, 1997; Fantl *et al.*, 1993). RTKs are integral membrane proteins, that traverse the membrane once, have an extracellular ligand binding amino-terminal tail and a carboxyl-terminal intracellular tail. Agonist stimulation of RTKs activates distinct second messenger pathways that leads to such mitogenesis events as cell differentiation and proliferation. The most prominent and best studied RTK-activated pathway is the Ras-MAPK pathway, mediated by the extracellular signal-

regulated kinases (ERKs), which is activated by EGF, FGF, PDGF, and IGF receptors (Egan and Weinberg, 1993; Medema and Bos, 1993).

The Ras-MAPK pathway, like other RTK-stimulated pathways, is characterized by a series of direct protein-protein interactions, mediated by specific binding domains, and by protein phosphorylation steps. For the prototypical EGF receptor, agonist binding to the single transmembrane receptor leads to dimerization of the receptor and autophosphorylation of tyrosine residues on the intracellular tail (Smithgall, 1995). The cytoplasmic adapter protein Shc binds the receptor at the phosphorylated tyrosine site. The adapter protein, Grb2, binds to the Shc protein receptor complex, or may bind the receptor directly at the phosphorylated tyrosine site, and at the same time recruits the Ras-guanine-nucleotide exchange factor Sos1 to the plasma membrane (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1993). Sos1 catalyzes a GTP for GDP exchange on the small GTP binding protein Ras21, inducing Ras activation (Buday and Downward, 1993; Chardin *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993). In short, Ras activation leads to the activation of a phosphorylation cascade terminating in a mitogenic response mediated by nuclear events. Ras activates the serine/threonine kinase Raf (known as a MAP kinase kinase kinase or MAPKKK) through a direct interaction (Haung *et al.*, 1993; Kyriakis *et al.*, 1992; Moodie *et al.*, 1993). The activated Raf phosphorylates the multifunctional threonine/tyrosine kinase MEK (1 or 2) (MAPK/extracellular signal-regulated kinase, known as a MAP kinase kinase or MAPKK) (Haung *et al.*, 1993; Kyriakis *et al.*, 1992; Moodie *et al.*, 1993) which in turn phosphorylates the MAP kinases (the ERK gene products; p42mapk or p44mapk) (Ahn *et al.*, 1992). The MAP kinases are localized in the cytoplasm and nucleus where they act upon the transcriptional machinery of the cell, ultimately regulating gene expression. Mutation or constitutive activation of components in the Ras-MAPK pathway can lead to tumorigenesis and have been used to elucidate the transduction mechanism of this pathway (Aaronson and Tronick, 1985; Kraus *et al.*, 1988; Luttrell *et al.*, 1997b; Tronick and

Aaronson, 1988). In addition the MAP kinases regulate components upstream in the signalling pathway by phosphorylation.

MAPK signalling is characterized by the prototypical MAPK pathway that involves the ERKs kinase. There are at least three subfamilies of MAPK present in mammalian cells. Two parallel pathways with structurally related but clearly distinct kinases have been identified in addition to the ERKs: the Jun N-terminal kinases (JNKs) (Derijard *et al.*, 1994) (also named stress-activated protein kinases, SAPKs) (Kyriakis *et al.*, 1994) and the p38-MAPKs pathways (Lopez-Illasaca, 1998). Like the ERK MAPK pathway, these two pathways involve a linear cascade of three Ser/Thr kinases. These pathways are not so much involved in cell growth and differentiation, but more involved in the cellular response to environmental stress.

Early evidence for the possibility of GPCR involvement in cell proliferation and differentiation came from studies which showed that alterations in the GPCR signalling mechanism could lead to oncogenesis. Constitutive activation of specific components in the GPCR pathway for the serotonin 1C, the m1, m3 and m5 muscarinic and the α_{1B} adrenergic receptors, either by continuous ligand stimulation or by structural alterations, leads to a fully transformed phenotype in NIH3T3 cells (Allen *et al.*, 1991; Gutkind *et al.*, 1991; Julius *et al.*, 1989). The mas oncogene is a putative GPCR that demonstrates oncogenic potential (Young *et al.*, 1986). GTPase-inhibitory mutations of both $G\alpha_s$ and $G\alpha_i$, that causes constitutive activation of this subunit, have been found in a subset of endocrine tumors (Dhanasekaran *et al.*, 1995; Landis *et al.*, 1989; Lyons *et al.*, 1990). Constitutively active mutants of $G\alpha_i$, $G\alpha_q$, $G\alpha_o$, $G\alpha_{12}$ and $G\alpha_{13}$ all behave as transforming genes (Dhanasekaran *et al.*, 1995). Components of the RTK-activated Ras-MAPK pathway have long been shown to lead to transformation and have been implicated in oncogenesis (Aaronson and Tronick, 1985; Kraus *et al.*, 1988; Tronick and Aaronson, 1988). Constitutive activation of the GPCR signalling mechanism can also lead to cell transformation and oncogenesis, and provided early

evidence that GPCRs are involved in proliferation, and that this may be mediated through a RTK type mechanism.

More direct evidence that GPCR activation can lead to cell mitogenesis has come from a number of studies. Proliferation and differentiation were shown to be initiated by tropic hormones through a cAMP dependent pathway, implicating GPCR involvement (Dumont *et al.*, 1989). Specific components involved in the proliferative effect of GPCRs have been identified for the thrombin receptor, in its stimulation of DNA synthesis in CHO-K1 cells (Pouyssegur *et al.*, 1988). PTX blocked the proliferative response in these cells in response to thrombin stimulation, but not in response to the stimulation of the RTK PDGF receptor, thus implicating a G_i -mediated mechanism (Pouyssegur *et al.*, 1988). Similarly, the highly potent lysophosphatidic acid (LPA) receptor, and other G_i -mediated GPCRs, were shown to produce a mitogenic response which was blocked by the tyrosine kinase inhibitor genistein (van Biesen *et al.*, 1996; van Corven *et al.*, 1989). Using antibodies raised against specific $G\alpha$ subunits, serum-induced DNA synthesis by the thrombin receptor was blocked by $G\alpha_{12}$, $G\alpha_q$ and $G\alpha_{12}$ antibodies (Aragay *et al.*, 1995; LaMorte *et al.*, 1992; LaMorte *et al.*, 1993). A recent study has shown, using gene knock-out studies, that certain GPCRs are essential for cell growth under physiological conditions (Nagata *et al.*, 1996). Activation of GPCRs leads to rapid phosphorylation and activation of MAP kinases. This has been shown for the catecholamine, acetylcholine, adenosine, thyrotropin releasing hormone, luteinizing hormone, angiotensin, bombesin, endothelins, LPA and α -thrombin receptors (Dhanasekaran *et al.*, 1995; Faure *et al.*, 1994; Koch *et al.*, 1994; Ohmichi *et al.*, 1994). The G_i -coupled dopamine D2 receptor has also been shown to lead to cell proliferation and differentiation by means of the MAPK pathway (Faure *et al.*, 1994; Luo *et al.*, 1998). These results clearly demonstrated that ligands acting on GPCRs can elicit a mitogenic response (Rozengurt, 1986; van Biesen *et al.*, 1996).

Intensive research into the GPCR activation of the mitogenic pathways has made it clear that

the MAPK pathway is involved. Activation of these proliferative pathways has been shown to be both PTX-sensitive and -insensitive, mediated by both the G_{α} and $G_{\beta\gamma}$ subunits, to be Ca^{2+} -dependent and -independent, and to be PKC-dependent and -independent. Furthermore G proteins of the $G_{i/o}$, $G_{q/11}$, $G_{12/13}$ and G_s families can all lead to cellular transformation and MAPK pathway activation (Dhanasekaran *et al.*, 1995; Dhanasekaran *et al.*, 1998). GPCRs have been shown to be able to activate all three of the identified MAPK cascades, the ERKs, JNKs and p38-MAPKs (Coso *et al.*, 1995; Crespo *et al.*, 1994; van Biesen *et al.*, 1996). The activation of these pathways has been shown to occur at different entry points in these sequential cascades. Signal integration and concurrent stimulation of multiple receptors and pathways leads to distinct and specific cellular responses (Gutkind, 1998a). The present data suggest that a number of distinct mechanisms exist which mediate the mitogenic effects of GPCRs. The most prominent pathways are summarized below.

The GPCR mechanism that leads to the Ras-MAPK pathway (ERKs) activation closely resembling the RTK pathway has been best characterized and contributes most significantly to GPCR mitogenesis (van Biesen *et al.*, 1996). This pathway can be activated by the α_2 -adrenergic, LPA and dopamine D2 receptors (Luo *et al.*, 1998), is PTX sensitive and mediated by the $G_{\beta\gamma}$ subunits of the G_i -coupled receptors. G_i -coupled GPCR regulation of adenylyl cyclase and PLC β has been shown to be neither necessary nor sufficient to account for mitogenesis (Coughlin *et al.*, 1989; Mohammadi *et al.*, 1992). Constitutive activation of components in GPCR signalling upstream of the MAPK pathway is sufficient for tumorigenesis (Mansour *et al.*, 1994; Schlessinger, 1993). This pathway has been shown to be regulated at a number of entry points by a variety of GPCRs. In addition a number of distinct pathways are utilized to affect mitogenesis.

Elucidation of the pathways and components that lead to GPCR-activated mitogenesis have been thoroughly reviewed (Dhanasekaran *et al.*, 1998; Gutkind, 1998a; Gutkind, 1998b). The main components of the G_i -coupled GPCR activation of the MAPK pathway include a Src-like kinase that

links the GPCR signalling to the traditional RTK pathway. The pathway, as it is known at present, is described (also see Figure 1.5). In short, after receptor ligand binding and G_i coupling and activation, the free $G\beta\gamma$ subunit (Crespo *et al.*, 1994) interacts directly with the wortmannin-sensitive phosphatidylinositol 3-kinase (PI3-K) lipid (Lopez-Illasaca, 1998; Lopez-Illasaca *et al.* 1997; Stoyanov *et al.*, 1995). PI3-K interacts upstream of the non-receptor tyrosine kinase Src or a Src-like kinase (Lopez-Illasaca *et al.*, 1997). The exact kinases responsible for tyrosine protein phosphorylation

FIGURE 1.5 GPCR activation of the Ras-MAPK pathway. The steps involved in the activation of the MAPK pathway by G_i -coupled receptors is illustrated (see text for detail).

after GPCR stimulation are not known at present, but non-receptor tyrosine kinases such as Src, Fyn, Lyn, and Yes are believed to be involved (Chen *et al.*, 1994; Ptasznik *et al.*, 1995; Wan *et al.*, 1996) as well as the recently identified Ca^{2+} and PKC-dependent protein tyrosine kinase, Pyk2 (Della Rocca *et al.*, 1997). At this point the GPCR and RTK pathways converge on Shc. Shc is phosphorylated by the Src-like kinase resulting in the recruitment by Shc of the Grb2-Sos complex to the membrane (Luttrell *et al.*, 1996; Ohmichi *et al.*, 1994; van Biesen *et al.*, 1995). Sos binds to the membrane-bound small G protein, Ras, and activates the previously described Ras-MAPK pathway in a RTK-like manner by activating Raf. A dominant negative mutant of Ras has been shown to interfere with the $\text{G}\beta\gamma$ activation of the MAPK pathway (Hawes *et al.*, 1995).

G_s coupled receptors have been shown to both activate and inactivate the MAPK pathway in a PTX insensitive manner. In some cells the $\text{G}\beta\gamma$ of the G_s protein activates PI3-K and leads to MAPK activation by means of Src and Ras as described above (Dhanasekaran *et al.*, 1998). In most cells, however, the action of G_s , mediated through the $\text{G}\alpha_s$ subunit, inhibits GPCR activation of the MAPK pathway. G_s activation leads to adenylyl cyclase stimulation and increases in cAMP levels, resulting in PKA activation (Dhanasekaran *et al.*, 1998). It is still unclear, but PKA is believed to phosphorylate Raf at Ser 43 and/or Ser671, thus preventing its activation by Ras and not initiating the Ras-MAPK cascade (Burgering and Bos, 1995; Mischak *et al.*, 1996; Wu *et al.*, 1993). Evidence suggest, however, that the MAPK pathway can also be stimulated by G_s . Increases in cAMP levels have also been shown to lead to the activation of PKA that stimulates Rap1, Raf and then MAPK for the G_s -coupled β -adrenergic receptor in S49 mouse lymphoma and PC12 cells (Vossler *et al.*, 1997; Wan and Huang, 1998).

In endocrine tissues G_s -coupled proteins have been shown to stimulate mitogenesis in a MAPK and PTX- independent manner by coupling to the adenylyl cyclase pathway (Dumont *et al.*, 1989; Vallar, 1996). Adenylyl cyclase activation leads to increases in cAMP levels and activation of

PKA (Vallar, 1996). Persistent activation of PKA leads to the activation of the cAMP responsive element-binding protein (CREBP) and binding to the cAMP responsive elements (CREs) to transactivate transcription of specific genes involved in cell proliferation (Dhanasekaran *et al.*, 1998). G_s -stimulated proliferation occurs only in those cells that are positively responsive to the cAMP-PKA signalling pathway, as found in endocrine tissues (Dhanasekaran *et al.*, 1998). In other cells, G_{α_i} mediates an inhibitory effect as described above. Note that, although the $G\beta\gamma$ has been shown to be unequivocally involved in mitogenesis, to date only constitutive activation or mutations of G_{α} subunits have been found to lead to tumorigenesis, as seen for the *gsp* oncogene (the mutationally activated form of G_{α_s}) (Dhanasekaran *et al.*, 1998).

Activation of G_q -coupled receptors, including the α_{1B} adrenergic and m1 muscarinic receptors, appears to lead to Raf activation and MAPK signalling by its effect on PLC in a PTX insensitive manner (Hawes *et al.*, 1995). G_q activation of the MAPK pathway, unlike that of G_i , appears to be mediated by the G_{α} subunit. The G_{α_q} subunit directly interacts and activates PLC which then may lead to Raf activation by at least two mechanisms: 1) increases in IP_3 leads to an increase in intracellular Ca^{2+} and $Ca^{2+}/CaMKII$ (calcium/calmodulin-dependent protein kinase II), and possibly the activation of the Src-like Pyk2 protein, which then activates the Ras-MAPK pathway by means of the transactivation of RTKs that then leads to the classic MAPK response (Daub *et al.*, 1996; Della Rocca *et al.*, 1997; Faure *et al.*, 1994), 2) diacylglycerol activates PKC which then activates the MAPK pathway at the level of Raf (Dhanasekaran *et al.*, 1998; Faure *et al.*, 1994). The G_i -coupled LPA receptor, in some cells, including CHO and COS, also require PI3-K or IP_3 activity, stimulated by a $G\beta\gamma$ interaction, for MAPK activation (Daub *et al.*, 1996; Hawes *et al.*, 1996; Lopez-Illasaca *et al.*, 1997). PI3-K and PLC activation may lead to the transactivation of the RTKs and activation of the Ras-MAPK pathway in a similar manner as described above (Daub *et al.*, 1996).

GPCR stimulation leads to a distinct pattern of expression of immediate early genes of the *jun*

and fos family (Coso *et al.*, 1995). The m1 muscarinic, but not the PDGF receptor, in NIH3T3 cells leads to high expression of c-jun which is not associated with MAPK activation (Coso *et al.*, 1995). Details of this pathway are emerging and are believed to closely resemble those seen with MAPK. Activation of certain GPCRs, mediated through free $\beta\gamma$ (Coso *et al.*, 1996) or the $G\alpha_{12}$ (Prasad *et al.*, 1995) subunits, leads to the activation of the JNK pathway. JNKs/SAPKs are structurally related, but distinct from the prototypic ERK MAPK pathway (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994). These kinases phosphorylate the amino-terminal domain of the c-jun protein thereby increasing its transcriptional activity. It appears that Ras does not activate the JNK pathway. Rather, the small GTP-binding proteins Rac1 and Cdc42 related to Ras, and belonging to the Rho subfamily (one of six subfamilies belonging to the Ras superfamily) are involved in regulating JNK activity. Analogous to the Ras-Raf interaction seen in the MAPK pathway, a Rac1-Pak or Cdc42-Pak activation is postulated to lead to a kinase cascade regulating JNK activity (Manser *et al.*, 1994). Activation of Pak leads to the sequential interaction of the tyrosine kinases MEKK, JNKK, JNK and eventual regulation of c-jun (Gutkind, 1998a).

In summary, for the G_i -coupled receptors, a number of mechanisms have been shown to lead to proliferation and differentiation. It is likely that the G_i -coupled dopamine D4 receptor may utilize one such pathway in its stimulation of mitogenesis (Chio *et al.*, 1994). LPA and α_{2A} adrenergic receptor stimulation and release of the free $G\beta\gamma$ leads to the activation of c-Src and the Src-kinase dependent activation of Shc, followed by Grb2-Sos recruitment and the activation of Sos (Luttrell *et al.*, 1996). However, another group has shown that Src and Shc are not necessarily involved in the LPA activation of the Ras-MAPK pathway, but have identified a 100 kDa protein that interacts with Grb2 and leads to Ras activation (Kraneneburg *et al.*, 1997). Activation of the Src-kinase by $G\beta\gamma$ may lead to the transactivation of the RTKs, or activation of Shc or focal adhesion kinase (FAK) which then leads to the recruitment of the Grb2-Sos heterodimer to the RTK resulting in Ras activation

(Luttrell *et al.*, 1997c). As described, stimulation of G_i -coupled receptors can lead to mitogenesis by means of the activation of the MAPK signalling pathway or through a G_{α_q} and PLC-mediated process. To date, this has been shown to be mediated by a MAPK (ERK) or JNK/SAPK for a number of GPCRs, including the G_q -coupled m1 muscarinic receptor (Kyriakis *et al.*, 1994). The LPA receptor, that is both G_i and G_q -coupled, activates the calcium dependent FAK family tyrosine kinase, Pyk2. Recently, it has been shown that the dopamine D2 receptor can also activate both of these pathways (Luo *et al.*, 1998).

Although it has been known that the β_2 -adrenergic receptor couples to G_s and activates adenylyl cyclase that leads to PKA activation, a study with surprising results showed that the receptor could switch to G_i coupling and activate the Ras-MAPK pathway after PKA phosphorylation of the receptor (Daaka *et al.*, 1997). This indicates that for the β_2 -adrenergic, and perhaps for other G_s -coupled GPCRs, activation of the Ras-MAPK pathway occurs in an identical manner to that seen for other G_i -coupled GPCRs.

β_2 -adrenergic and LPA receptor activation of the MAPK pathway appears to require endocytosis of the receptor. Dominant negative suppressor mutants of β -arrestin and dynamin, that have been shown to inhibit endocytosis, also inhibit MAP kinase activation at the level of Raf in HEK-293 cells (Daaka *et al.*, 1998; Luttrell *et al.*, 1997a). These results suggest that GPCR endocytosis is a necessary step in the activation of the Ras-MAPK pathway for at least some GPCRs. It is thought, that like the RTKs, the receptor forms a multiprotein complex which includes Raf and that Raf internalization is required for it to activate the MAPK pathway (Daaka *et al.*, 1998). In fact for the IGF receptor, and other RTKs, β -arrestin binding is also required for the endocytosis of the RTK receptor (Lin *et al.*, 1998). Furthermore, arrestin-mediated endocytosis enhances mitogenic signalling (Lin *et al.*, 1998). A very recent study has demonstrated that the non-receptor tyrosine kinase Src binds to the arrestin protein through its SH3 domain (Luttrell *et al.*, 1999). Impaired cSrc

binding to arrestin interferes with endocytosis of the β_2 -adrenergic receptor and MAPK activation (Luttrell *et al.*, 1999). Thus, for GPCRs, arrestin binding desensitizes the receptor and attenuates certain types of signalling, but enhances endocytosis, by targeting the receptor to clathrin-coated vesicles which can enhance mitogenic signalling.

1.5.2 SH3 and Other Modular Binding Domains in Signalling and Endocytosis

1.5.2.1 Modular Binding Domains

Some of the protein-protein interactions crucial in RTK and GPCR signalling as well as in receptor endocytosis are mediated by modular binding domains (for review see Cohen *et al.*, 1995; Dalgarno *et al.*, 1997; Pawson, 1995; Pawson and Schlessinger, 1993; Williams *et al.*, 1998). These domains are small (between 50-110 amino acids), non-catalytic, and structurally-conserved units that recognize and bind to specific amino acid motifs in a non-covalent manner. They make up part of the protein, yet they can maintain their structure and function in isolation of the full length protein. Structurally and functionally unrelated proteins may use the same modular binding domains to mediate specific protein-protein interactions. To date a number of distinct modular binding domain structures have been identified and include the SH2, SH3, PH, WW, PTB, and PTZ domains (Pawson and Scott, 1997). Proteins may be composed of one or a number of these binding domains and may contain multiple domains of the same type.

The SH2 and SH3 domains were first discovered as homology domains in the tyrosine kinase product of the v-Src (*Rous sarcoma virus*) oncogene along with the catalytic tyrosine kinase domain SH1 (Koch *et al.*, 1991). It was soon discovered that these domains could be found in a number of unrelated proteins. In RTK signalling they were found to be important in mediating interactions with the receptor and intermediary proteins that lead to Ras-MAPK signalling. SH2 domains are conserved regions of about 100 amino acids in length. They recognize and bind to phosphorylated tyrosines on

FIGURE 1.6 Schematic of several SH2 and SH3 domain containing proteins. The Grb2, Nck and Crk adapter proteins are depicted. These proteins contain no catalytic domains and are composed of SH2 and SH3 domains.

the target protein although exceptions to this have been found (see Vihinen and Smith, 1998). The three amino acids immediately carboxyl-terminal to the phosphorylated tyrosine determine, for the most part, selectivity of binding. Affinity for this type of interaction is relatively high, with a dissociation constant (K_d) of about 20-100 nM. More than 60 SH3 domains are known (for review see Dalgarno *et al.*, 1997). SH3 domains have been identified in kinases, lipases, GTPases, structural proteins, viral regulatory proteins and adapter proteins. SH3 domains are about 60 amino acids in length and recognize proline-rich sequences that induce a left-handed polyproline type II helix conformation, with few exceptions containing in its core a PXXP motif.

PH domains have been found to be important in GPCR signalling. The interaction between the G $\beta\gamma$ dimer and β ARKs is mediated by PH domain (Touhara *et al.*, 1994; Touhara *et al.*, 1995). The characteristics of PH domains are less well understood than SH2 and SH3 domains. They are regions of about 110 amino acids found in signalling and cytoskeletal proteins. In general, related PH domains have low sequence homology (21-25% identity), but contain a striking degree of structural similarity (Scaife and Margolis, 1997). The structure is composed of orthogonal β sheets around a central hydrophobic cleft and α helix (Ferguson *et al.*, 1994). The carboxyl-terminal portion of the PH domain and several amino acid residues outside of the PH domain are involved in β ARK interactions (Touhara *et al.*, 1994). In addition to protein-protein interactions, PH domains are also

believed to function in protein-lipid interactions (Harlan *et al.*, 1994).

The WW domain is a globular structure of 35–40 amino acids (Bork and Sudol, 1994). Its structure is made up out of a three-stranded antiparallel β -sheet. Two conserved tryptophans (WW) are 20–22 amino acids apart (Bork and Sudol, 1994). A hydrophobic pocket, composed of a leucine, tyrosine and the second tryptophan, make up the ligand binding interface (Sudol, 1996a; Sudol, 1996b). The WW domain recognizes proline-rich regions with a xPPxY binding sequence (Sudol, 1996a). The tyrosine residue is required for WW domain binding. Several identified ligands have been identified and contain the amino acid sequence PPPPY . The possibility of WW and SH3 domains competing for the same ligand has been proposed, but requires a tyrosine in the SH3 binding sequence. The WW domain may thus regulate the SH3 interaction (Ahn and Kunkel, 1993; Nguyen *et al.*, 1998). To date, the WW domain has not been implicated in GPCR function.

1.5.2.2 Modular Binding Domains in RTK and GPCR Cell Signalling and Endocytosis

SH3 interactions have been found to be involved in regulating enzymatic activity, in mediating interactions between proteins, protein recruitment, cellular localization and receptor-mediated endocytosis, for both RTK and GPCR signalling pathways and in the interaction with viral proteins (for review see Gutkind, 1998a; Selbie and Hill, 1998).

SH2 and SH3 domains are involved in several critical steps in RTK and GPCR activation of the Ras-MAPK pathway. Disruption of these interactions, either by mutation of residues in the binding site or in the binding domain, leads to attenuation or abolition of MAPK signalling (Pawson, 1995). The interaction of Shc with the RTK, such as the EGF receptor, is mediated by the SH2 domain of Shc, that binds to a phosphorylated tyrosine on the activated receptor (Rozakis-Adcock *et al.*, 1992). Grb2 is an adaptor protein that has no catalytic activity and contains one SH2 domain flanked by two SH3 domains (Lowenstein *et al.*, 1992). Grb2 either binds directly to the

phosphorylated RTK (Lowenstein *et al.*, 1992) or to the phosphorylated Shc protein by means of its SH2 domain (Rozakis-Adcock *et al.*, 1992). Some RTKs, like the PDGF receptor, contain multiple autophosphorylation sites which can each bind a specific SH2 domain (Valius and Kazlauskas, 1993). The interaction of Grb2 with the guanine nucleotide exchange factor Sos is mediated by its SH3 domains which binds to a PXXP motif in the carboxyl-terminal region of Sos (Lowenstein *et al.*, 1992). Phosphorylation of Sos by MAP kinases negatively modulates the Grb2-Sos interaction leading to its dissociation and/or preventing its association (Corbalan-Garcia *et al.*, 1996).

In GPCR signalling, SH2 and SH3 domain interactions are important in linking the signal from the activated GPCR to the small G protein Ras, in Ras-MAPK signalling. PI3-K, Grb2, Src, Shc and Sos, all implicated in GPCR signalling, utilize SH2 and SH3 interactions to mediate protein-protein interactions (Lopez-Illasaca *et al.*, 1997; Luttrell *et al.*, 1996). Unlike in RTK signalling, no direct SH2 or SH3 domain interaction with the GPCR have been identified that is required for Ras-MAPK activation. These interactions, however, are important for signalling events downstream from the $G\alpha$ and $G\beta\gamma$ subunits. The Shc interaction with the non-receptor tyrosine kinase, Src or Src-like proteins, is mediated by SH2 interactions (Luttrell *et al.*, 1996). The interaction between Shc, Grb2 and Sos are mediated by the same SH2 and SH3 interactions as those seen in RTK signalling (Chen *et al.*, 1996b; van Biesen *et al.*, 1996).

Aspects of clathrin-mediated endocytosis, important in GPCR and RTK internalization, involve SH3 interactions. The GTPase mechanoprotein dynamin, that is important in pinching off the clathrin-coated vesicle from the membrane surface (Sweitzer and Hinshaw, 1998), contains several sites for SH3 binding (Scaife and Margolis, 1997). To date three dynamin genes have been isolated: one encoded for exclusively in neuronal tissues, the other found both in neuronal and testis or just in the periphery (Scaife and Margolis, 1997). Dynamin may also be involved in signal transduction (Urrutia *et al.*, 1997). Dynamin has a proline-rich region that contains several SH3 binding motifs in

the carboxyl-terminal portion of the protein that are able to bind to distinct subsets of SH3 domain containing proteins (Scaife and Margolis, 1997). Strong dynamin interactions with Grb2, PLC γ , the p85 subunit of PI3-K have been reported (Gout *et al.*, 1993; Scaife *et al.*, 1994; Seedorf *et al.*, 1994). Dynamin is activated by PKC phosphorylation and SH3 interactions with dynamin increase its GTPase activity, thus helping to regulate its activity (Herskovits *et al.*, 1993; Robinson *et al.*, 1993).

In RTK signalling, Grb2 interacts with dynamin and has been shown to recruit dynamin to the activated EGF (Seedorf *et al.*, 1994) and PDGF receptors (Scaife *et al.*, 1994), or to receptor-associated proteins such as the insulin receptor substrate (IRS-1) (Ando *et al.*, 1994) or FAK (Kharbanda *et al.*, 1995) by means of SH2 and SH3 interactions. It has been demonstrated that Grb2 is required for EGF receptor endocytosis (Wang and Moran, 1996).

Src and the Src-like kinases, classified as non-receptor tyrosine kinases, are required for GPCR-mediated activation of Ras. This family includes Src, Yes, Fgr, Yrk, Fyn, Lyn, Hck, Lck and Blk proteins. Stimulation of Src, Yes, Fyn and Lyn have been shown to occur after the stimulation of several GPCRs (Chen *et al.*, 1994; Luttrell *et al.*, 1996; Ptasznik *et al.*, 1995). They contain an SH3 domain, and an SH2 domain followed by a catalytic domain on the carboxyl-terminal portion of the protein. Kinase activity is reduced when a conserved tyrosine residue (Tyr52 in Src) is phosphorylated and bound by the SH2 domain. Regulation of the protein is mediated by the disruption of this interaction (Alexandropoulos and Baltimore, 1996). A very recent report has shown that an SH3-mediated interaction of c-Src with the β -arrestin molecule, important for GPCR desensitization and endocytosis, is critical for GPCR activation of the Ras-MAPK pathway (Luttrell *et al.*, 1999). Arrestin binds directly to the GPCR and targets the receptor to clathrin-coated pits (Ferguson *et al.*, 1996b; Goodman *et al.*, 1996). An interaction between the SH3 domain of c-Src and an amino-terminal SH3 binding motif in arrestin, brings this non-receptor tyrosine kinase to the receptor complex (Luttrell *et al.*, 1999).

The SH3 domain containing integral membrane protein amphiphysin (amph-2) binds dynamin, recruiting it to the membrane (David *et al.*, 1996; Grabs *et al.*, 1997; Lichte *et al.*, 1992). In addition amphiphysin is able to bind to synaptojanin, an enzyme with 5' phosphatase activity, by means of its SH3 domain (Micheva *et al.*, 1997; Ramjaun *et al.*, 1997). Synaptojanin is believed to regulate amphiphysin-dynamin binding by interfering with this interaction and preventing the recruitment of dynamin to the membrane (Wigge and McMahon, 1998). Amphiphysin is predominantly found in synaptic vesicles in the brain. The interaction of amphiphysin with dynamin and synaptojanin is mediated by SH3 interactions, which appears to be very specific for dynamin and synaptojanin, since few other proteins associate with amphiphysin *in vivo* (David *et al.*, 1996; Wigge and McMahon, 1998). A dominant negative amphiphysin, with a mutation in the SH3 domain, prevents amphiphysin binding to dynamin and clathrin-mediated endocytosis (Shupliakov *et al.*, 1997). It appears that amphiphysin is recruited to the clathrin-coated pit by its interaction with clathrin (McMahon *et al.*, 1997). This interaction, although not SH3 mediated and occurring at non-overlapping binding sites, can nonetheless be disrupted by dynamin (McMahon *et al.*, 1997). Expression of only the amphiphysin SH3 domain, but not the SH3 domains of Grb2, PLC γ or spectrin, interfered specifically with endocytosis in COS-7 cells (Wigge *et al.*, 1997). Amphiphysin is required for the GTPase activity of dynamin. It is clear that for GPCR functioning, SH3-mediated interactions play an essential role in its regulation.

The PH modular binding domain has been implicated in the phosphorylation of GPCRs by certain GRKs. β ARK1 and β ARK2 both contain a PH domain that makes up part of the G $\beta\gamma$ binding site (Touhara *et al.*, 1994; Touhara *et al.*, 1995). β ARK interaction with G $\beta\gamma$ targets the kinase to the membrane and GPCR, thus promoting phosphorylation of the receptor. The presence of a lipid cofactor, such as PIP₂, that with G $\beta\gamma$ concurrently binds to the PH domain of β ARK, is required to promote full phosphorylation of the GPCR (DebBurman *et al.*, 1996; Pitcher *et al.*, 1996; Touhara

et al., 1995). Interfering with PH binding results in a disruption of the G β γ / β ARK interaction and results in a reduction in homologous phosphorylation. The dynamin mechanoprotein, required for some clathrin-mediated endocytosis, also contains a PH domain (Scaife and Margolis, 1997). PLC (γ and δ), found to be important in GPCR-mediated signalling of G $_q$ -coupled receptors also contains a PH domain (Scaife and Margolis, 1997). Since studies have shown that phosphorylation of the GPCR receptor enhances endocytosis (Moro *et al.*, 1993b) and that endocytosis has been shown to be required for GPCR activation of the Ras-MAPK pathway (Daaka *et al.*, 1998), disruption of the PH domain in both β ARK and dynamin would be expected to interfere with this GPCR function. In addition, disruption of the PH domain in PLC, which mediates an alternate pathway of GPCR activated mitogenesis (Hawes *et al.*, 1995), would interfere with inositol phospholipid binding (Harlan *et al.*, 1994) and mitogenesis. The guanine nucleotide exchange factor Sos, that participates both in RTK and GPCR signalling, also contains a PH domain important in its regulation (McCollam *et al.*, 1995).

The conserved modular binding domains, such as the SH2, SH3 and PH domains, are important in a number of critical steps in both RTK and GPCR signalling. Nature has used these conserved modules to mediate similar interactions in a diverse array of proteins. Furthermore, these protein interactions allow for the cross regulation of pathways to allow for precise signal integration and very specific cellular responses.

1.5.3 SH3 Domains in the Dopamine D4 Receptor

1.5.3.1 SH3 binding motifs in the Dopamine D4 receptor and other GPCRs

Although the importance of modular binding domains, in particular the SH2, SH3 and PH domains, has been well established in GPCR signalling, these interactions have been shown to occur downstream of the G protein, or as in the case of the c-Src interaction with the GPCR, mediated by

an intermediary protein such as arrestin (Luttrell *et al.*, 1999). To date, a number of direct protein-protein interactions have been demonstrated for GPCRs, including G proteins, arrestins and a number of kinases. Only a few examples exist of direct protein interactions with GPCRs mediated by modular binding domains. This includes the SH2 mediated interaction of Shp2 with the angiotensin AT₁ receptor (Marrero *et al.*, 1998) and the interaction of the Na⁺/H⁺ exchanger regulatory factor (NHERF) by means of its PDZ domain with the carboxyl-terminal tail of the β₂-adrenergic receptor (Hall *et al.*, 1998). No direct SH3 mediated interaction with the GPCRs have as of yet been identified. This is in contrast to the RTKs, which rely extensively on these direct interactions for both signalling and endocytosis. The GPCRs employ and share many components of the RTK signalling mechanism. It seems plausible that direct binding of SH2 and SH3 domains with the GPCR may have evolved in regulating some of the GPCRs effects.

The third intracellular loop and carboxyl-terminal domain of most GPCRs are long intracellular sequences that contain potential sites for tyrosine phosphorylation and proline-rich areas which could serve as SH2 or SH3 binding motifs respectively. For the dopamine D4 receptor, the third intracellular loop and the polymorphic repeat region within the loop is extremely proline rich. Proline rich regions like that found in the dopamine D4 receptor have been suggested to be SH3 binding sites. In particular the dopamine D4 receptor contains multiple PXXP sequences, found to constitute the majority of SH3 binding sites. These PXXP motifs occur in about 50% of the surveyed GPCRs.

The dopamine D4 receptor, although this may not be the case for other GPCRs, is not a likely candidate for the other types of identified modular binding domains. SH2 domains require a phosphorylated tyrosine residue for binding. The dopamine D4 receptor contains no tyrosine residues in the third intracellular loop or the carboxyl-terminal tail to mediate this interactions. WW domains bind to proline-rich regions, and have been reported in some cases to bind to the same sites as SH3

domains (Sudol, 1996b). WW domains, however, also require a tyrosine residue in their recognition motif (PpXY) (Sudol, 1996b). Although, the possibility of direct protein interaction with the dopamine D4 receptor utilizing other modular protein binding domains cannot be excluded, an SH3 mediated protein interaction with the dopamine D4 receptor appears to be a possible and potential interaction.

1.5.3.2 SH3 Domains and SH3 Binding Domains

In addition to identifying important intracellular events mediated by modular binding domains, such as the SH3 domain, a great deal of research has been completed in characterizing specific modular binding domains, their binding motifs and the factors that affect the strength of their interaction. For SH3-mediated interactions, much of the research evolved around identifying peptide sequences that bind to SH3 domains (Lemmon *et al.*, 1994; Lim *et al.*, 1994b; Pisabarro and Serrano, 1996; Pisabarro *et al.*, 1998; Posem *et al.*, 1998; Ren *et al.*, 1993; Sparks *et al.*, 1994; Sparks *et al.*, 1996; Yu *et al.*, 1994). These studies provided insights into the binding requirements and strength of protein interaction, but may also lead to peptide drugs or drugs that mimic the structure of SH3 binding motif that could be used to pharmacologically manipulate SH3-mediated events. These include such diseases as AIDS, asthma, cancer inflammatory disease, leukemia and osteoporosis.

SH3 domains are conserved regions of about 55-70 amino acids. The structure of SH3 domains has been resolved by nmr spectroscopy and x-ray crystallography in the absence of ligand or complexed with either peptide ligands or protein ligands. The basic structure of the SH3 domains is a five antiparallel stranded β -sandwich that form two perpendicular β sheets (Feng *et al.*, 1994; Yu *et al.*, 1994). In addition to these β -strands, a charged RT (Arg-Thr) loop and a Src loop are common elements. Different SH3 domains differ in their secondary structure, but generally are configured to bring conserved residues into proximity of each other to form an aromatic patch which forms the

ligand binding site (see Dalgarno *et al.*, 1997). Little structural alterations upon ligand binding occurs. Several SH3 binding domains may occur in the same protein. This can allow multiple, simultaneous interactions, thus forming a cluster of several proteins.

The interaction between the SH3 domain and SH3 ligand is hydrophobic in nature. SH3 domains recognize and bind to proline-rich regions in the target protein. With very few exceptions they recognize a minimal PXXP motif where the prolines are absolutely conserved and help to form a left-handed polyproline type II helix. A sequence of 7 to 10 amino acids is absolutely required and shows the most conservation for binding and helps to determine some of its affinity for SH3 domains (Dalgarno *et al.*, 1997; Ren *et al.*, 1993). Additional sequences flanking the core binding sequence provide additional specificity and affinity for the interaction. Although the flanking region is less well conserved than the core sequence, it helps to increase the overall binding affinity (Dalgarno *et al.*, 1997; Feng *et al.*, 1995). This makes prediction of interactions more difficult. The dopamine D4 receptor, as well as a large number of other GPCRs is proline rich in the third cytoplasmic loop, making these receptors potential candidates for SH3 binding.

Ligand binding to SH3 binding domains can occur in two orientations. The short left-handed polyproline type II helical conformation (Adzhubei and Steinberg, 1993) has a certain amount of symmetry that allows it to bind ligands in two orientations, which are classified as Class I or II depending on the amino- and carboxyl-orientation of the protein (Feng *et al.*, 1994; Feng *et al.*, 1995; Yu *et al.*, 1994). In general the position of an Arginine (R) residue (amino or carboxyl) determines if a ligand is class I or II (RXLPPLP is class I, XPPLPXR is class II). A class I ligand binds with its amino terminus at the RT loop, while a class II ligand binds with its carboxyl-terminus at the RT loop. Specificity comes from the flanking region which is found on opposite sides of the core sequence for class I and II SH3 ligands.

A complete turn of the helix requires three residues (Adzhubei and Steinberg, 1993). The

protein sequence of the binding motif takes on a triangular configuration, of which two sides are in contact with the SH3 domain and the third side stabilizes the PPII helix (Pawson and Schlessinger, 1993). For class I and II ligands the XPpXP motif describes the core sequence. The capital P denotes a proline that is highly conserved and contacts the SH3 domain, the lower case p is less conserved and acts as a scaffolding residue promoting the PPII conformation. The two prolines denoted by capital P are residues found on the same plane.

FIGURE 1.7 Class I and II ligands. The binding properties of class I and class II binding ligands are illustrated to the SH3 domain (c-Src). An arginine (R) located amino-terminal to the PXXP sequence or carboxyl-terminal to the PXXP sequence determines whether the ligand is class I or class II respectively. The conserved c-Src amino acids in the SH3 binding pocket are shown in the ovals.

Several examples of non-PXXP-mediated SH3 interactions have been identified. The most notable being the intramolecular interaction of the Src SH3 domain to the upper lobe of the kinase (Gorina and Pavletich, 1996; Sicheri *et al.*, 1997; Xu *et al.*, 1997). The SH3 domain binds within a linker region that links the SH2 domain to the catalytic domain (Pisabarro *et al.*, 1998). Although the SH3 binding site does not contain the prolines normally seen, the binding site still takes on a PPII type conformation which is sufficient for binding. SH3 binding has been shown to occur in a few cases where the typical PXXP motif was not present, but the left-handed polyproline helix conformation was nevertheless adopted (Adzhubei and Steinberg, 1993; Sicheri *et al.*, 1997; Xu *et al.*, 1997).

In contrast to SH2 binding, phosphorylation of serines or threonines adjacent to the proline-

rich core sequence of the SH3 binding site may reduce SH3 interactions (Chen *et al.*, 1996a). Phosphorylation of these sites can either prevent interactions or promote dissociation (Chen *et al.*, 1996a). Although these phosphorylation sites may disrupt and regulate SH3 interactions, they are not a requirement for SH3 binding sites or SH3 binding.

In general, these factors are required for the SH3 ligand binding of short peptide sequences: a high proline content to promote the PPII conformation, complementary interactions between noncore sequences, and optimized protein-ligand interactions with the proline-rich core. Since these ligands tend to be highly conserved in the core region even between different SH3 domains, there is little selectivity of binding. Binding affinity of these short peptide sequences ranges between 5 to 100 μ M (Dalgarno *et al.*, 1997). Additional flanking sequences increase both selectivity and affinity. Although less well conserved, these flanking sequences also show consensus sequences, but these have not been well characterized. Full strength interaction and selectivity are demonstrated by full ligand-SH3 interactions. These interactions can be as strong as 0.25 μ M (Lee *et al.*, 1995). Secondary interactions within the variable RT loop help to determine SH3 ligand affinity and selectivity, increasing interaction strength synergistically (see Dalgarno *et al.*, 1997).

1.6 Summary

The dopamine receptor belongs to the super-family of GPCRs. These receptors demonstrate a complex and sophisticated mechanism of signalling. Where originally considered separate of RTK signalling, it is now clear that these two pathways employ many of the same mechanisms and proteins to elicit specific signalling. Modular protein binding domains have been shown to be essential for both GPCR and RTK signalling. In addition, these modules mediate protein-protein interactions not only in signalling, but in such receptor regulating events as endocytosis. In fact, it has become clear that receptor endocytosis and Ras-MAPK signalling are intimately linked for PTX-sensitive activation

of Ras. Some direct protein interactions with RTKs are mediated by the modular binding domains. In the case of GPCRs, however, the identified modular protein interactions occur with an intermediary protein that binds to the receptor. The dopamine D4 receptor is extremely proline rich, especially in a unique highly polymorphic region of the third intracellular loop. Numerous PXXP sequences exist that have been shown to form the core SH3 domain binding site. The D4 receptor appears to be a candidate for SH3 binding. This type of binding may be important for signalling events, regulation of the receptor or required for creating specific protein complexes required for either signalling or processes such as endocytosis. If the dopamine D4 receptor utilized SH3 interactions, then it is likely that other GPCRs have “adopted” this mechanism for their function.

1.7 Thesis Overview

The thesis is presented as a series of experiments, divided into individual chapters, according to the research objective of those particular experiments. A general “methods” section precedes these chapters. A brief introduction, describing information about the procedures employed and other relevant material is presented before the methods specific to the experiment. A combined results and discussion section at the end of each chapter details specific aspects and conclusions of the set of experiments. An amalgamated discussion and future research are presented in the final chapters incorporating the results of all the chapters.

Chapter 2

General Materials and Methods

2.1 Materials

2.1.1 General Chemicals

All common chemicals and reagents, unless otherwise specified, were purchased from Sigma Chemicals Co. (St. Louis, MO), Aldrich Chemical Company Inc. (Milwaukee, WI) or B.D.H. Chemicals Ltd. (Toronto, ON). Bovine serum, albumin, IGEPAL CA-630 (NP-40), polyxyethylenesorbitan monolaurate (Tween-20), Triton X-100, diethiothreitol (DTT), potassium 2-(N-morpholino]ethanesulfonic acid (KMES), sodium azide, sodium orthovanadate, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',-N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), CHAPS, digitonin and N,N,N,N-tetramethyl-ethylenediamine (TEMED) were obtained from Sigma Chemical Co. (St. Louis, MO).

Chloroform and monobasic potassium phosphate were obtained from Mallinckrodt Chemicals Ltd. (St. Louis, MO). β -Mercaptoethanol and sodium dodecyl sulphate (SDS) were purchased from Schwarz/Mann Biotechnology (ICN, Aurora, IL). Ethanol and methanol were purchased from Commercial Alcohols Inc. (Brampton, ON). Potassium acetate and magnesium acetate were purchased from J. T. Baker Chemicals (Toronto, ON). Diethylpyrocarbonate (DEPC), ethidium bromide, N-[2-hydroxyethyl]piperazine-N'-[2-thanesulfonic acid] (HEPES) and Tris (hydroxymethyl) aminomethane (Tris) were purchased from Gibco (Burlington, ON). 5-bromo-4-chloro-3-idolyl- β -D-galactoside (X-gal) was purchased from Molecular Probes Inc. (Eugene, OR). Ammonium persulfate, hydrochloric acid, and dimethylsulfoxide (DMSO) were obtained from Caledon Laboratories (Georgetown, ON). Glycerol, calcium chloride, isopropyl alcohol, dibasic potassium phosphate,

chloroform and glacial acetic acid were acquired from ACP Chemicals Inc. (Montreal, PQ).

2.1.2 Bacterial and Tissue Culture

Bacto tryptone, bacto peptone agar and yeast extract were purchased from Difco Laboratories (Detroit, MI). Ampicillin, tetracycline and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, MO)

α -Minimum essential media (α -MEM), Ham's F10 media and Dulbecco's modified Eagle's medium were obtained from Gibco BRL Ltd. (Grand Island, NY) and Central Media Preparation Service (University of Toronto, ON). Optimem, fetal bovine serum (FBS), horse serum (HS), Geneticin™ (G418 sulfate), lipofectin, and trypsin were obtained from Gibco BRL Ltd. (Grand Island, NY). All tissue culture supplies were purchased from Nunc (Roskilde, Denmark), Dupont NEN Life Science Products (Mississauga, ON), Corning (Corning, NY), Fisher Scientific (Fair Lawn, NJ), Sarstedt Inc. (Newton, NC) or Beckton Dickinson Labware (Lincoln Park, NJ).

2.1.3 Vectors and Strains

The pBluescript SKII (-) and pAlter Ex-1 vectors were purchased from Stratagene (La Jolla, CA). PGEX-2T and PGEX-3X were obtained from Pharmacia Biotech AB (Uppsala, Sweden). The vectors pcDNA3 and pRc/RSV were purchased from Invitrogen (San Diego, CA). The yeast two-hybrid vectors pAS I and pACT II were a gift of Dr. T. Pawson (University of Toronto, Toronto, ON). The PXCJL-2 and PJM17 vectors were a gift from Dr. F. Graham (McMaster University, Hamilton, ON).

Saccharomyces cerevisiae strains Y-153 and Y-187 were a gift from Dr. T. Pawson (University of Toronto, Toronto, ON). Chinese hamster ovary cells (CHO K1), COS-1, COS7, HEK-293, SKNMC. were obtained from the American Type Culture Collection (Rockville, MD). The

Escherichia coli (*E. coli*) XL-1, ES1301 mutS and JM109 strains were purchased from Stratagene (La Jolla, CA). *E. coli* BL-21 was obtained from Pharmacia Biotech AB (Uppsala, Sweden).

2.1.3 Molecular Biology

SeaKem (high melt) and SeaPlaque (low melt) agarose were purchased from FMC Bioproducts (Rockland, ME). Acrylamide was obtained from Sangon Ltd. (Toronto, ON). N,N'-methylene-bis-acrylamide and urea was obtained from Biobasic Inc. (Toronto, ON). Bromophenol blue and xylene cyanol FF were obtained from Sigma Chemical Co. (St. Louis, MO).

Standard restriction endonucleases, unless specifically detailed, were purchased from New England Biolabs (Beverly, MA), Gibco BRL Ltd.(Grand Island, NY), Pharmacia (Baie d'Urfé, PQ) or Boehringer Mannheim (Laval, PQ). T7 polymerase, Klenow fragment of DNA polymerase I, calf intestinal phosphatase (CIP) and T4 DNA ligase were obtained from Gibco BRL Ltd.(Grand Island, NY). Mung Bean was purchased from New England Biolabs (Beverly, MA). Sequenase v2.0 was purchased from Amersham (Oakville, ON).

Adenosine triphosphate (ATP) was purchased from Boehringer Mannheim (Laval, PQ) and dNTPs (dATP, dCTP, dGTP and dTTP) were purchased from Pharmacia (Baie d'Urfé, PQ). 1kb DNA Ladder was purchased from Gibco-BRL (Grand Island, NY). Materials required for small and large scale plasmid preparation were obtained from Qiagen (Chatsworth, CA). Oligonucleotides were synthesized at Gibco-BRL (Grand Island, NY).

2.1.4 Drugs and Radiochemicals

Drugs for pharmacology or functional assays were obtained as follows: dopamine, forskolin, guanylimidophosphate (Gpp[NH]p) were obtained from Sigma Chemical Co. (St. Louis, MO), clozapine from Sandoz Pharma Ltd. (Basel, Switzerland), haloperidol from RBI (Natick, MA). [³H]

Spiperone (120 Ci/mmol) was purchased from Amersham (Oakville, ON). GTP γ ³⁵S was acquired from Dupont NEN Life Science Products (Mississauga, ON), 35S L-cysteine [³⁵Cys] from ICN (Aurora, IL).

2.1.5 Protein Analysis

Protein A sepharose beads, aprotinin, leupeptin, pepstatin, glutathione, phenylmethylsulfonyl fluoride (PMSF) and brilliant blue protein stain were purchased from Sigma Chemical Co. (St. Louis, MO). PVDF membranes, 8-16 % Tris-glycine gels and protein marker were obtained from Novex Experimental Technologies (San Diego, CA). EN³HANCED was obtained from Dupont NEN Life Science Products (Mississauga, ON).

2.1.6 Kits

The following kits were obtained: small scale and large scale DNA preparation kits from Qiagen Inc. (Santa Clara, CA), ECL western blot detection kit and 7-Deaza-GTP Sequencing Kit from Amersham Life Science (Oakville, ON), TnT Coupled Reticuloctye System, Canine Pancreatic Microsomal Membranes and Altered Sites®II *in vitro* Mutagenesis Systems from Promega, and the Chameleon™ Double-Stranded Site-Directed Mutagenesis Kit from Stratagene, and the GST-Gene Fusion System from Pharmacia Biotech AB, PhosphoPlus MAPK Antibody Kit from New England Biolabs Inc. (Beverly, MA).

2.2 General Methods

2.2.1 Molecular Biology

Standard molecular biology techniques were followed as described in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989).

2.2.1.1 Preparation of Competent *E. coli* Bacteria

The preparation of competent *E. coli* XL-1 bacterial cells was started by scraping, with a sterile metal tip, a glycerol stock of the bacterial cells (800 µl bacterial culture with 150 µl glycerol stored at -80 °C) or a single colony from a Luria-Bertani (LB) (1 % w/v yeast extract, 0.5 % w/v bacto tryptone and 1 % w/v NaCl in double distilled water (ddH₂O), sterilized) agar plate (1.2 % w/v agar) supplemented with 10 µg/ml tetracycline and inoculating 4 ml of 10 µg/ml tetracycline-containing LB media in a 15 ml Falcon tube. The cells, grown overnight at 37 °C in a shaking incubator (275 rpm), were transferred to a 76 ml of LB media in a 250 ml sterile Erlenmeyer flask and grown for another 1-2 h. The culture was chilled on ice for 10 min and centrifuged at 4500 X g for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in 20 ml KMES buffer (60 mM CaCl₂, 5 mM MgCl₂, 5 mM MnCl₂ and 20 mM KMES, pH 5.8). The tube was placed on ice for 1-1.5 h and centrifuged again at 4500 X g for 10 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in 4 ml KMES buffer and stored at 4 °C for no more than 10 days.

2.2.1.2 Transformation of Competent *E. coli* with Plasmid DNA

To transform *E. coli* XL-1 bacteria, competent XL-1 bacteria (100 µl) were combined with plasmid DNA (0.5-1 µg/µl) in an eppendorf tube and chilled on ice for 15-45 min. The mixture was then heat shocked in a 42 °C water bath for 1.5-2 min and placed on ice for at least 2 min. The transformed cells were then pipetted onto a 100 mm LB agar plate (supplemented with 50 µg/ml

ampicillin) and spread evenly with a sterilized glass spreader. The plates were incubated overnight at 37 °C.

2.2.1.3 Small-Scale DNA Preparation

A single transformed colony from LB agar plates or a glycerol stock was used to inoculate 4 ml LB media supplemented with 50 µg/ml ampicillin. The culture was grown overnight at 37 °C with shaking (275 rpm).

Preparation of plasmid DNA was performed in order to isolate and purify the DNA of transformed bacteria. Three methods were used to prepare small quantities of DNA. 1) 1.5 ml of the overnight culture was transferred to an eppendorf tube and spun in a microcentrifuge at 13 000 rpm for 30 s. The supernatant was aspirated and the bacteria were resuspended by vortexing the pellets in 100 µl of ice cold resuspension buffer (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0). 200 µl of lysis buffer (0.2 N NaOH, and 1% SDS) and the contents were mixed by inverting the tubes until the contents were clear. 150 µl of ice-cold neutralization buffer (5 M potassium acetate, pH 4.8) was added and the tube was gently vortexed. The tubes were placed on ice for 3-5 min and then centrifuged at 13 000 rpm for 5 min. The supernatant was transferred to a new eppendorf tube containing 450 µl of cold 1:1 (v/v) phenol/chloroform and vortexed vigorously and then centrifuged at 13 000 rpm for 10 min. The top, aqueous phase, was extracted and placed in a new tube. The DNA in the aqueous phase was precipitated with 2 volumes of ice cold 100 % ethanol. The tube was inverted several times and placed at -20 °C for 20 min and then centrifuged at 13 000 rpm for 20 min. The supernatant was discarded and the DNA pellet was washed with 1 ml of ice-cold 70 % ethanol followed by centrifugation at 13 000 rpm for 10 min. The ethanol was aspirated and the pellet allowed to dry. The pellet was resuspended in 50 µl of ddH₂O containing 50 µg/ml RNase A. 2) DNA was purified using a rapid alkaline lysis method (Zhou *et al.*, 1990). Briefly, 1.5 ml of the

overnight culture was centrifuged for 10 s at 13 000 rpm. The supernatant was decanted and the bacteria pellet was resuspended in the residual media by vortexing. 300 µl of TENS (TE buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA) with 0.1 M NaOH and 0.5 % SDS) was added and the mixture vortexed until the solution appeared clear. 150 µl of 3 M sodium acetate (pH 5.2) was added and the lysates were mixed by inversion followed by centrifugation (13 000 rpm for 5 min). The supernatant was transferred to a new eppendorf tube and the plasmid DNA was precipitated with ice cold 100 % ethanol and placement at -80 °C for 10 min. The tubes were centrifuged at 13 000 rpm for 5 min and the DNA pellet was rinsed with 1 ml of ice cold 70 % ethanol, followed by centrifuging (13 000 rpm for 5 min). The supernatant was removed and the pellet allowed to dry. The DNA was resuspended in 50 µg/ml RNase A. 3) Finally, for applications requiring high purity plasmid DNA, the QIAGEN spin miniprep system was used and the manufacturers instructions were carefully followed.

2.2.1.4 Large-Scale DNA Preparation

Large-scale plasmid DNA preparation was performed using the solutions, columns (QIAGENtip 500) and protocols provided by Qiagen (Chatsworth, CA). 500 ml LB media (supplemented with 50 µg/ml ampicillin) was inoculated with a single transformed colony from LB agar plates or a glycerol stock. The culture was grown for 16 h at 37 °C with shaking (275 rpm) and then harvested and processed according to the manufactures instructions. The resulting purified plasmid DNA pellet was air dried and finally resuspended in 1 ml of ddH₂O. The concentration and yield of DNA was determined by measuring the absorbance at 260 nm of a 1:100 dilution in a spectrophotometer (Hitachi U-2000, Tokyo, Japan). A ratio of A₂₆₀/A₂₈₀ of 2:1 or better indicated acceptable DNA purity.

2.2.1.5 Restriction Enzyme Digests

For complete restriction digests, restriction endonuclease enzymes (1 to 5 units/ μ g DNA) were combined with plasmid DNA (0.5-1.0 μ g) in a final volume of 20 μ l containing the manufacturer's suggested 1 X buffer. Digestions were carried out for at least 1 h at the recommended incubation temperature for the particular enzyme.

2.2.1.6 Visualization of DNA

DNA ($\sim$ 1 μ g), directly from DNA plasmid preparations or restriction digests, were combined with 1 μ l loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % glycerol). Restriction digest buffer (NEB), if not in the sample, was added to a final concentration of 1 X. The samples and a 1 kb ladder (Gibco-BRL) were then loaded into wells of an agarose gel (0.8 % SeaKem (high melt) or SeaPlaque (low melt) agarose in TAE buffer (40 mM Tris, 20 mM glacial acetic acid and 1 mM EDTA) with 10 mg/ml ethidium bromide) submerged in TAE and size separated by gel electrophoresis (75-125 V). The DNA was visualized using a UV transilluminator (Bio/Can Scientific/UV 26, Mississauga, ON). The size of the DNA fragments were estimated against the 1 kb DNA ladder.

2.2.1.7 Subcloning Techniques

Restriction digests and modification of the 5' and 3' ends of the DNA were performed in order to manipulate the DNA fragment and appropriate vector and then ligate these to each other.

The filling-in of 5' overhanging ends (5' $\rightarrow$ 3' polymerase reaction) or the digestion of 5' or 3' overhanging ends (5' $\rightarrow$ 3' or 3' $\rightarrow$ 5' exonuclease reaction) were performed on the DNA to eliminate incompatible restriction sites prior to ligation and ensuring that the DNA maintained the proper reading frame if required. After heat inactivation (70 °C for 20 min, reannealing at 37 °C for 15 min)

of the restriction enzyme in the restriction digest, 3' DNA overhangs were removed by the digestion with the Klenow fragment of DNA polymerase I (1 U/ μ g DNA) at 37 °C for 30 min. For 5' DNA overhangs digestion mung bean nuclease (1 U/ μ g DNA) was added to the DNA in 1 X mung bean nuclease and incubated at 30 °C for 30 min. In order to fill in 5' DNA overhanging ends, Klenow (1 U/ μ g DNA) and 33 μ M of each dNTP were added after restriction enzyme inactivation and incubated at 37 °C for 30 min. The reactions were terminated by the addition of EDTA to 10 mM, heat inactivated, and then reannealed except for the mung bean nuclease which was inactivated by a phenol/chloroform extraction and the DNA recovered by alcohol precipitation.

The 5' end of the vector was dephosphorylated to prevent religation and recircularization during the subcloning procedure. After heat inactivation of the restriction digest enzymes and/or Klenow, the vector DNA was incubated with 1 μ l (10 U) calf intestinal phosphatase (CIP) and incubated for 1 h at 37 °C. The reaction was stopped by the addition of EDTA to 1 mM and heat inactivated. After the incubation with CIP, the DNA was extracted by adding 10 μ l phenol/chloroform (1:1 v/v). The aqueous phase was carefully removed and prepared for gel electrophoresis (see above).

The DNA fragments, from the above procedures, were separated by gel electrophoresis (0.8 % SeaPlaque (low melt) agarose in TAE buffer). The desired DNA fragments were excised from the gel and placed in eppendorf tubes. The tubes containing the DNA fragments in the low melt gel were heated to 60 °C until the gel was liquidified. The ligation was carried out, by typically adding 1 μ l of the vector DNA and 9 μ l of insert DNA (4:1 molar ratio of insert:vector) with 30 μ l of ddH₂O. The mixture was allowed to cool to room temperature prior to the addition of 10 μ l 5X T4 DNA ligase buffer and 0.5-1 μ l (5-10 U) of T4 DNA ligase. The mixture was placed in a 16 °C water bath for 15 min to overnight. Of this mixture, 25 μ l was used to transform competent *E. coli* XL-1 cells as described above.

2.2.1.8 Sequencing

Sequencing was performed using the 7-Deaza-dGTP sequencing kits and Sequenase v2.0 (United States Biochemical, Cleveland, OH) according to the manufacturer's instructions. For sequencing 1-4 µg of plasmid DNA was used and 10 ng/µl of the appropriate primer solution was used. Primers directed against the T7 or T3 sequences, or oligonucleotides targeted to regions within the dopamine D4 receptor sequence were utilized.

The sequence reactions were separated on a 8 % acrylamide sequencing gel (6 % acrylamide, 17.5 mM N,N'-methylene-bis-acrylamide, 6.82 M urea, 0.054 % ammonium persulfate, 0.02 % TEMED in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA)) which was prewarmed for 1 h at 50 W. The sequencing samples were denatured (80 °C for 2 min) and placed on ice prior to loading onto the gel. The gels were run 3-5 h and then soaked in a solution of 12 % methanol and 10 % glacial acetic acid for 1 h. The gel was transferred to Whatman No. 1 filter paper and dried under vacuum for 1 h using a gel drier (Bio-Rad). Dried gels were exposed to BioMax MR Film (Kodak) overnight prior to developing.

2.2.2 Construction of Recombinant Plasmids

D4.4(220-336) encoding most of the third intracellular loop of the human dopamine D4.4 receptor was subcloned into the XbaI/EcoNI sites of ppoly(A)luc(T7) such that most of the luciferase gene from ppoly(A)luc(T7) was removed to provide a start codon and a poly A tail for the *in vitro* expression of the third intracellular loop of D4.4. The full length cDNAs of D4.2(1-387), D4.4(1-419) and D4.7(1-467) (Van Tol *et al.*, 1992) were subcloned into pBluescript SKII (-). Human D4.4 deletion mutants (see Figure 3.1, p. 85) D4.(Δ254-315) (Asghari *et al.*, 1994), D4.(Δ221-315) (Asghari *et al.*, 1994) and D4.(Δ221-337) were created by removing the indicated amino acids from the D4.4(1-419) construct. D4.2(249-280) only codes the first (α) and last repeat (ζ) of the human

D4.2 receptor, and was subcloned into the XbaI/EcoRV sites of ppoly(A)luc(T7). The human dopamine D3 receptor (1-400) (a gift from Dr. J.-C. Schwartz and Dr. P. Sokoloff), the human beta-adrenergic β_1 receptor (1-477) (a gift from Dr. M. Caron) and the human m4 muscarinic receptor (1-479) (a gift from Dr. T. Bonner) were subcloned into pBluescript. The region of the rat D4 receptor(224-305) that is homologous to the human D4 receptor repeat and flanking region was subcloned into the EcoRV/XbaI site of ppoly(A)luc(T7) to create D4Rat(224-305). All constructs made for *in vitro* expression were placed under the control of the T7 promoter.

Glutathione S-transferase (GST) fusion protein constructs, encoding either the entire protein or only the SH3 domains, were made. GST-Nck 3SH3, encoding the three SH3 domains (1-255) of the human Nck protein; GST-Crk SH3 (N) encoding the amino-terminal SH3 domain of human Crk-II (133-184); and GST-Crk SH3 (C) (238-290) encoding the carboxyl-terminal SH3 domain of human Crk-II were subcloned into PGEX-2T. GST-Grb2 encoding the full length human Grb2 protein (1-217), GST-Grb2 SH3 (N) encoding the amino-terminal SH3 domain (1-58) and GST-Grb2 SH3 (C) encoding the carboxyl-terminal SH3 domain (159-217) have been described (Gout *et al.*, 1993; Lowenstein *et al.*, 1992). Grb2 (P49L) was created by substituting a conserved proline with a leucine at amino acid residue 49 of the human Grb2 protein. GST-Abl SH3 (65-123 of p210 Bcr-Abl), GST-p85 SH3 encoding the SH3 domain of the bovine p85 α protein (1-86), GST-PLC γ encoding the human PLC γ SH3 domain (792-851), GST-spectrin encoding the chicken alpha chain spectrin protein's SH3 domain (967-1025) and GST-c-Src SH3 encoding the chicken c-Src SH3 domain construct has been described (Gout *et al.*, 1993; Liu *et al.*, 1996; Puil *et al.*, 1994).

D4.7, D4.(Δ 221-315) and D4.(Δ 221-337) were subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen) and fused either to the HA (hemagglutinin) epitope (YPYDCPDYA) or the signal-Flag epitope sequence at the amino-terminal extracellular domain of the receptors as described previously for the opioid receptors (Chu *et al.*, 1997).

2.2.3 *In Vitro* Protein-Protein Interaction Assay

Proteins expressing the different GPCRs, or parts thereof, were labeled by T7 polymerase with ^{35}S L-cysteine [^{35}Cys] (ICN) using a rabbit reticulocyte *in vitro* transcription/translation system (TNT Coupled Reticulocyte Lysate Systems, Promega) according to the manufacturer's instructions. *In vitro* product was quantified according to the manufacturer's instructions by liquid scintillation of the purified radiolabeled protein. GST fusion proteins were prepared according to the GST Gene Fusion System protocol (Pharmacia Biotech) either in XL-1 or BL-21 *E.coli* cells. Cells were grown in LB media to an $\text{OD}_{600} > 0.6$. Protein synthesis was induced with 1 mM isopropylthiogalactopyranoside for 2 h. The cultures (1.5 ml) were spun down and resuspended in lysis buffer (Phosphate-buffered saline (PBS), 1 % TritonX-100, 1 % Tween 20, 10 mM DTT, 2 $\mu\text{g/ml}$ Aprotinin and 2 $\mu\text{g/ml}$ Leupeptin) and sonicated (power 70 for 10 s; Biosonik sonicator, Bronwill Scientific). The lysate was spun down (30 s, 500 X g). 50 μl of a 50 % slurry of glutathione beads was added to the lysate and was then incubated with shaking at room temperature for five minutes. The beads were spun down (30 s, 500 X g) and rinsed three times with fresh lysis buffer (100 μl) and finally resuspended in 300 μl lysis buffer. The concentration of the purified GST fusion protein was determined by eluting off the GST fusion protein from the glutathione beads using glutathione (10 mM) and measuring the absorbance at 280 nm. Freshly prepared *in vitro* transcribed [^{35}Cys] labeled protein (2 μl ; 1.5 nM) was added to the 300 μl glutathione bead preparation and incubated with gentle shaking at 4 °C for two hours. The beads were spun down, the supernatant discarded and the beads were rinsed three times with fresh ice cold lysis buffer (100 μl). Finally, 30 μl of 1X sample buffer (62.5 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 0.005 % bromophenol blue, 10 % glycerol, 2.5 % β -mercaptoethanol) was added, heated to 90 °C for five minutes and run on a 8-16 % Tris glycine gel (NOVEX). The gels were fixed, stained with Brilliant Blue (Sigma), soaked in EN³HANCED (Du Pont), dried on filter paper and exposed to autoradiography film.

2.2.4 Tissue Culture

CHO-K1 cells were grown in α -minimal essential medium supplemented with 2.5 % FBS and 2.5 % HS. COS-1 and COS-7 were maintained in α -MEM supplemented with 10 % FBS. HEK-293 were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum. SKNMC cells were maintained in Ham's F10 medium supplemented with 8 % FBS. The cells were incubated (Napco incubator, model 5430) as monolayers on tissue culture dishes at 37 °C in a humidified 5 % CO₂ atmosphere. Cells were expanded by first washing with 5 - 10 ml of 1 X PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4) and then dissociating the cells by rinsing with 1 X PBS containing 0.25 % trypsin and 1 mM EDTA for 2 min. The trypsin containing 1 X PBS was removed, and the cells were resuspended in the appropriate media and replated at 10-20 % confluency. Freezer stocks of stably transfected cell lines were prepared by resuspending cells in the appropriate media supplemented with 10 % DMSO and stored at -80 °C. Cells were transferred to liquid nitrogen for long term storage.

Clonal cell lines were selected by growing transfected cells in media containing the selection reagent G418 (400 μ g/ml active G418 for stable CHO cells). Individual colonies were picked from plates following 2-5 weeks of selection. These picks, or clonal cell lines, were expanded on separate plates. Cells used for Scatchard analysis and histoimmunochemistry procedures were collected by rinsing and scraping in 1 X PBS and pelleting at 250 X g for 10 min at 4 °C).

Chapter 3

SH3 Binding Motifs Within G Protein-Coupled Receptors

3.1 Introduction

As discussed in chapter one, the dopamine D4 receptor has a number of attributes that distinguish it from the other dopamine receptors and GPCRs. The D4 receptor displays higher affinity, compared to the D2 receptor, for the atypical neuroleptic clozapine, is highly polymorphic in the human population, and is extremely proline rich in the third cytoplasmic loop. There are multiple sites in the third cytoplasmic loop that conform to the minimally required PXXP motif, necessary for SH3 binding. It is known that some GPCRs activate the Ras-mediated MAP kinase pathway by means of a G protein-mediated pathway (Koch *et al.*, 1994; Luttrell *et al.*, 1996). It is known that GPCRs interact directly with G proteins, GRKs and other kinases, but to date no direct interaction with an SH3 domain containing protein has been demonstrated for any member of this superfamily.

The binding characteristics of a number of well established SH3-mediated protein-protein interactions have been detailed (Cheadle *et al.*, 1994; Feng *et al.*, 1995; Graham *et al.*, 1998; Knudsen *et al.*, 1995; Lee *et al.*, 1995; Lemmon *et al.*, 1994; Lim *et al.*, 1994b; Pisabarro *et al.*, 1994; Pisabarro and Serrano, 1996; Posern *et al.*, 1998; Ren *et al.*, 1993; Sparks *et al.*, 1994; Sparks *et al.*, 1996; Yu *et al.*, 1994). These findings have shown that most SH3 interactions are mediated by a protein sequence that contains a minimal PXXP sequence. In addition the amino acids that flank this motif have been firmly established for both class I and class II binding sites for specific SH3-mediated interactions. Although some general binding trends have been established for this interaction, the different SH3 interactions indicate that the amino acid sequence beyond the core sequence is

extremely variable in nature. Furthermore, the same SH3 domain can interact with binding motifs that are quite dissimilar from each other in amino acid sequence (Sparks *et al.*, 1996; Yu *et al.*, 1994). If an amino acid sequence in a protein matches a known SH3 binding motif, the probability is high that it will bind to the known SH3 domain (Ren *et al.*, 1993). Unfortunately, no prediction can be made as to whether a PXXP motif that displays a flanking amino acid sequence that shows no homology to a known SH3 binding motif will in fact bind an SH3 domain. Therefore this interaction has to be tested for experimentally.

Several studies did suggest the possibility of a direct GPCR interaction with SH3 domain containing proteins. Ren *et al.* in identifying a ten amino acid peptide sequence required for binding to SH3 domains, noted that the rat m4 muscarinic acetylcholine receptor had a sequence in the third cytoplasmic loop that could bind the SH3 domain of Abl (Ren *et al.*, 1993). No subsequent studies were completed in order to confirm binding of the third intracellular loop region or of the entire m4 receptor to Abl.

A comprehensive scan of the GPCR superfamily revealed that the dopamine D4 receptor is unique in its extremely proline rich region in the third cytoplasmic loop. A large number of GPCRs, however, did show a number of PXXP motifs in this region. In our attempt to show the ability of the D4 receptor to interact with SH3 domains, we also wanted to establish if this phenomenon was unique to the D4 receptor, or if the GPCRs in general, or a category of GPCRs may use SH3 binding in their function.

The superfamily of GPCRs is subdivided into five subfamilies (A-E). Class A, the largest subfamily includes the amine, peptide, hormone, rhodopsin, olfactory, protanoid, nucleotide, cannabis, platelet activating factor, gonadotrophin, melatonin and viral receptors. Class B includes the secretin receptors. Class C contains the metabotropic glutamate receptors. Class D contains the pheromone receptors. Class E contain the dictyostelium receptors (Horn *et al.*, 1998).

A number of methods have been employed in order to determine and confirm SH3 binding interactions. *In vitro* interaction assays (Anafi *et al.*, 1996; Bokoch *et al.*, 1996; Jackman *et al.*, 1995; Sudol, 1994), phage display of short peptide sequences (Cheadle *et al.*, 1994; Phizicky and Fields, 1995; Sparks *et al.*, 1994), yeast two hybrid techniques (Anafi *et al.*, 1996; Clemens *et al.*, 1996; Hu *et al.*, 1995; Lussier and Larose, 1997) and *in vivo* immunoprecipitation techniques (Bokoch *et al.*, 1996; Hu *et al.*, 1995; Lu *et al.*, 1997; Motto *et al.*, 1994) have all been used successfully to confirm and characterize SH3 interactions. The *in vitro* interaction assay has a number of advantages. The specific proteins are synthesized *in vitro* ensuring that the interaction detected is between the two proteins of interest. Constructs expressing either the entire SH3 domain containing protein or a specific SH3 domain or the region containing the SH3 binding motif can be expressed and tested for protein interaction. Thus, the specific SH3 domain and binding region can be firmly established. Mutagenesis of specific amino acids in the SH3 domain or SH3 binding domain can be quickly tested to see if they disrupt the interaction to confirm that the interaction is SH3 mediated and to establish the specific SH3 domain and binding region. The binding properties of the interaction can be established because the amount of protein used in the *in vitro* interaction assay can be accurately estimated. SH3 interactions observed using the *in vitro* interaction assay demonstrate the ability of a protein to interact with SH3 domains which can then be confirmed using an *in vivo* interaction assay (Bokoch *et al.*, 1996; Liu *et al.*, 1996; Motto *et al.*, 1994; Rivero-Lezcano *et al.*, 1994; Wang *et al.*, 1996). The demonstration of an *in vitro* interaction between two proteins, however, does not necessarily indicate that this is the endogenous interaction that occurs within the cell. Under physiological conditions these proteins may not interact because of a competing, higher affinity, SH3 binding interaction, the lack of expression of one or both proteins in the same cell or a lack of expression of the proteins in the same cellular compartments. Furthermore, the function of the interaction cannot be determined using the *in vitro* interaction assay. The *in vitro* interaction assay

is a quick method to demonstrate and characterize SH3 binding and is a useful first step to screen for potential endogenous interactions.

The first objective of the experiments was to determine the propensity of PXXP motifs in the superfamily of GPCRs. A comprehensive scan of the GPCRDB database (Horn *et al.*, 1998) was completed to determine the percentage of GPCRs that contain such a motif, the location of the motifs and other characteristics of the distribution pattern. The next objective was to determine if the dopamine D4 receptor could interact with SH3 domains using an *in vitro* protein-protein interaction assay. Furthermore, the pharmacological characteristics of this binding was assessed by means of a saturation binding analysis and competition analysis.

3.2 Methods

3.2.1 Scan of GPCRs for PXXP motifs

A comprehensive scan of the GPCRs was completed using several Internet tools. A study of the GPCRDB database (Horn *et al.*, 1998) on the world wide web site <http://www.gpcr.org/7tm> was completed. The sequence and alignments of a vast number of GPCRs is listed here. The occurrence and frequency of the PXXP motif in the different GPCR families was examined. Briefly, individual prolines and prolines that were separated by two intervening amino acids were identified for each of the GPCRs in the database. PXXP sequences that occurred in a GPCR subfamily and/or were conserved between species were noted. The location of the PXXP sequences in the receptors were identified. Distribution trends and properties were surveyed. For the dopamine receptors, the occurrence of the PXXP motifs in the different receptors was examined.

3.2.2 *In vitro* Competition and Saturation Analysis

For the *in vitro* protein-protein interaction assay the constructs and methods as described in Chapter 2, General Materials and Methods (p. 78), were used. Specific methods pertaining to the saturation analysis are detailed below.

For the competition assay, equivalent amounts of GST-Nck 3SH3 and equivalent amounts of *in vitro* labeled [³⁵Cys]D4.4(220-336) protein (1 μ l; ~1 nM) were co-incubated. A tenfold higher amount (10 μ l; ~10 nM) of unlabeled *in vitro* product D4.4(220-336) or D4.(Δ 221-315) was added either thirty minutes prior, at the same time, or thirty minutes after the addition of the labeled protein. The interaction was analyzed as described above.

For the saturation assay, equivalent molar amounts of *in vitro* labeled [³⁵Cys] protein (2 μ l; ~5 nM) was incubated with increasing concentrations of GST, GST-Nck 3SH3 or GST-Grb2 fusion protein to determine the strength of interaction between the various D4 constructs or D4 deletion mutants with Nck 3SH3 or Grb2. The volume of glutathione beads and lysis buffer was kept constant for the various dilutions of GST fusion protein for each saturation curve. After incubation, the beads were washed with two volumes of 200 μ l lysis buffer. Samples were run as described (p. 78). and exposed to a phosphoimaging cassette overnight. The amount of *in vitro* product immobilized by GST fusion protein was quantified using the STORM 680 phosphoimager (Molecular Dynamics). The scans were analyzed with ImageQuant for Windows NT software (Molecular Dynamics) and approximations for the kinetic values (K_d and B_{max}) were obtained using Graphpad Prism 2.01 software.

3.3 Results and Discussion

3.3.1 Putative SH3 binding motifs within the GPCRs and Dopamine Receptors

Numerous studies have been performed to characterize the consensus sequence for SH3 binding sites by using combinatorial peptide libraries or by identifying the specific binding requirements of known SH3 protein interactions (Alexandropoulos *et al.*, 1995; Anafi *et al.*, 1996; Cheadle *et al.*, 1994; Knudsen *et al.*, 1995; Lee *et al.*, 1995; Lim *et al.*, 1994b; Pisabarro *et al.*, 1994; Pisabarro and Serrano, 1996; Ren *et al.*, 1993; Sparks *et al.*, 1994; Sparks *et al.*, 1996; Wang *et al.*, 1996; Yu *et al.*, 1994). From these studies the binding characteristics of known SH3 domains to specific consensus sequences have been well detailed. Sequences of about ten amino acids which have a PXXP motif have been found to be necessary but not sufficient for SH3 binding.

Analysis of the dopamine D4 receptor sequence identified a number of putative SH3 binding domains conforming to the minimally conserved PXXP sequence (Figure 3.1). The dopamine D4

FIGURE 3.1 Depicted is the complete amino acid sequence of the third cytoplasmic loop of the human dopamine D4.4 receptor. The repeat units ($\alpha\beta\zeta$) are boxed. Horizontal lines highlight the different PXXP motifs. Bold lettering is used to indicate the SH3 binding motifs that meet the more stringent consensus sequence (Yu *et al.*, 1994). The amino acid residues of the third intracellular loop fragment D4.4(220-336) and the amino acid residues removed in the deletion mutants (D4.(Δ 254-315), D4.(Δ 221-315) and D4.(Δ 221-337)) are indicated with arrows.

receptor is remarkable since it is extremely proline rich in the third cytoplasmic loop and displays the largest number of putative SH3 binding domain motifs of any of the known GPCRs. The D4.2 variant contains thirteen PXXP motifs, many of them overlapping and found in the polymorphic repeat units, while the other human D4 variants contain more PXXP sites depending on the number of repeat units

and the specific sequence of the units. Some of the sites exist at the junction of two repeats. For all the human D4 variants, four PXXP motifs are found directly amino-terminal to the repeat, two PXXP motifs straddle the intersection of the amino-terminal region and the first repeat unit (α), and one PXXP motif is located carboxyl-terminal to the last repeat (ζ).

In the human dopamine D4 receptor there are two potential SH3 binding domains that conform to the more stringent p Φ PpXP consensus sequence (Yu *et al.*, 1994). One that is just amino-terminal to the first repeat (aa 241-246) and one that straddles the flanking region and the first couple of amino acids of the first repeat (aa 246-251). Several of the PXXP motifs found amino- and carboxyl-terminal to the repeat region in the human dopamine D4 sequence are conserved in the rat and mouse D4 sequences, despite the absence of the repeat sequence in these species and the high divergence in amino acid sequence that exist in the third intracellular loop. The conservation of these PXXP motifs across species suggests a functional role.

A comprehensive survey of the majority of the known GPCRs identified the PXXP motif in about half of these receptors. The PXXP motifs are rare in the transmembrane domains of these receptors, but are most often seen in the amino-terminal tail, in the third intracellular loop and in the carboxyl-terminal tail. Not all of these PXXP sites in these receptors would be expected to be actual binding sites for SH3 domains, since the PXXP motif would be expected to occur at least once in a 400 amino acid stretch if all amino acids are present at about equal proportions.

The PXXP motif is found in most of the catecholaminergic receptors (Figure 3.2). Only the β_2 -adrenergic and dopamine D1 receptor contain no PXXP motif in the third intracellular loop and the amino- and carboxyl-terminal-tails of the receptor. The prevalence of this motif in the catecholaminergic mammalian receptors is about 1 in 120 aa in the amino-terminal tail, never in the transmembrane domains, and 1 in 40 aa in the third intracellular loop and carboxyl-terminal tail. The more stringent p Φ PpXp motif (Yu *et al.*, 1994) is only found in the human dopamine D4 receptor at

FIGURE 3.2 Depicted are the GPCR sequences and their alignments of the catecholamine receptors. The amino acid sequence of the human α -adrenergic, human β -adrenergic, human, mouse and rat dopaminergic and the m4 muscarinic receptors are shown. The PXXP motifs, potential SH3 binding sites, are indicated in boxes. The transmembrane domains are indicated. The human D4 repeat region is shaded.

amino acid positions 241 to 246, and 246 to 251.

The absence of this motif in the transmembrane domains of the catecholamine receptors reflects the propensity of Pro residues to disrupt the α -helical structure and thus the integrity of the receptor, especially since the transmembrane domains form the binding pocket for catecholamines (for review see Shenker, 1995). The high incidence of this motif in the third intracellular loop and the carboxyl-terminal tail in this receptor family suggests that some of these sites may be genuine SH3 binding domains that may play a role in receptor signalling and/or transport, especially since the third intracellular loop and carboxyl-terminal tail have been found to be important in functional coupling and other protein interactions (Shenker, 1995). Many of the PXXP motifs found in the third intracellular loop tend to be conserved between species for the same receptor subtype, this despite the fact that the hydrophilic loops are the most variable regions of these receptors. This conservation across species also suggests a functional importance.

3.3.2 Interaction of the dopamine D4 receptor with Nck and Grb2

Putative SH3 binding motifs can be found in the third intracellular loop of the human dopamine D4 receptor, in particular in the polymorphic repeat sequence and the immediate surrounding sequence (Figure 3.1). To determine whether the third intracellular loop of the D4 receptor can interact with the SH3 domain containing proteins Nck and Grb2 we synthesized a radiolabeled fragment of the third intracellular loop of the D4.4(220-336) receptor *in vitro* by means of a transcription-translation system. The three SH3 domains of Nck and the entire Grb2 protein were expressed as GST fusion proteins. In the interaction assays, GST by itself did not interact with the radiolabeled receptor fragments, nor did the radiolabeled luciferase control protein, which contains a PXXP motif, interact with any of the GST-SH3 fusion proteins tested (Figure 3.3). The third intracellular loop fragment D4.4(220-336) showed strong interaction with both the Nck and Grb2

FIGURE 3.3 Identification of the different dopamine receptors that interact with the adapter proteins Nck and Grb2. Different [^{35}Cys] *in vitro* labeled receptor product (~ 1.5 nM) or control (luciferase) (top panel) was incubated with the GST, GST-Nck 3SH3 or GST-Grb2 fusion proteins (30 μM) (lower panels). The immobilized *in vitro* product was run on a protein gel and exposed to film (24 h exposure). Molecular masses are indicated in kDa.

SH3 adapter proteins. Although the D4.4(220-336) fragment ran at a larger than expected size (~ 30 kDa instead of ~ 21 kDa), extensive sequencing revealed no out of frame mutations, indicating that this was a gel running artefact. The third intracellular loop fragment of the dopamine D4 rat receptor (D4Rat(224-305)) which does not contain the repeat sequence as seen in the human D4

FIGURE 3.4 Identification of the different parts of the human dopamine D4 receptor that interact with the adapter proteins Nck and Grb2. Different [³⁵Cys] *in vitro* labeled receptor product (~1.5 nM) or control (luciferase) (top panel) was incubated with the GST, GST-Nck 3SH3 or GST-Grb2 fusion proteins (30 μM) (lower panels). The immobilized *in vitro* product was run on a protein gel and exposed to film (24 h exposure). Molecular masses are indicated in kDa.

receptor, but does contain PXXP motifs as seen in the flanking sequences to the repeat of the human dopamine D4 receptor, was also able to interact with Nck and Grb2.

In addition to the third intracellular loop of the human D4.4 receptor, several full length receptor products were tested for their ability to interact with Nck and Grb2 using the *in vitro* assay.

The full length D4.2, D4.4 and D4.7 human dopamine receptors, which contain two, four, and seven repeats respectively, and the human dopamine D3 receptor also showed strong interaction with both Nck and Grb2. The D4.4 receptor shows a shift in relative size when immobilized with Grb2, but not with Nck, a result of the Grb2 protein running at the same relative size on the protein gel.

In order to determine which part of the third intracellular loop of the human D4 receptor is involved in binding to Grb2 and Nck, we made several constructs of the full length D4.4 receptor with deletions of different parts of the third intracellular loop and a construct expressing only the two repeats. The construct D4.(Δ 254-315), in which almost the entire repeat sequence was deleted, maintained Nck and Grb2 interaction (Figure 3.4). The interaction with D4.(Δ 221-315), which has the repeat sequence and the PXXP motifs amino to the repeat sequence deleted, but expresses a PXXP motif carboxyl to the repeat sequence, could still interact with Nck and Grb2, but this was significantly reduced compared to the interaction seen with the full length D4.4 receptor. Finally, the

FIGURE 3.5 A schematic of the different parts of the dopamine D4 receptor tested is represented. *In vitro* interaction of the different D4 constructs tested is indicated (+++) very strong, (++) strong, (+) weak, (+/-) very weak.

D4.(Δ 221-337) construct, in which all PXXP motifs were deleted, showed a marked reduction in interaction with both Nck and Grb2. The repeat sequence by itself (known as the $\alpha\zeta$ sequence D4.(249-280)) did not interact with Nck or Grb2. The results are summarized in the schematic illustrating the relative parts of the receptors deleted and the relative strength of binding (Figure 3.5).

3.3.3 Saturation analysis of the dopamine D4-SH3 interaction

We used a saturation analysis to quantify the strength of interaction between the D4.4 constructs and Nck or Grb2. We used increasing concentration of the GST fusion protein, with a maximal concentration of 300 μ M, while a constant amount of *in vitro* labeled receptor was added. The interaction of the third intracellular loop D4.4(220-336) fragment, as well as the full length D4 constructs, showed saturable binding with both Nck 3SH3 and Grb2 (Figure 3.6). The calculated amount of labeled receptor in the saturation analysis (B_{max}) bound at the high concentrations of GST fusion protein was comparable to the total amount of labeled receptor added to the incubation mixture. Approximations for the affinity of the interactions were obtained from replicate experiments

FIGURE 3.6 Saturation analysis of D4.4 with Nck and Grb2. A constant amount of *in vitro* labeled protein (5 nM) was incubated with increasing concentrations of GST-Nck 3SH3 (A) or GST-Grb2 (B). The immobilized *in vitro* product is shown as a scan and the quantified results are depicted graphically.

of the saturation analysis (Table 3.1). Nck had a K_d of about 40 μM and Grb2 had a K_d of about 10 μM for the third intracellular loop D4.4(220-336) fragment. The full length receptors showed strong interaction with Nck 3SH3 and Grb2, although this interaction was weaker than that seen with

	GST	GST-Nck 3SH3	GST-Grb2
	K_d μM	K_d μM	K_d μM
CONTROL	no binding	no binding	no binding
D4.4(220-336)	no binding	40	10
D4.2		200	200
D4.4		200	200
D4.7		200	50
D4.(Δ254-315)		30	50
D4.(Δ221-315)		>600	>600
D4.(Δ221-337)	no binding	no binding	no binding

TABLE 3.1 The affinity (K_d) of the various D4 constructs for Nck or Grb2, estimated from replicate experiments, are summarized. The binding constant of GST, GST-Nck and GST-Grb2 to various D4 receptors and D4 deletion mutants is given.

just the third intracellular loop by itself. Of the three receptor variants it seems that the D4.7 displays the strongest interaction, with a K_d of about 50 μM for Grb2, while the D4.2 and D4.4 receptors have an affinity of about 200 μM for both Nck and Grb2. The deletion mutant D4.(Δ 254-315), which has most of the repeat region removed, showed no reduction in binding (K_d 30-50 μM). The deletion mutant D4.(Δ 221-254), which has the repeat region and the sequence containing the amino-terminal PXXP motifs removed, has a significantly reduced interaction (K_d >600 μM). The deletion mutant D4.(Δ 221-337), which has the repeat region as well as the sequence containing the amino- and carboxyl-terminal PXXP motifs removed, displayed no binding.

3.3.4 Pharmacological profile of the dopamine D4-SH3 interaction

To determine whether the observed interaction between the SH3 domains and the D4 receptor followed the classic pharmacological rules for a specific and reversible interaction we also performed a competition analysis with GST-Nck 3SH3 and the third intracellular loop D4.4(220-336) fragment.

We competed the GST-Nck 3SH3/[³⁵Cys]D4.4(220-336) interaction with a 10-fold excess of unlabeled D4.4(220-336) or D4.(Δ254-315). The GST-Nck 3SH3/[³⁵Cys]D4.4(220-336) interaction can be significantly reduced by the addition of a 10-fold excess of unlabeled D4.4(220-336) or D4.(Δ254-315) interacting fragment prior, during or after the interaction with [³⁵Cys]D4.4(220-336) has been established (Figure 3.7).

FIGURE 3.7 Competition analysis of D4.4(220-336) and the deletion mutant D4.4(Δ254-315). *In vitro* labeled [³⁵Cys]D4.4(220-336) was immobilized with either GST or GST-Nck(3SH3) and competed off with a 10-fold excess of unlabeled D4.4(220-336) or D4.4(Δ254-315) which was added thirty minutes prior (pre-), at the same time (co-) or thirty minutes after (post-) the addition of the labeled receptor product to the GST- fusion protein. The immobilized *in vitro* product is shown. Molecular masses are indicated in kDa.

3.3.5 Interaction of the dopamine D4 receptor with other SH3 containing proteins

The third intracellular loop D4.4(220-336) fragment also interacted with the GST fusion proteins containing SH3 domains of Abl, Crk, p85 kinase α and c-Src. No interaction was seen with the SH3 domain containing fragments of PLC γ and spectrin (Figure 3.8). The interaction between D4.4(220-336) and Nck and Grb2 was significantly stronger than with the other SH3 domain containing proteins, or parts thereof, tested (data not shown).

The carboxyl-terminal SH3 domain of Crk displayed a moderate interaction with the

FIGURE 3.8 *In vitro* interaction of D4 with various SH3 domain containing proteins. *In vitro* [^{35}Cys] labeled D4.4(220-336) protein (1.5 nM) was incubated with equivalent amounts of various GST fusion proteins (~30 μM) expressing either the whole protein or only the SH3 domain containing fragments of various SH3 domain containing proteins (see materials and methods for abbreviations). The top panel shows the relative amounts of GST fusion protein (Brilliant Blue Stain) used to precipitate the different *in vitro* labeled proteins (96 h exposure) shown in the lower panels. Molecular masses are indicated in kDa.

dopamine third intracellular loop D4.4(220-336) fragment, however, the amino-terminal SH3 domain of Crk was unable to interact with the third intracellular loop of the D4.4 receptor.

The panel of proteins tested are all SH3 domain containing proteins. They are involved in signal transduction, kinase regulation or function as adapter proteins. These protein are cytosolic proteins that may be able to interact with the dopamine D4 receptor. The possible function of the D4-SH3 interaction has not been determined. This interaction may be important for signal transduction, for recruiting other proteins to the receptor or may be involved in receptor targeting or

such processes as endocytosis. Without a known function for the D4-SH3 domain interactions, any of the SH3 domain containing proteins that co-localizes with the dopamine D4 receptor may be a candidate for interaction with this receptor.

Grb2, Crk, Abl, Src, and p85 have all been implicated in RTK signal transduction. Grb2 is an adapter protein composed of two SH3 and one SH2 domains. Grb2 is important in recruiting SOS to the activated RTK. Abl is a cytosolic protein tyrosine kinase (PTK) that has one SH3 domain and one SH2 domain. The SH3 domain has been implicated in the negative regulation of the Abl kinase (Franz *et al.*, 1989; Pisabarro *et al.*, 1998). The major substrate for Abl is Crk II. Crk II contains one SH2 and two SH3 domains. Crk II is tyrosine phosphorylated after RTK activation. P85 is a subunit of the PI3-K protein and contains two SH2 and one SH3 domains. It has been shown to interact with RTKs by means of its two SH2 domains. The interaction of D4 with these SH3 domain containing proteins needs to be confirmed *in vivo*. It is possible that other SH3 domain containing proteins, from the ones tested here, interact with D4 endogenously.

3.3.6 Interaction of D4.4 with Grb2 requires both SH3 domains for maximal binding

Grb2 consists of two SH3 domains separated by a single SH2 domain. The amino-terminal SH3 domain of Grb2 displayed almost no interaction with the third intracellular loop D4.4(220-336) fragment while the carboxyl-terminal SH3 domain displayed only an intermediate interaction as compared to the full length protein (Figure 3.9). Mutagenesis of Pro⁴⁹ to Leu⁴⁹, which has been shown to abolish interactions with the amino-terminal SH3 domain (Motto *et al.*, 1994), also displayed a significant reduction in binding to D4.4(220-336).

The above results indicate that the interaction of the dopamine D4 receptor with Grb2 is a cooperative interaction. The affinity of the carboxyl-terminal SH3 domain of Grb2 for D4 is much less than that of the entire Grb2 protein, while the amino-terminal SH3 domain displays no affinity

FIGURE 3.9 *In vitro* interaction of GPCRs with different Grb2 SH3 domains. *In vitro* [35 Cys] labeled D4.4(220-336), beta-adrenergic β_1 receptor, m4 muscarinic receptor and control (luciferase) proteins (~ 1.5 nM) were incubated with equivalent amounts of various GST fusion proteins (~ 30 μ M) expressing either the whole protein or only the SH3 domain containing fragments of Grb2 (see materials and methods for abbreviations). The top panel shows the relative amounts of GST fusion protein (Brilliant Blue Stain) used to precipitate the different *in vitro* labeled proteins (96 h exposure) shown in the lower panels. Molecular masses are indicated in kDa.

for D4 when expressed by itself. The full strength interaction is only observed when both SH3 domains are expressed. The Grb2 (P49L) mutant tested confirms this observation. The mutation is in the amino-terminal SH3 domain which has been shown not to interact with the D4 receptor (Figure 3.9). This mutation, however, reduces the affinity of Grb2 for D4 to a level comparable to just the carboxyl-terminal SH3 domain by itself. The dopamine D4 deletion mutants also confirm the requirement of two SH3 binding motifs for maximal binding affinity to Grb2. The expression of the sequence containing the PXXP motifs both amino- and carboxyl-terminal to the repeat region is required for the strongest interaction with Grb2 indicating that the binding is mediated by two SH3 domains. Grb2 has previously been shown to bind in cooperative manner to the HPK1 protein (Anafi *et al.*, 1997).

3.3.7 Other GPCRs also show an ability to bind Grb2 and Nck

The full length proteins of the dopamine D3, D4, m4 muscarinic and β_1 -adrenergic receptors (Figure 3.3 and 3.9) could all interact with Grb2. The relative strength of the interaction between the adaptor proteins Grb2 with the receptors is D4>D3>M4> β_1 . The m4 muscarinic and β_1 -adrenergic receptor showed virtually no detectable interaction with the other SH3 domain containing fusion proteins, including the carboxyl-terminal Grb2 SH3 domain and the Grb2 (P49L) mutant protein. The interaction of SH3 domains with the different catecholamine and muscarinic receptors indicate that GPCRs can interact with these domains. It is likely that this type of interaction is applicable to other GPCRs.

3.3.8 GPCRs can interact with SH3 domains

The *in vitro* interaction assay demonstrates that some of the identified PXXP sequences found in the dopamine D4 receptor as well as other GPCRs can interact with SH3 domains. The different

human dopamine D4 variants were all able to interact with the adaptor proteins Nck and Grb2. The rat D4 sequence which has PXXP motifs in the third intracellular loop, but does not exhibit the repeat region, was also able to interact with the SH3 adapter proteins. These results indicate that the interaction is not limited to the D4 receptor and that the VNTR polymorphism is not necessary for this interaction.

The interaction between the dopamine D4 receptor and the SH3 domain containing adapter proteins is specific, saturable and competitive in nature. The interaction of the receptors and the SH3 domains is specific as demonstrated by the lack of an interaction with just the GST fusion protein. Furthermore, the luciferase control protein which contains a PXXP motif, is unable to interact with the SH3 domains tested. In addition, not all the D4 constructs that contain PXXP sites interact with SH3 domains and the GPCRs only interact with a subset of the SH3 domains tested. This is clearly demonstrated by the construct expressing only the first and last repeat sequence (D4.2(249-280) (α_5)) which has six PXXP motifs, but did not show any interaction with the adapter proteins Nck and Grb2. The lack of interaction is not due to the short length of the construct because amino acid sequences of less than ten amino acids are able to interact with the SH3 domains (Ren *et al.*, 1993; Sparks *et al.*, 1994). The left-handed polyproline PPII helix can be formed as a short peptide. The interaction between the dopamine D4 receptor and the SH3 adapter proteins is dose dependent and saturable. These results indicate that the interaction is specific and thus mediated by a specific site. Furthermore, the interaction can be competed off with an excess of ligand.

The observed interaction is not mediated by the SH2 domain of the adapter proteins. There are no putative phosphotyrosine residues in the third intracellular loop of the dopamine D4 receptor, while the whole receptor only has a site in TM5. Therefore, the interaction between the third intracellular loop construct and Nck and Grb2 could not have been mediated by the SH2 domain. Furthermore, the Nck construct tested only expresses the three SH3 domains of the adaptor protein

and not the SH2 domain. These proteins have no other functional domains, indicating that the interaction is SH3 mediated.

The interaction between the dopamine D4 receptor and the SH3 domains of Nck and Grb2 are mediated by the third intracellular loop. The third intracellular loop is sufficient for binding as indicated by the D4 construct expressing only the third intracellular loop. Removal of a large portion of the third intracellular loop that contains all the PXXP sequences disrupts the interaction of the full length receptor with the SH3 domains. Therefore the third intracellular loop is necessary and sufficient for the SH3-mediated interaction.

The SH3 interaction of the human dopamine D4 receptor appears to be mediated by the sequences amino-terminal and carboxyl-terminal to the repeat. The repeat sequence is not necessary for the interaction since the rat D4 receptor and the human dopamine D4 construct not expressing the repeat sequence both showed strong interaction for the SH3 domain containing proteins, while the repeat sequence by itself did not interact with these proteins. From the saturation analysis it appears that the constructs not expressing the repeat sequence showed a higher affinity of interaction as compared to the full length wild type receptor constructs, except for the interaction observed between the D4.7 construct and Grb2. Removal of both the amino-terminal sequence and repeat sequence reduced the SH3 binding affinity about 20-fold, but did not eliminate binding. Subsequent removal of the carboxyl-terminal sequence, in addition to the amino-terminal and repeat sequence, eliminated all SH3 interaction, indicating that both the amino- and carboxyl-terminal sequences are required for maximal binding. This indicates that the dopamine D4 receptor interacts with two distinct sites in the adapter protein. This has been previously demonstrated for the Grb2 protein and HPK1 protein (Anafi *et al.*, 1997).

In conclusion the *in vitro* interaction assay suggests that at least some of the dopamine receptors can interact with SH3 domains. The interaction is dose-dependent, saturable and specific.

Chapter 4

Functional Characterization of Deletion Mutants

The experiments presented in this section involved a collaborative effort with Drs. Mark von Zastrow and Ross Vickery (Nina Ireland Laboratory, Departments of Psychiatry and Cellular and Molecular Pharmacology, University of California, San Francisco). James Oak (M. Sc. thesis, Department of Pharmacology University of Toronto) and Dr. H. -C. Guan were instrumental in the receptor binding, GTP γ ³⁵S, cAMP and MAPK assays.

4.1 Introduction

The *in vitro* experiments using the deletion mutants demonstrated that removal of the sequence that contained all the PXXP motifs in the third intracellular loop of the dopamine D4 receptor abolished the D4-SH3 interaction seen with the full length receptor. Furthermore, expression of only the carboxyl-terminal PXXP motif in the dopamine D4 receptor gave SH3 binding that was intermediate in strength compared to that of the full length receptor and that of the deletion mutant containing no PXXP motifs. As discussed in chapter one, the third intracellular loop, and in particular the amino- and carboxyl-terminal segments of the third intracellular loop proximal to the transmembrane domains of the GPCR have been found to be critical for G protein-coupling and specificity. In the following sets of experiments the functionality of the receptor with respect to ligand binding and G protein-coupling is established. The ability of the various dopamine D4 deletion mutant receptors to inhibit forskolin-stimulated cAMP levels is also assessed. Recent experiments have indicated that G_i-coupled GPCRs are able to stimulate the MAPK pathway (Koch *et al.*, 1994; Luttrell *et al.*, 1997b). SH3 domain-mediated interactions are essential in this pathway, as exemplified by the Grb2-Sos association that leads to Ras-MAPK activation. We assessed the ability of the

dopamine D4 receptor and the deletion mutant receptors to stimulate this pathway. Finally, research completed by von Zastrow et al. has shown that the dopamine receptors are subject to endocytosis. The D1 and D2 receptor display distinct patterns and mechanisms of internalization (Vickery and von Zastrow, 1999). The involvement of the SH3 domain containing proteins Grb2 and dynamin have been implicated in this function. Experiments were performed to characterize the ability of the dopamine D4 receptor and the deletion mutant D4 receptors to undergo this internalization process.

The deletion mutant D4.4.(Δ 221-337) resulted in the removal of not just the repeat region of the dopamine D4 receptor, but also the immediate flanking sequence. For the catecholamine receptors critical amino acid residues required for binding are located in the transmembrane domains and on the extracellular surface of the receptors (Missale *et al.*, 1998). The third intracellular loop of GPCRs is, in general, not involved in ligand binding. The deletion mutants made would not be expected to affect ligand binding. In contrast to ligand binding, the third intracellular loop is critical for G protein-coupling for the D2-like dopamine receptors. Previous studies have only suggested small differences in binding and coupling between the different dopamine D4 receptor variants (Asghari *et al.*, 1995; Asghari *et al.*, 1994). These studies suggest that the repeat region does not contribute significantly to G protein-coupling. G protein-coupling appears to be mediated by only a few amino acids at the extreme ends of the third intracellular loop that are proximal to TM 5 and TM6 (Wess, 1997). The intervening sequence is not believed to contribute significantly to G protein coupling. The deletion mutant, that had a large portion of the third intracellular loop removed, was tested for its ability to couple to G proteins.

Normal functioning for GPCRs, and presumably the dopamine D4 receptor, involves ligand binding which results in a conformational change in the receptor. The activation of the receptor allows the appropriate heterotrimeric G protein to couple. In the GDP bound form, the $G\alpha$ subunit is associated with $\beta\gamma$ and interacts with the receptor. Upon binding, a GDP for GTP exchange occurs

on the $G\alpha$ subunit which is believed to result in the dissociation of the activated $G\alpha$ subunit from the receptor and the $G\beta\gamma$ subunit (for review see Wess, 1998). For the dopamine D4 receptor, the inhibitory $G\alpha_i$ subunit that dissociates from the receptor acts to inhibit the activity of adenylyl cyclase. Ligand binding to the receptor is reduced in affinity when the non-hydrolysable GTP analogue guanylimido-diphosphate (Gpp(NH)p) is added. Gpp(NH)p is exchanged for GDP on the $G\alpha$ subunit upon G protein-coupling to the ligand-activated receptor, after which the activated G protein dissociates from the receptor. Gpp(NH)p is not released from the $G\alpha$ subunit, preventing the reassociation of the activated heterotrimeric G protein to the receptor, as a consequence reducing the ligand binding affinity of the uncoupled receptor. A reduction in affinity of the receptor, in the presence of Gpp(NH)p is taken as evidence for G protein coupling.

Functional coupling of GPCRs can also be confirmed by measuring the incorporation of $GTP\gamma^{35}S$. Proper coupling of the heterotrimeric G protein to the receptor results in the exchange of this non-hydrolysable radioactive GTP analogue for GDP upon receptor activation. Proper functioning of the receptor can also be assessed by measuring the effect of the receptor on cAMP levels. Activation of the wild type dopamine D4 receptor will normally inhibit forskolin-stimulated cAMP levels. The reduction in cAMP levels is evidence of successful coupling of the heterotrimeric G_i protein to the receptor and functional coupling to adenylyl cyclase.

Dopamine D4 receptor activation has been shown to result in mitogenesis by means of activation of the MAPK pathway (unpublished results). Ligand stimulation of the wild type receptor results in receptor activation and the phosphorylation of critical kinases involved in the MAPK pathway. Evidence from other PTX-sensitive GPCRs suggests that this activation of the pathway is mediated by the $G\beta\gamma$ subunit which can through a variety of mechanisms, involving for example P13-K, c-Src and Ras, activate the MAPK pathway (Gutkind, 1998b; Koch *et al.*, 1994; Luttrell *et al.*, 1996). The deletion mutant receptors were assayed for their ability to stimulate the MAPK pathway.

The dopamine D1 and D2 receptors have been shown to undergo internalization (Vickery and von Zastrow, 1999). This has not yet been established for the dopamine D4 receptor. Experiments were conducted to determine if the D4 receptor is subject to internalization and if the deletion mutants show differences in internalization from that of the wild type receptor.

4.2 Methods

4.2.1 Radioligand Receptor Binding

Ligand binding studies were completed as previously described (Asghari *et al.*, 1994; Sanyal and Van Tol, 1997). In brief, CHO-K1 cells expressing the D4.7 or the deletion mutant receptors were homogenized (Polytron, Brinkmann Instruments, Westbury, NY; maximum, 15 s) at 4 °C in binding buffer [50 mM Tris-HCl (pH 7.4), 1.5 mM CaCl₂, 5 mM MgCl₂, 5 mM KCl, 5 mM EDTA and 120 mM NaCl]. The homogenates were centrifuged for 20 min at 34 000 X g @ 4 °C, and the pellets were resuspended in binding buffer at a protein concentration of approximately 50-200 µg/ml. For the saturation binding analysis, 250 µl of the homogenates were incubated in duplicate with increasing concentrations (10-1000 pM) of [³H]spiperone (98 µCi/mmol) in 250 µl binding buffer (4 X final concentration). Competition binding analysis was performed by co-incubation of 300 pM [³H]spiperone and increasing concentrations (10⁻¹¹ - 10⁻⁴ M) of dopamine in either the presence or absence of 400 µM guanylimido-diphosphate (Gpp(NH)p). Nonspecific binding was determined by co-incubation of [³H]spiperone with 1 µM haloperidol. The samples were incubated in a final volume of 1 ml for 2 h at room temperature and then filtered using a combicell harvester (Skatron Instruments, Lier, Norway) and washed through glass fibre filters (with 50 mM Tris-HCl, pH 7.5, 2 X 9 s). Filters were transferred to vials with 5 ml scintillation cocktail and incubated overnight. Radioligand bound to the filters was detected by liquid scintillation counting (Beckman LS6000SC). The density of [³H]ligand-binding sites (B_{max}) and dissociation constants of ligands (K_d) were

determined by Scatchard analysis. The ligand binding data were analyzed by the nonlinear least squares curve-fitting program Ligand. A 12-point saturation binding plot (0.01 to 1 nM [^3H] spiperone) were used to determine the K_d (nM) and B_{max} (DPM).

4.2.2 GTP γ ^{35}S Binding Assay

Membrane preparations were tested for agonist-stimulated GTP γ ^{35}S binding. Membranes were incubated (30 °C, 30 min) in duplicate with dopamine (1 nM to 100 μM) in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM KCl, 4 mM MgCl_2 , 1.5 mM CaCl_2 , 120 mM NaCl, 10 μM GDP and 0.1-0.2 nM GTP γ ^{35}S (1250 Ci/mmol; NEN) in a total volume of 340 μl . Incubations were terminated by rapid filtration onto GF/A filters and the amount of protein-bound GTP γ ^{35}S was determined by scintillation counting. The disintegration per minute (DPM) values were corrected for non-specific binding which was determined with 10 μM GTP γ S. The Data was fit to a sigmoidal dose-response curve using Graph Pad Prism v2.0.

4.2.3 cAMP Measurements

cAMP measurements were completed as described previously (Asghari *et al.*, 1995; Sanyal and Van Tol, 1997). In brief, cells expressing the recombinant receptors were plated onto six-well, 35-mm dishes 2 days before the experiment and grown to 80-100% confluency ($\sim 1.0 \times 10^6$ cell/plate). These cells were washed with 2 ml HBBS buffer (118 mM NaCl, 4.6 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM D-glucose, 20 mM HEPES, and 0.3 mM isobutyl-1-methylxanthine, pH 7.2). To determine the potency of agonists, D4.7 and D4 deletion mutant expressing cell lines were incubated in triplicate with 10 μM forskolin in the presence or absence of 10 μM dopamine. The cells were incubated for 30 min at 37 °C in a final volume of 2 ml. At the end of the incubation period, the medium was removed by aspiration and the cells were harvested in 1ml ice-cold permeabilization

buffer [0.05 % (v/v) Triton X-100 in HBBS buffer]. The samples were scraped, transferred to eppendorfs, vortexed and spun for 5 min at 14 000 rpm (4 °C) in a microcentrifuge. The supernatant was collected and the cAMP concentration was assayed by cAMP radioimmunoassay (RIA) (Asghari *et al.*, 1995).

4.2.4 Radioimmunoassay

The cAMP concentration was assayed by radioimmunoassay as described in the protocol provided with the cAMP antiserum (Sigma, A-0670) and previously described (Asghari *et al.*, 1995). In brief, lysates of cells treated above were diluted 15-25-fold with ice-cold 50 mM sodium acetate buffer (pH 6.2). 100 µl of the sample was transferred in duplicate to 5 ml polypropylene test tubes and succinylated by the addition of 20 µl succinylation reagent (200 mg succinic anhydride dissolved in 1 ml anhydrous acetone, followed by the addition of 360 µl triethylamine). 2 ml of ice-cold 50 mM sodium acetate (pH 6.2) was added and mixed. 100 µl was transferred (in duplicate) to new 5 ml tubes and 100 µl cAMP antiserum was added (100 µl of 0.5 mg/ml solution prepared in 0.1 % BSA). After vortexing and incubation of the samples for 2 h at 4 °C, 100 µl of cAMP (adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl [¹²⁵I] iodotyrosine methyl ester, 0.045 µCi/ml in 50 mM sodium acetate, pH 6.2) was added. This was vortexed and incubated at 4 °C overnight. Next, 100 µl of 10 % BSA was added to each tube followed by 2 ml of ice-cold ethanol. Tubes were vortexed and centrifuged at 3500 X g for 20 min at 4 °C. The supernatant was removed by aspiration and the amount of radioactivity retained in the pellet was determined by gamma counting. The amount of radioactivity was converted to a corresponding amount of cAMP from a standard curve with a range of 0-5000 fmol of cAMP/100 µl sample volume assayed in parallel.

4.2.5 MAPK Assay

A MAPK assay, which detected the phosphorylation of Erk1 (p42) and Erk2 (p44) MAP kinases after receptor stimulation, was completed as described by the manufacturer (PhosphoPlus MAPK Antibody Kit, New England Biolabs Inc., MA) with the following changes. Cells were plated on 6-well plates and grown to about 80 % confluency. Prior to carrying out the drug incubations, the cells were incubated overnight with serum-free α -MEM. Two hours prior to the experiment, the media was again replaced with fresh serum-free α -MEM. To measure receptor-mediated stimulation of Erk1 and Erk2 phosphorylation, cells were incubated with α -MEM or α -MEM and 10 μ M dopamine for 5 min at 37 °C.. Incubations were stopped by washing cells with 2 ml ice-cold 1 X PBS, followed by the addition of 200 μ l sample buffer (62.5 mM Tris-HCl (pH 6.8), 2 % w/v SDS, 10 % glycerol, 50 mM DTT, 0.1 % w/v bromophenol blue).

4.2.6 Receptor Internalization Assay

Immunofluorescence microscopy was used to visualize HA and Flag epitope tagged receptors that were stably transfected into HEK-293 cells. Cells grown on glass coverslips were preincubated for one hour with HA.11 (3 μ g/ml; Berkeley Antibody Co., Richmond, CA) or M1 (3 μ g/ml; Kodak, New Haven, CT) monoclonal antibody to allow the antibodies to bind the epitope-tagged receptors exposed at the plasma membrane. The experiments were stopped by fixing, then the cells were permeabilized and visualized using fluorochrome-labeled secondary antibodies as described previously (Chu *et al.*, 1997).

4.3 Results and Discussion

4.3.1 Pharmacological Characterization of Deletion Mutants

Saturation binding analysis with [3 H] spiperone indicated that the D4.7 receptor and the two deletion mutant receptors, D4.(Δ 221-315) and D4.(Δ 221-337), showed saturable, concentration dependent binding with affinities in the range of 100 to 300 pM and a $B_{\max}$ of about 2 pmol/mg protein. Competition of [3 H] spiperone binding by dopamine indicated no significant differences between the D4.7 receptor and the deletion mutant receptors D4.(Δ 221-315) and D4.(Δ 221-337). All receptors tested showed a high affinity for dopamine binding, in the order of 1-5 nM, which could be shifted by the addition of Gpp(NH)p to a lower affinity (Figure 4.1). Cells expressing only the expression vector pcDNA3 displayed no measurable [3 H] spiperone binding.

FIGURE 4.1 Binding characterization of the D4.7 receptor and the D4 deletion mutant receptors. Competition binding analysis of [3 H] spiperone binding on membranes isolated from the cell lines D4.7, D4.(Δ 221-315) or D4.(Δ 221-337) by dopamine in the presence (□) and absence (■) of Gpp(NH)p was done as described in the "experimental procedures". The logarithm of the concentration of the drug in molar (M) used is indicated on the x-axis. The amount of specific binding expressed as disintegrations per minute (DPM) is indicated on the y axis. The assay was done in parallel for the different receptors in the presence and absence of Gpp(NH)p using the same membranes preparations. The results are representative of two independent experiments.

Removal of the repeat region and the immediate surrounding sequence did not impair the ability of the dopamine D4 receptor to bind either the agonist dopamine or the antagonist spiperone, indicating that the ligand binding site is unaffected. The ability of the non-hydrolysable GTP analogue Gpp(NH)p to shift the receptor to a lower affinity for dopamine indicates that the receptor

successfully couples to G protein. Gpp(NH)p is exchanged for GDP on the $G\alpha$ subunit, and prevents the reassociation of the activated G protein with the receptor, thus decreasing the receptor's ligand binding affinity. These results suggest that the deletion mutants bind ligand normally and couple to G protein.

4.3.2 GTP γ ³⁵S Coupling of the Deletion Mutant D4 Receptors

Functional coupling of the dopamine D4.7, D4.(Δ 221-315) and D4.(Δ 221-337) receptors was measured by dopamine-induced GTP γ ³⁵S binding. These binding assays demonstrated a concentration-dependent dopamine-mediated binding of GTP γ ³⁵S to these receptors (Figure 4.2). Cells expressing only the expression vector pcDNA3 displayed no measurable induction of GTP γ ³⁵S binding by dopamine.

FIGURE 4.2 GTP γ ³⁵S Coupling of the deletion mutant dopamine D4 receptors. Dopamine-stimulated GTP γ ³⁵S binding by the D4.7 and deletion mutant receptors is expressed as % over basal binding. The logarithm of the concentration of the drug in molar (M) used is indicated on the x-axis. The amount of GTP γ ³⁵S binding expressed as % over basal binding is indicated on the y axis. The results are representative of two independent experiments.

Successful coupling of the heterotrimeric G protein results in the incorporation of the radioactive GTP. These results are in agreement with the results seen with a shift in binding in the presence of Gpp(NH)p, suggesting that the receptor, upon agonist binding, successfully binds G protein. Thus the receptor should be able to affect the traditional second messenger systems mediated

by the $G\alpha$ and $G\beta\gamma$ subunits.

4.3.3 Modulation of Intracellular cAMP Levels by Mutant D4 Receptors

Intracellular cAMP levels were measured in CHO-K1 cells expressing either the D4.7 receptor or the deletion mutant D4.(Δ 221-315) or D4.(Δ 221-337). Forskolin-stimulated increases in cAMP levels were blocked by dopamine in cells expressing the D4.7 receptor, but not in cells expressing the deletion mutant receptor D4.(Δ 221-337) (Figure 4.3). Stimulation of the D4.(Δ 221-315) deletion mutant with dopamine showed a decreased ability to inhibit forskolin-stimulated cAMP levels. These results suggest that the dopamine D4 receptor is not functional with respect to adenylyl cyclase activity. This is in contrast to the coupling results, which indicate that the deletion mutants have not lost their ability to couple to G proteins and would be expected to have normal activity.

FIGURE 4.3 Modulation of Intracellular cAMP Levels by Mutant D4 Receptors. Inhibition of forskolin-stimulated cAMP levels by dopamine in CHO-K1 stable cell lines expressing the D4.7 or the deletion mutant receptors is shown. Intracellular levels of cAMP (M) was measured after incubation of cells with forskolin (10 μ M) in the presence (dashed bar) or absence (open bar) of dopamine (10 μ M) for 30 min. The average of three independent experiments assayed in duplicate are shown. The CHO-K1 cell line expressing pcDNA3 was used as control.

4.3.4 Activation of the MAPK Pathway by the Dopamine D4 receptor

A MAPK assay was performed to assess the ability of the D4 receptor and D4 deletion

mutants to activate the MAPK pathway. Stimulation of the D4.7 receptor in CHO-K1 cells by dopamine (10 μ M) resulted in strong activation of the MAPK pathway as determined by the immunodetection of the phosphorylated Erk1 and Erk2 MAP kinases (Figure 4.4). The deletion mutants D4.(Δ 221-315) and D4.(Δ 221-337) demonstrated a very weak activation of the MAPK pathway that is comparable to the control cells, treated with dopamine, that did not express the dopamine D4 receptor construct.

FIGURE 4.4 Activation of MAPK Pathway by the Dopamine D4 receptor. Immunoblot detection of the phosphorylated Erk1 or Erk2 MAPK proteins after stimulation of the dopamine receptors or deletion mutants with dopamine (10 μ M) in CHO-K1 cells. In all of these experiments the cells stably expressing the eukaryotic vector pcDNA3 in CHO-K1 cells served as control. The blot is representative of three independent experiments. The CHO-K1 cell line expressing pcDNA3 was used as control.

These results indicate that the deletion mutant receptors are not able to activate the MAPK pathway. Several possible reasons for this may include an inability of the G protein to uncouple from the deletion mutant receptor, consequently the G $\beta\gamma$ cannot affect the MAPK pathway. Alternatively, expression of the receptor may have been altered which would affect the receptors ability to activate both adenylyl cyclase and MAPK. It is also possible that the deletion of the SH3 motifs from the dopamine D4 receptor may have eliminated the receptors ability to bind SH3 domain containing proteins that may be essential for the direct activation, through a non-G protein-mediated pathway, of MAPK.

4.3.5 Internalization of D4.4 and the D4.4 Deletion Mutants

The internalization and localization of D4.7 and the deletion mutants D4.(Δ 221-315) and D4.(Δ 221-337) were analyzed using an antibody uptake experiment in untreated cells. Plasma membrane-bound receptor was first labeled with a primary antibody, then after incubating the cells, the internalization of the receptor-antibody complex was visualized by immunofluorescence. The D4.7 receptor was localized predominantly in the plasma membrane (Figure 4.5 A) while the deletion mutant that removed all the potential SH3 binding domains, D4.(Δ 221-337), was predominantly localized in endosomal vesicles (Figure 4.5 C). The D4.(Δ 221-315) deletion mutant, which still

FIGURE 4.5 Receptor Internalization Assay. Immunofluorescence microscopy was used to visualize Flag epitope tagged versions of the D4.7 (A), D4.(Δ 221-315) (B) and D4.(Δ 221-337) (C) receptors that were transiently transfected into HEK-293 cells. The subcellular distribution of the receptors were visualized using fluorochrome-labeled secondary antibody (see "experimental procedures" for details). The HA epitope tagged receptors showed a similar distribution (data not shown).

contains a potential SH3 binding domain carboxyl-terminal to the repeat region showed an intermediate phenotype for internalization (Figure 4.5 B). Radioligand receptor binding analysis on HEK 293 cells exhibited equivalent amounts of expressed receptor for the deletion mutant receptors and the wildtype D4 receptor (~500 fmol).

The visualization of the receptor inside the cell and not at the plasma membrane indicates that

the receptor is rapidly internalized. The receptor is successfully transported to the cell surface after expression as demonstrated by the successful labeling of the receptor at the cell surface by the antibody.

The rapid internalization of the deletion mutant dopamine D4 receptor indicates that the region of the third intracellular loop containing the SH3 motifs is important in cellular localization of the receptor at the plasma membrane. Removal of this region severely affects the ability of the receptor to remain at the cellular surface. This occurs even in the absence of ligand, indicating that this process is constitutive. Normally, the dopamine D4 receptor is localized on the plasma membrane and exhibits very low levels of internalization, even upon receptor stimulation.

The third intracellular loop of the α_{2A} -adrenergic receptor has recently also been shown to be important for maintaining receptor expression at the cell surface (Edwards and Limbird, 1999). Removal of the third intracellular loop resulted in a constitutive internalized receptor indicating that, like the dopamine D4 receptor, the third intracellular loop is important for receptor localization.

The internalized deletion mutant dopamine D4 receptor could account for the functional impairment observed with the cAMP and MAPK assay. An internalized dopamine D4 receptor would be unable to bind ligand at the extracellular surface upon cellular stimulation and thus would affect receptor function. It is likely that the internalized receptor resulted in the lack of an effect on adenylyl cyclase and may also account for the inability of the receptor to activate the MAPK pathway. The ligand binding study and the GTP γ ³⁵S assay would not be affected by the internalized phenotype, since both these assays are completed in a cell free system that are unaffected by receptor localization. The deletion mutant that still expresses the carboxyl-terminal PXXP motif and exhibits an intermediate internalization phenotype also displayed a partially impaired ability to inhibit forskolin-stimulated cAMP levels. These results suggest that the receptor may still have normal cAMP function, but that this is compromised by the internalization of the receptor. Impairment of MAPK activation

of the deletion mutant receptor may also be attributed to the internalized receptor phenotype. Alternatively, the impaired MAPK activity may be the direct result of an impairment in SH3 binding as observed for the adapter protein Grb2. This is in contrast to the results by Luttrell et al. (1999) that indicate receptor internalization is a prerequisite for MAPK activation. These results suggest that the receptor must undergo a specific type of internalization or that important protein interactions with the GPCR must be made for it to activate the MAPK pathway. Receptor-SH3 binding may be important for the direct interaction of components in the MAPK pathway, or it may be important in receptor localization that are both impaired in the deletion mutants.

The results of the functional assays indicate that both deletion mutant dopamine D4 receptors tested maintained their ability to bind to ligand and couple to G proteins. However, these receptors showed an impaired ability to inhibit cAMP levels and stimulate the MAPK pathway. Receptor internalization, possibly because of the removal of the PXXP motifs in the third intracellular loop of the receptor, found to bind to SH3 domain containing proteins, may account for the receptor's reduced functional capabilities. Only removal of all the PXXP motifs results in maximal internalization and the strongest functional impairment of the receptor.

Chapter 5

Interaction of SH3 Domains and the Dopamine D4 Receptor in a Living Cells

The yeast two-hybrid experiments presented in this section involved a collaborative effort with Dr. Mordechai Anafi, postdoctorate fellow in Dr. T. Pawson lab (Mount Sinai hospital, Toronto).

5.1 Introduction

The ability of SH3 domains to interact with the dopamine D4 receptor in the *in vitro* assay system indicates that the requirements for SH3 binding have been met. The results of the described studies have shown that the dopamine D4 receptor can bind to several SH3 domain containing proteins in a specific, saturable, dose-dependent and competitive manner. In the case of Grb2, this interaction can be disrupted by a single specific amino acid mutation in just one of the SH3 domains. Furthermore, the interaction has been localized to a specific region in the third intracellular loop of the dopamine D4 receptor. The functional studies suggest that this same region, involved in SH3 binding, is also involved in the internalization of the receptor and possibly in MAPK activation by the receptor.

In vitro, Nck and Grb2 demonstrated a high affinity for the dopamine D4 receptor. These results, however, do not necessarily demonstrate that Nck and Grb2 represent the endogenous SH3 binding partners for the dopamine D4 receptor. In the *in vitro* assay, only specific proteins of interest are presented to each other at concentrations and conditions which likely differ markedly from those normally observed in the cell. Therefore, the Nck and Grb2 interactions need to be confirmed *in vivo*. It is also possible that Nck and Grb2 are not the endogenous ligands and that the true physiological SH3 binding partner of the dopamine D4 receptor binds the receptor with a higher affinity, precluding the established, but lower affinity, Nck and Grb2 interactions. A comprehensive screen of all the

different SH3 domain containing proteins and their affinity for the D4 receptor was not performed using the *in vitro* assay. The possibility that proteins such as amphiphysin, shown to be involved in GPCR endocytosis, interact with the D4 receptor needs to be tested.

An endogenous SH3 interaction with the dopamine D4 receptor may also be regulated by receptor and/or cellular processes. Protein concentration and localization may determine the ability of these proteins to interact with the dopamine D4 receptor. The expression of both proteins in the same cell and cellular compartment is a necessary prerequisite to binding. Receptor occupancy, conformation and phosphorylation state of the receptor may also affect the interaction. Conformational changes brought on by ligand binding may alter the ability of the receptor to bind SH3 domain containing proteins by covering or uncovering the SH3 binding site, in a similar manner as that seen with G protein-coupling. Phosphorylation of serines and threonines adjacent to the PXXP motif has been shown to disrupt or prevent SH3 binding (Chen *et al.*, 1996a). Second messenger-dependent kinases may phosphorylate the D4 receptor in a heterologous manner thus regulating SH3 interactions. It is important to identify the endogenous SH3 domain containing proteins that interact with the dopamine D4 receptor, to determine the conditions which allow this interaction in the cell and how this affects cellular function.

Co-immunoprecipitation provides convincing evidence of endogenous protein interaction. Disruption of this interaction would then be expected to alter cellular function. Ideally, immunoprecipitation of the naturally expressed protein would result in the copurification of the interacting proteins. Often, as is the case with the dopamine D4 receptor, cellular expression levels of the protein are so low that immunoprecipitation of the naturally present receptor is insufficient to detect the receptor, let alone the interacting protein complex. Different expression techniques are then required to increase the cellular expression levels of the protein. Although an interaction between two proteins may be confirmed this way, the increased expression levels of a specific protein may not be

physiologically representative of the system and may “force” the interaction.

The yeast-two hybrid system has been used to identify and confirm protein-protein interactions as well as identify novel protein interactions (for review see Fields and Sternglanz, 1994; Young, 1998). Proteins of interest are expressed as fusion proteins in yeast to determine if they interact. The conditions are more physiologically representative of those in the cell, than those used in the *in vitro* system. The presence of a full complement of proteins in an intracellular environment more closely resemble the conditions inside the cell and more likely to maintain the integrity of the protein interactions. The yeast two-hybrid system allows for the expression and proper folding of the protein of interest.

5.1.1 Interaction In Yeast

The yeast two hybrid system has been successfully used to demonstrate and confirm SH3 interactions (Anafi *et al.*, 1996; Hu *et al.*, 1995; Lussier and Larose, 1997). Briefly, the two proteins of interest are expressed as fusion proteins coupled to two components of the yeast Gal4 transcription activator protein: a DNA binding domain (BD) and a DNA activation domain (AD) respectively (for review Young, 1998). Successful interaction of the proteins of interest brings together the DNA binding and DNA activation domains into close proximity of each other, which after being transported to the nucleus of the yeast, allows the complex to bind to the DNA upstream activator sequence (UAS) and activate or drive the expression of the reporter gene (Gal4 UAS reporter gene). Successful interaction between protein species is indicated by a blue stain when the lacZ reporter gene is expressed. The assay is highly sensitive and can detect interactions in the range of (K_d) 10^{-6} M (Durfee *et al.*, 1993). The yeast two-hybrid assay may be more sensitive than other protein-protein interaction assays. The well established interaction between Ras and Raf was observed in the yeast two-hybrid system, but had not been detected by co-immunoprecipitation techniques (Van Aelst *et*

al., 1993). Proteins as short as 8-10 amino acids and up to 750 amino acids can be used (Golemis *et al.*, 1997). The proteins of interest can be swapped between the DNA binding domain and the activation domain. This has the advantage of confirming interactions and may avoid problems of self activation often associated with this system. The yeast two-hybrid system can be used to identify the area of the protein that is involved in the interaction by constructing hybrid proteins. The yeast two-hybrid system can also be used to screen a yeast expression library to identify novel protein interactions with the protein of interest.

There are a number of limitations to the yeast two-hybrid system which may prevent the demonstration of a protein-protein interaction. Not all proteins are properly expressed in yeast. Short, hydrophilic protein fragments are more likely to be properly expressed than large proteins with hydrophobic portions. Proteins that are membrane associated or proteins that undergo post-translational modifications are often not properly expressed (Young, 1998). Many proteins expressed in this system result in self activation, making the confirmation of a protein-protein interaction impossible. In this case the fusion protein by itself, either expressed as the DNA binding domain or activation domain, is sufficient to drive expression of the reporter gene. Another problem encountered is that the assay may not always detect known interactions. This may be due to problems with the conformation of the protein, competition with endogenous yeast ligands or problems of expression that may interfere with the ability to detect an interaction. False positives do not appear to be caused by non-specific binding of proteins (Fields and Sternglanz, 1994), such that positive interactions likely represent specific protein-protein interactions. Despite these problems, the yeast two-hybrid system is a robust interaction assay that has been successfully used to demonstrate known interactions and identify novel interactions. In fact, the yeast two-hybrid system has been successfully used to demonstrate protein-protein interactions for GPCRs, by expressing short hydrophilic fragments of the receptor (Kajkowski *et al.*, 1997).

5.1.2 Co-Immunoprecipitation

In co-immunoprecipitation experiments, specific antibodies directed against one of the proteins of interest is used to immobilize and purify the protein, and the interacting protein, from the cellular lysate by means of a secondary antibody conjugated to agarose beads. The purified proteins are then transferred to a western blot. Protein interaction is demonstrated by the successful labeling of the putative interacting protein with a specific antibody directed against that protein.

In some cases, the protein of interest is expressed at such low endogenous levels, that it cannot be detected. Researchers have relied on different expression techniques to increase the amount of protein in cultured cell lines. The use of different expression vectors and the use of transient, stable, adeno- or baculoviral expression techniques have all been used to express proteins at significantly higher levels. Furthermore, these expressed proteins can also be engineered to carry an epitope tag for which commercially available, highly specific, antibodies exist. This ensures the purification and identification of the specific protein of interest and may circumvent the problems associated with developing a specific antibody in cases where this proves to be difficult (as is the case with the dopamine D4 receptor). Although this allows for the purification of the protein and may demonstrate the putative interaction, it may also “force” certain interactions which are normally not observed because of alterations in protein concentration. However, these expression techniques have proven to be useful in the identification and characterization of many physiological relevant protein-protein interactions.

Co-immunoprecipitation techniques demonstrate protein-protein interactions in a physiologically representative system (Phizicky and Fields, 1995). Mammalian protein expressed in mammalian cells are subject to the proper expression and post-translational modifications. Membrane-bound receptors, such as the GPCRs, are properly folded and inserted into the membrane. Furthermore, the interaction occurs in a cell system that contains a full complement of proteins that

compete or are required for the observed interaction (this is less true when proteins levels are increased using recombinant expression techniques). Co-factors that may be necessary for specific protein interactions are present in the *in vivo* system. In addition, the functional effects of receptor activation and phosphorylation on protein-protein interactions can be investigated. Protein modification, by the disruption of the putative binding site should eliminate the observed specific interaction.

Since the endogenous SH3 domain containing protein that binds the dopamine D4 receptor is unknown, a method of screening a panel of different SH3 domain containing proteins is required to identify the SH3 domain(s) of highest affinity and that display endogenous interactions. Co-immunoprecipitation by purifying the SH3 domain with the dopamine D4 receptor and vice versa would lend support that the identified proteins interact *in vivo*. A number of likely interacting candidates was identified from the 60 or so different SH3 domain containing proteins identified to date. Several criteria were used to include these proteins, including cellular localization and known participation in GPCR function. Disruption of the interaction resulting in a functional effect would support the validity of the interaction.

5.2 Methods

5.2.1 Yeast Two-Hybrid Assay

The yeast two-hybrid assay was performed as previously described (Anafi *et al.*, 1996; Durfee *et al.*, 1993). pASI vectors, encoding fusions between the DNA-binding domain of *GAL4* and the different SH3 domains, and pACTII vectors encoding the transcriptional activation domain of *GAL4* fused to different fragments described above were transformed into *Saccharomyces cerevisiae* strains Y-153 and Y-187, respectively. Yeast transformation was performed by the lithium acetate method (Ausubel *et al.*, 1990) except that 10 % dimethyl sulfoxide was included during 42 °C heat shock. Co-

expression of pASI and pACTII vectors were achieved by mating Tyr-153 and Tyr-187 yeast strains, each containing the appropriate vector. β -Galactosidase activity was detected on 5-bromo-4-chloro-3-indoyl β -D-galactoside plates after permeabilizing the yeast by liquid nitrogen treatment.

The sequences encoding the third intracellular loop of the human D4.2, D4.4 and D4.7 dopamine receptors (219-304, 219-336 and 219-384 respectively) were subcloned into the BamHI site of the yeast two- hybrid expression vector pACTII. The Crk SH2 domain (10-105 of Crk-II) and the Sos tail were subcloned into the BamHI/EcoRI site of pACTII (Anafi *et al.*, 1997). The entire Grb2 protein was subcloned into SmaI/EcoRI site of pACTII.

The mouse Abl SH3 domain (65-123 of p210 Bcr-Abl), the Grb2 amino- (N) or carboxyl- (C) terminal domains (1-59 and 158-217 respectively), the human PLC γ (792-851) and the mouse Crk carboxyl- (C) terminal SH3 domain (235-293) were subcloned into the SmaI/BamHI site of the pASI vector (Anafi *et al.*, 1996). The entire human Grb2 sequence and the Nck 3SH3 (1-255) sequence, encoding only the three SH3 domains, was subcloned into the BamHI/EcoRI site of pASI. The third intracellular loop of the human D4.2(219-304), D4.4(219-336) and D4.7(219-384) dopamine receptors were subcloned into the SmaI/SfiI sites of the yeast two-hybrid expression vector pASI.

5.2.2 Immunoprecipitation Assay

5.2.2.1 Vector Constructs

The region encoding the third intracellular loop of the dopamine D4.4 receptor was subcloned into the EcoRV/XbaI site of the modified eukaryotic expression vector pcDNA3 (Invitrogen) (Anafi *et al.*, 1996) such that a start codon followed by an HA tag (YPYDVDPYA amino acid sequence inserted into the HindIII/BamHI site of pcDNA3 vector), followed by the coding sequence of D4.4(220-336) and a stop codon allowed for the expression of the receptor fragment, creating the D4.4(220-336)(HA)pcDNA3 construct. The third intracellular loop fragment of D4 was excised using

an Xba I/Sac I digest from the existing D4.4(220-336) construct (see chapter 2, page 76) and inserted into the Eco RV/Xho I site of pcDNA3 after appropriate modifications of the restriction sites.

A full length D4.4 receptor (SSFD4) eukaryotic expression construct, with a flag tag sequence for antibody recognition and a signal sequence to increase expression levels of D4 was created. A Hind III/Xba I fragment of the D4.4 receptor was inserted into the Hind III/Xba I site of the eukaryotic expression vector pRc/RSV (Invitrogen, San Diego, CA, U.S.A.) (Asghari *et al.*, 1995). Subsequently, a modified influenza hemagglutinin signal sequence (MKTIIALSYIFCLVFA) and an eight amino acid "Flag" tag epitope sequence (DYKDDDDA) (called SSF) (generously donated by B. K. Kobilka) (Guan *et al.*, 1992) was inserted into the 5' coding sequence of the D4.4 cDNA as a Hind III/Nco I fragment.

The D4.4(219-336)(HA)RSV construct was created by excising the activation domain, HA tag, D4.4 third intracellular loop region, and the terminator sequence from the existing D4.4 pACTII construct (see chapter 5, page 121 with a Hind III digest and inserting the sequence into the Hind III site of the pRc/RSV vector. The full length dopamine D4.4 receptor was subcloned into the HindIII/XbaI site of the pRc/RSV vector. The dopamine D4.4 receptor was subcloned into a recombinant adenovirus (AdRSVD4.4) as previously described (Graham and Prevec, 1991; Knapp *et al.*, 1998). As controls, a pRc/RSV (RSV), Crk-IIpcDNA3-HA (Anafi *et al.*, 1996), β GAL in pRc/RSV (β GALRSV) and the luciferase gene expressed in the adenovirus construct (AdRSVLuc) were created (Knapp *et al.*, 1998).

5.2.2.2 Expression of the D4 Constructs

In vitro expression of the constructs was achieved as described in chapter 2 (page 78) using the TNT coupled Reticulocyte Lysate System (Promega). Where appropriate, canine pancreatic microsomal membranes (Promega) were added to the transcription/translation reaction according to

the manufacturers instructions.

Transient expression of the different constructs was achieved by means of electroporation, calcium chloride transfection, or lipofection into the COS-1, COS-7, SKNMC or HEK-293 cell lines (American Type Culture Collection). For lipofection, lipofectin (GibcoBRL) was used and the manufacturers instructions were followed. Briefly, in one polystyrene tube 1.5 ml of optimem (GibcoBRL) and 10 μ l lipofectin was added. To the other tube 1.5 ml optimem and DNA (3 or 10 μ g) in 100 μ l 1 X PBS was added. The two tubes were incubated for 30 min, mixed together at RT for 15 min and then added to a 50 % confluent 100 mm plate of cells with 3 ml 1 X PBS. The plates were incubated for 4-5 h and then 3 ml of α -MEM media supplemented with 20 % FBS was added. For the calcium chloride method, 0.6 ml of 2.5 M CaCl_2 was added to 5.4 ml TE and 20 μ g of template DNA. The DNA solution was added drop-wise to 6 ml of 2 X HBS while gently flicking the tube. The mixture was incubated for 20-30 min at RT and then pipetted up and down once. To the confluent plates, the media was removed and 1 ml of the precipitated DNA solution was added to the cells and incubated for 15 min at RT. 10 ml of media was added and incubated at 37 °C for 24 h. After 24 h, the media was removed, the cells were rinsed with 1 X PBS and media was added for an additional 24-60 h after which cells were harvested. All plates were grown to 100 % confluency prior to collection.

Electroporation was used for stable and transient transfection of cells. Cells were harvested by first aspirating the media, the cells were rinsed for 1-2 min in fresh 1 X PBS (pH 7.2) containing 2 mg/ml trypsin and then the cells were collected in 1 X PBS. The cells were then centrifugated for 10 min at 700 X g, the supernatant discarded and the cells resuspended in 1 X PBS. The cells (5×10^7 cells/ml in 1 X PBS) were transfected at 4 °C with the appropriate construct (40 μ g of DNA/ 5×10^7 cells) in a 2 mm cuvette using the BTX 600 Electro Cell Manipulator (capacitance = 400 μ F, $V=174$ V and resistance @ R1). The transfected cells were seeded at 5×10^6 cells/100 mm plate and

grown in the appropriate medium.

Expression levels for transient expression conditions were checked with an X-Gal histochemical assay. Cells simultaneously transformed with the β -galactosidase gene in the eukaryotic expression vector RSV were assayed for blue stain expression. The medium was aspirated and 1 ml of 4 % paraformaldehyde was added to each well in a 6 well plate and left for 5 min at 0 °C. The cells were rinsed two times with 1 X PBS and then 10 ml β Gal stain (1 M Na_2HPO_4 , 1 M NaH_2PO_4 , 1 M MgCl_2 , 50 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 50 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 20 mg/ml X-GAL) was added and incubated at 37 °C. Expression was confirmed by the appearance of blue stain.

The SSFD4 vector was stably expressed in CHO-K1 cells (American Type Culture Collection). CHO-K1 cells and subclones stably expressing D4 receptors were grown in α -MEM supplemented with 2.5 % fetal calf serum and 2.5 % HS at 37 °C and 5 % CO_2 . Transfected cells were continuously grown in the presence of G418 (400 $\mu\text{g/ml}$). After clonal selection and expansion of several G418-resistant cells, stable expression of D4 receptors was determined by [^3H] spiperone binding (see chapter 4 page 104).

Recombinant adenoviral expression of the dopamine D4 receptor (AdRSVD4.4) into HEK-293 cells and SKNMC cells has been previously described (Knapp *et al.*, 1998). Briefly, the shuttle plasmid pXCJL-2, containing the transgene of interest, was cotransfected with the plasmid pJM17 into HEK-293 cells and overlaid with agarose. Plaques were picked, verified by restriction digestion and subjected to plaque purification followed by amplification in HEK293 cells and subsequent purification by two rounds of CsCl density ultracentrifugation. CsCl purified virus was dialyzed using a Pierce Dialysis Cassette (Pierce) and viral stocks were aliquoted and stored at -70 °C. Titers were determined with a HEK-293 plaque assay. Stocks were assayed for wild type adenoviruses using HeLA plaque assays and PCR with E1 specific primers (Zhang *et al.*, 1993; Zhang *et al.*, 1995).

5.2.2.3 Immunoprecipitation and Immunoblotting Conditions

The sources for the different antibodies and the amounts used for immunoprecipitation (ip) and immunoblotting (ib) are detailed below. The M1 monoclonal antibody (Kodak; 5.0-10 µg/ml ip, 1.0-2.0 µg/ml ib, NB. 1 mM CaCl₂ was added to all solutions when using this antibody) and the anti-Flag probe rabbit polyclonal antibody (Santa Cruz, 0.2 µg/ml ib) were used against the Flag tag sequence. From Santa Cruz the following antibodies directed against the dopamine D4 or SH3 domain containing proteins were obtained: anti-D4 goat polyclonal antibodies directed against the carboxyl-terminal tail of the dopamine D4 receptor (sc-1438 and sc-1439; 0.625 µg/µl ib), mouse monoclonal anti-Grb2 antibody (1.0-2.0 µg/ml ip, 0.5-1.0 µg/ml ib) and rabbit anti-Grb2 polyclonal antibody (1.0-2.0 µg/ml ip, 0.5-1.0 µg/ml ib). Protein A sepharose (25-50 µl), anti-mouse agarose (25-50 µl), anti-goat agarose (25-50 µl), goat anti-mouse horse radish peroxidase antibody (1:5000-1:10 000), anti-rabbit horse radish peroxidase antibody (1:5000-1:10 000) and anti-goat horse radish peroxidase (1:5000-1:10 000) were obtained from Sigma Immunochemicals. From Boehringer-Mannheim, mouse monoclonal anti-HA antibody directed against the HA tag (15 µg/ml ip, 4 µg/ml ib) was obtained.

The following antibodies were gifts: the anti-D4 monoclonal antibody (1 µl/ml ip, 1:2000 ib, a gift from Dr. D. Hartman, Hoffman-LaRoche (Lanau *et al.*, 1997a)), the anti-HA antibody (Anafi *et al.*, 1996) (a gift from T. Pawson; 1:5 ib of ascite) and the Nck antibody (Garrity *et al.*, 1996) (a gift from T. Pawson; 5-50 µl/ml ip, 1:200 ib of ascite)

The following lysis buffers were used to solubilize and purify the dopamine D4 receptor or the third intracellular loop fragment: Triton lysis buffer (0.1 % triton, 150 mM NaCl, 10 % glycerol, 50 mM Tris-HCl (pH 7.5), 0.1 mM NaVO₃, 0.1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin), NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 % Nonidet P-40, 0.1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin), 3 % NP-40 lysis buffer (100 mM Tris-HCl, 300 mM NaCl,

2 mM CaCl_2 , 2 mM MgCl_2 , 3 % Nonidet P-40, 2 mM NaF, 2 mM Na Orthovanadate, 20 % glycerol, 200 μM PMSF, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin and 10 $\mu\text{g/ml}$ pepstatin) protein solubilization buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 7.1), 1 mM DTT, 100 mM NaCl, 0.9 % detergent (CHAPS or digitonin), 0.1 mM PMSF, 2 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin), chaps digitonin lysis buffer (150 mM NaCl, 0.5 % CHAPS, 0.5 % digitonin, 50 mM Tris-HCl (pH 8.0), 0.1 mM PMSF, 2 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin), *in vitro* lysis buffer (1 % Triton, 1 % Tween20, 10 mM DTT, 10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin in 1 X PBS). 1 mM CaCl_2 was added to the above lysis buffers when immunoprecipitating using the M1 antibody (an excess of CaCl_2 was added for the lysis buffer containing EDTA immediately prior to adding the primary M1 antibody).

When the transfected cells reached 100 % confluency they were harvested by aspirating the media, rinsing with ice cold 1 X PBS (two times) and then adding the appropriate lysis buffer to the tissue culture plates (1-3 ml for 100-150 mm plates) for 30 min on ice. The cell lysates were scraped, collected in eppendorfs, gently shaken for 10-30 min and centrifuged (10 min at 15 000 X g). The supernatant was transferred to fresh tubes. The supernatant was gently shaken for 1-2 h with the appropriate primary antibody and for an additional hour or overnight with the appropriate secondary antibody linked to agarose beads or protein A sepharose beads (25-50 μl slurry). The agarose/sepharose bead complex was purified by centrifugation (1 min at 500 X g) and the supernatant removed by aspiration. The beads were washed three times with ice cold lysis buffer, centrifuged (500 X g) and supernatant was removed by aspiration. All steps were performed at 4 °C.

In an alternate protocol, after the cells were collected in lysis buffer, they were placed in a glass homogenizer, subjected to twenty strokes and centrifuged at 300 000 X g for 1 h (4 °C). The supernatant was transferred to a separate tube. The pellet was redissolved in lysis buffer, homogenized, as above, and gently shaken for 1 h to dissolve the membranes. To both the supernatant and the membrane portions of the sample, the primary antibody was added and the

immunoprecipitation protocol described above was performed. All steps were performed at 4 °C.

Sample buffer (2 X Tris-glycine sample buffer; 0.125 M Tris-HCl, pH 6.8, 20 % (v/v) glycerol, 2 % (w/v) SDS, 0.05 % bromophenol blue, 0.5 % (v/v) β -mercaptoethanol) was added to the isolated bead complex or cell lysates and the mixture was incubated for 5 min at 80 °C. The cell lysates or the purified supernatant were separated on a SDS-poly acrylamide gel electrophoresis. The proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with blotto (5 % w/v skim milk and 0.02 % sodium azide in TBST (0.2 % Tween 20 dissolved in TBS)) for 2 h to overnight with shaking. Specific proteins were detected by immunoblotting with the appropriate primary antibody in blotto (1 h) followed with four washes (5 min) with TBS. The appropriate peroxidase-linked secondary antibody dissolved in TBS was added to the membrane for 1 h, the membrane rinsed with TBS (4 times for 5 min). The HRP-antibody labeled bands on the membrane were visualized using an ECL kit (Amersham) according to the manufacturer's instructions and autoradiography.

In some immunoprecipitation experiments, 2-4 h prior to collection of the cells, either serum free media or serum free media with 2-10 μ M quinpirole was added to the cells. For those cells treated with quinpirole, 2-10 μ M quinpirole was also added to the appropriate lysis buffer.

In the case that the membrane was probed with another antibody, the membrane was stripped in a stripping solution (10 mM Tris pH 7.5, 100 mM β -mercaptoethanol, 2 % SDS) for 3 min at 70 °C, blocked using blotto and the immunoblotting procedure described above was repeated.

5.3 Results and Discussion

5.3.1 Interaction of SH3 domains with the dopamine D4 receptor in *Saccharomyces cerevisiae*

To determine if the identified SH3 binding domains in the human D4 receptor could indeed bind SH3 domain containing proteins in a cellular system we employed the yeast two-hybrid assay. We tested whether the receptor fragments encoding the third intracellular loop of D4.2(219-304), D4.4(219-336) and D4.7(219-384) fused to the *GAL4* activation domain (pACT II) could interact with SH3 domains fused to the *GAL4* DNA-binding domain (pAS I) to activate transcription of the yeast reporter gene. All three D4 constructs showed a moderate interaction with the Nck fragment containing all three SH3 domains (Figure 5.1 and Table 5.1). D4 did not interact with the other SH3 domain containing proteins Grb2 (N) (1-59), Grb2 (C) (158-217), Abl (65-123), PLC γ (792-851) and

FIGURE 5.1 Interaction of the dopamine D4 receptor and SH3 domain containing proteins assayed in yeast. The yeast two hybrid system was used to check for an interaction between the dopamine D4 receptor variants and several SH3 domain containing proteins or parts thereof. The D4 constructs and the control Sos tail were expressed in the pACT II vector (left to panel). The SH3 domain containing proteins were expressed in the pAS I vector (above panel). Depicted is a representative scan of replicate experiments.

pAS I Constructs	pACTII Constructs				
	Sos tail	Crk SH2	D4.2 (219-304)	D4.4 (219-336)	D4.7 (219-384)
Grb2 (N)	+++	+	-	-	-
Grb2(C)	+++	++	-	-	-
Nck 3SH3	++++	-	++	++	++
Abl	-	+++	-	-	-
PLC γ	-	-	-	-	-

TABLE 5.1 Summary of results of the yeast two-hybrid system. The interactions seen with the yeast two hybrid system are summarized. Interactions are indicated in strength (++++ very strong, +++ strong, ++ moderate, + weak, - no interaction).

Crk (235-293). The full length Grb2 protein construct resulted in self activation and therefore could not be tested for its ability to interact with D4. The number of repeats did not result in clear detectable differences in the relative strength of interaction. Strong interactions were seen in the positive control group testing the established SH3-mediated Sos/Grb2 and Sos/Nck interaction, as well as for Grb2/Crk and Abl/Crk (Anafi *et al.*, 1996; Hu *et al.*, 1995; Rozakis-Adcock *et al.*, 1993). The D4 constructs expressed in the pAS I vector did not interact with the D4 constructs expressed in the pACT II vector.

These results confirm the *in vitro* interaction observed between the dopamine D4 receptor and Nck. All three D4 variants tested interacted with approximately the same strength. Surprisingly, the Grb2 constructs did not interact with the dopamine D4 receptor in the yeast two hybrid system. The Grb2 constructs used in the yeast two-hybrid assay expressed either the amino- or carboxyl-terminal SH3 domain, but as demonstrated in the *in vitro* assay, only the expression of both SH3 domains of Grb2 resulted in a high affinity interaction with the dopamine D4 receptor. This interaction was significantly reduced when only one of the SH3 domains was expressed or the Grb2 mutant (P49L)

was tested in the *in vitro* assay. Therefore, the detection of this interaction using the yeast two-hybrid assay likely requires the expression of the full length protein expressing both SH3 domains simultaneously. Our attempts at expressing the full length Grb2 protein both in the pAS I or pACT II construct resulted in self activation, therefore the Grb2-D4 interaction could not be confirmed.

5.3.2 Immunoprecipitation of the dopamine D4 receptor

In order to demonstrate D4-SH3 interactions *in vivo* we employed commonly used co-immunoprecipitation techniques. A number of different conditions were used in order to increase dopamine D4 expression, allow for membrane purification of the receptor and to create conditions that favour co-immunoprecipitation of the membrane-bound GPCR and a cytosolic protein. Endogenous expression levels of the dopamine D4 receptor and the SH3 domain containing proteins, Nck and Grb2, in a number of cultured cell lines, were assayed. The expression levels of the D4 were increased by expressing the receptor or the third intracellular loop receptor fragment in different expression vectors and cell lines. Transient (electroporation, calcium chloride and lipofection), stable and adenoviral expression techniques were used to increase the expression of the D4 receptor. Different solubilization buffers were assayed for their ability to purify the receptor from the membrane and to maintain the D4-SH3 interaction as determined using *in vitro* techniques. Epitope tagged antibodies, and commercially available dopamine D4 receptor antibodies were used to purify the D4 receptor. A number of different antibodies directed towards SH3 domain containing proteins were used to demonstrate D4-SH3 interactions. The effect of D4 agonist on the D4-SH3 interaction was also evaluated.

The levels of endogenous dopamine D4 receptor expression were checked in both whole cell lysates and immunopurified samples from several different cell lines (data not shown). No D4 expression was detectable in the CHO-K1, HEK-293, COS-1 and COS-7 cell lines. This is consistent

with binding studies and mRNA studies that show no endogenous expression of D4 in these cell lines (Asghari *et al.*, 1995; Knapp *et al.*, 1998; Van Tol *et al.*, 1991). The levels of D4 receptor in SKNMC cells, which shows moderate D4 expression as determined using mRNA levels (Van Tol *et al.*, 1991), was not detectable using the available D4 antibodies. Three antibodies directed specifically to the D4 receptor were employed. Two commercial polyclonal antibodies from Santa Cruz directed against the carboxyl-terminal portion of D4 and a D4 monoclonal antibody (a gift from Dr. D. Hartman, Hoffman-LaRoche) were not able to detect endogenous dopamine D4 receptor expression using the immunoprecipitation conditions and concentrations described in methods section.

In order to increase the cellular expression of the dopamine D4 receptor and to detect the receptor with commercially available antibodies directed against specific tag sequences, a number of D4 expression vector constructs were created. The third intracellular loop fragment of the dopamine D4 receptor was expressed in the pcDNA3 vector with an HA tag (D4.4(220-336)(HA)pcDNA3) and the D4 fragment as expressed in the pACT II vector (successfully used in the yeast two-hybrid studies) with the HA tag, was expressed in the pRc/RSV vector (D4.4(219-336)(HA)RSV). The full length dopamine D4.4 receptor was expressed with a flag tag and a signal sequence to increase expression (SSFD4). The D4 receptor was also expressed using adenoviral techniques. Successful expression and immunoprecipitation of the D4 receptor was achieved with the SSFD4 construct and to a much lesser extent with the constructs expressing the third intracellular loop of the D4.4 receptor, D4.4(220-336)(HA)pcDNA3 and D4.4(219-336)(HA)RSV. The dopamine D4 receptor could not be detected in the SKNMC or HEK-293 cells expressing the D4 using the adenoviral expression technique and using the Santa Cruz or Hartman D4 antibody. This is likely the result of the immunoprecipitation and/or immunoblotting conditions used and/or the low affinity of the available D4 antibodies for the D4 receptor, since expression levels of the dopamine D4 receptor have been shown to be high using this technique (~50 pmol/mg protein) (Knapp *et al.*, 1998).

Successful immunoprecipitation and detection of the D4 receptor was achieved in CHO-K1 cells stably expressing the SSFD4 construct using standard immunoprecipitation techniques and the NP-40 lysis buffer (Figure 5.2). The construct expresses a signal sequence that serves to increase

FIGURE 5.2 Immunoprecipitation of the flag tagged dopamine D4 receptor. A NP-40 lysis buffer was used to immunoprecipitate the dopamine D4 receptor from CHO-K1 cells (100 mm plate) stably expressing a signal sequence, flag tagged D4 (SSFD4) receptor with the M1 antibody. Standard immunoprecipitation techniques were used (see methods). The receptor was immunoprecipitated using the M1 antibody directed against the flag tag. Cell lysates or immunoprecipitated samples were run on SDS-PAGE and transferred to PVDF membranes. The receptor was visualized by immunoblotting with the M1 antibody and using ECL..

expression of the receptor and is cleaved off after successful insertion into the membrane. This is confirmed by the successful immunoprecipitation using the M1 antibody (Kodak) that only recognizes the flag tag sequence if there is no sequence amino-terminal to the tag sequence. These results demonstrate that the receptor is expressed and that it can be successfully solubilized and purified from the membrane. Binding experiments and cAMP assays with the signal sequence tagged receptor shows a fully functional receptor with three-fold higher levels of expression than just the D4.4RSV construct (data not shown). The dopamine D4 receptor could not be detected in the whole cell lysate, but only after purification of the receptor by immunoprecipitation, indicating that expression levels for the D4 are low. These results demonstrate that the dopamine D4 receptor shows increased

expression and normal function and that the receptor can be successfully immunoprecipitated from the membrane using the M1 antibody and the NP-40 lysis buffer.

Nck and Grb2 expression levels were shown to be extremely high in CHO-K1, COS-1, COS-7, HEK-293 and SKNMC cells (data not shown). High endogenous levels of these proteins could be detected in the whole cell lysate samples using the same conditions to solubilize the dopamine D4 receptor (NP-40 lysis buffer). This indicates that the endogenous expression levels of the SH3 domain containing proteins is much higher than the transfected dopamine D4 receptor levels.

The conditions that allowed for successful purification of both dopamine D4 receptor and the adapter proteins Nck and Grb2 were used to determine if these proteins could be co-purified. CHO-K1 cells expressing the SSFD4 construct were solubilized using the NP-40 lysis buffer. Either the D4 receptor was immunoprecipitated with the M1 antibody, or the adapter proteins were immunoprecipitated with their specific antibody. Although both D4 and the adapter proteins Nck and Grb2 were successfully immunoprecipitated with their respective antibodies, neither D4 nor the adapter proteins were able to co-immunoprecipitate each other (data not shown). Neither D4 could purify Nck or Grb2 nor Nck or Grb2 could purify the D4 receptor under these conditions. These results do not necessarily demonstrate that these adapter proteins do not interact with the D4 receptor, but may indicate that the conditions used may not maintain the interaction or that the techniques used are unable to detect the interaction.

There are numerous reasons why the interaction between the dopamine D4 receptor and the SH3 domain containing proteins Nck or Grb2 could not be demonstrated using the above conditions. The *in vitro* and yeast-two hybrid studies strongly suggest that the D4 receptor interacts with these proteins, however, it may be that *in vivo* other SH3 domain containing proteins compete with Nck and Grb2 for the D4 interaction. The possibility also exists that the conditions used interfered with the interaction or were not sensitive enough to detect the interaction. Furthermore, the receptor state may

affect the D4-SH3 interaction. In order to determine if the D4 receptor interacts with Nck and/or Grb2, different expression techniques, cell lines, antibodies, solubilization and immunoprecipitation techniques need to be used.

Although the NP-40 lysis buffer allowed for the successful purification of the dopamine D4 receptor from the membrane, the conformation of the solubilized receptor or the strength of the detergent may have interfered with the interaction of D4 with Nck and/or Grb2. A number of solubilization buffers were assayed for their ability to purify the receptor from the membrane and maintain interaction with Nck and Grb2. The triton, the digitonin, the *in vitro* and the 3% NP-40 lysis buffers were all able to solubilize the dopamine D4 receptor *in vivo* with varying degrees of success (data not shown). Of these buffers, the NP-40 and the *in vitro* lysis buffers were able to maintain D4-Grb2 interaction in the *in vitro* assay using GST-Grb2 to purify the *in vitro* [³⁵Cys] labeled D4 receptor. In addition a chaps/digitonin and a chaps lysis buffer were also able to maintain *in vitro* D4-Grb2 interaction, however, the chaps buffer could not immunoprecipitate the D4 receptor *in vivo*. A range of concentrations of NP-40 in the NP-40 solubilization buffer was used to determine the lowest concentration that allowed for D4 immunoprecipitation (1.0 %, 0.5 %, 0.25 %, 0.125 %, 0.0625 % and 0.3125 %), in order to decrease the stringency of the buffer (Figure 5.3). A concentration of NP-40 as low as 0.0625 % in the solubilization buffer still allowed for robust D4 immunoprecipitation, but further reduction of the NP-40 concentration eliminated D4 detection. None of the buffers used, however, were able to co-immunoprecipitate D4 with Grb2 and/or Nck *in vivo*.

The success of some of these buffers in the *in vitro* system to co-purify D4 and Grb2 suggests that the SH3 binding domain and the conformation of the dopamine D4 receptor is not perturbed enough to prevent interaction (ie. these detergents are gentle enough to maintain the D4-Grb2 and/or -Nck interaction). The *in vitro* assay was used to co-immunoprecipitate *in vitro* labeled [³⁵Cys] D4 (SSFD4 used as template) and Grb2-GST or Nck-GST with the specific antibodies directed against

FIGURE 5.3 Immunoprecipitation of the dopamine D4 receptor with varying concentrations of NP-40 detergent in the lysis buffer. Varying concentrations of NP-40 lysis buffer were used to immunoprecipitate D4 from CHO-K1 or CHO-K1 cells stably expressing the SSFD4 construct (100 mm plate) with the M1 monoclonal antibody. The immunoprecipitated samples size separated on SDS-PAGE and transferred to PVDF membranes. The membranes were immunoblotted with the M1 antibody and visualized using ECL.. Molecular mass is indicated in kDa.

these proteins. Although, both Grb2 and Nck were successfully immunoprecipitated with their respective antibodies, D4 was not co-purified by the antibody adapter protein complex, as determined by autoradiography of the labeled D4. The antibodies directed against both Nck and Grb2 are targeted to the SH3 domain areas of these proteins and may possibly interfere with the D4-SH3 interaction, thus preventing an antibody-adapter protein-D4 complex. This would also make these antibodies unsuitable to use in the *in vivo* interaction assay as immunoprecipitating antibodies. The M1 antibody directed to the flag tag of the SSFD4 receptor could not detect the D4 receptor, although the receptor could be visualized as evidenced by autoradiography. The flag tag sequence is preceded by a signal sequence meant to increase cellular expression of the receptor. In the *in vitro* system this sequence is likely not cleaved off, making the flag tag sequence inaccessible for the M1 antibody to bind to. A modification of the last amino acid in the flag tag sequence has made this sequence inappropriate for other commercially available antibodies to recognize. We could, therefore, replicate the interaction using the *in vitro* system only when using the GST pull down assay, but not with the available antibodies.

The Nck and Grb2 antibodies, although appropriate for detecting the denatured proteins on an immunoblot, apparently are inappropriate to use as immunoprecipitating antibodies since they likely interfere with the D4-SH3 interaction. The M1 antibody only recognized the SSFD4 receptor construct when expressed *in vivo*. Therefore, the *in vitro* system could not be used to evaluate the effectiveness of the antibodies. Furthermore, these results also suggest that *in vivo*, using the available antibodies, coimmunoprecipitation will not work if using the Nck and Grb2 antibodies, since these will likely disrupt the interaction. Antibodies directed against the dopamine D4 receptor or a tagged version of the receptor are more likely to purify a D4-SH3 complex. In addition, the endogenous levels of Nck and Grb2 are extremely high as compared to even the transfected levels of the dopamine D4 receptor. These adapter proteins have already been shown to interact with a number of proteins present at high endogenous levels, including RTKs and cytosolic proteins such as Src and Sos. Only a small proportion of these proteins may interact with the D4 receptor population at any one time. The amount of D4 receptor purified by these proteins may be too low to detect using the available immunoprecipitation techniques.

To determine if membrane purification of the receptor affects solubilization and purification of the dopamine D4 receptor, we *in vitro* expressed D4 in the presence of microsomal membranes. The *in vitro* system occurs in a membrane free system, and solubilization of the receptor in this system as opposed to the cellular system may result in differences in conformation, although this is unlikely to alter the structure of the third intracellular hydrophilic loop region. Addition of microsomal membranes to the *in vitro* translation assay resulted in greatly reduced levels of translated receptor. This prevented the assessment of the effect of membrane associated receptor on the interaction using the different lysis buffers. Furthermore, it did not allow for an *in vitro* assay to determine whether the addition of an agonist to a membrane expressed receptor might alter the ability of the receptor to bind to the SH3 domain containing proteins.

Stimulation of the dopamine D4 receptor with the dopamine receptor agonist quinpirole (2-10 μ M) 2-4 h prior to the harvesting of the cells did not result in changes in the amount of immunopurified receptor. The stimulated receptor was also unable to co-immunoprecipitate the SH3 domain containing proteins in the *in vivo* system. Increasing the incubation times of the solubilized cell lysate antibody mixture from 1-2 h to a period of greater than 16 h did not alter the amount of dopamine receptor purified, or result in co-immunoprecipitation of a D4-SH3 complex.

The different D4 constructs were expressed in a number of different cell lines using various transfection techniques (data not shown). Stable expression of the SSFD4 construct in the CHO-K1 cell line resulted in the highest expression of the D4 receptor as determined using immunoprecipitation techniques. Expression of the third intracellular loop fragment of the D4 receptor in the D4.4(220-336)(HA)pcDNA3 and D4.4(219-336)(HA)RSV constructs in the COS-1, COS-7 and HEK-293 cell lines only showed low expression levels of the D4 fragment. The highest level of expression of these receptor fragments was achieved with the calcium chloride transfection method, with lower amounts seen with the electroporation and lipofection techniques. Expression of the third intracellular loop fragments, shown to interact in both the *in vitro* and yeast-two hybrid assays, and likely expressed as a cytosolic protein fragment *in vivo*, was unable to interact with the Nck and Grb2 adapter proteins. Adenoviral expression of the dopamine D4 receptor results in high dopamine D4 receptor expression levels as determined by binding in both SKNMC and HEK-293 cells (Knapp *et al.*, 1998), but the receptor could not be detected with the available D4 antibodies.

A number of antibodies directed against the dopamine D4 receptor or tagged versions of the receptor and the adapter proteins Nck and Grb2 were assayed. The M1 antibody directed against the flag tag sequence of the SSFD4 vector, was able to purify the dopamine D4 receptor most effectively. A rabbit polyclonal flag antibody (Santa Cruz) was unable to purify the flag tagged receptor, likely the result of the difference in flag tag sequence as mentioned previously. The commercial D4

antibodies (Santa Cruz) and the D4 antibody (Dr. D. Hartman, Hoffman-LaRoche) were unable to immunoprecipitate or immunoblot the D4 receptor in our hands. The affinity of these antibodies are likely low, since the Santa Cruz antibodies are recommended for immunoblotting procedures only. The HA tag was also used to tag the D4 receptor. Low amounts of HA tagged D4 receptor were purified using this antibody. In addition to the mouse monoclonal antibody directed against the Grb2 protein, a rabbit polyclonal Grb2 antibody was used in order to prevent detection of the light and heavy chain of the mouse antibody when visualizing with the secondary peroxidase linked antibody, which might obscure the detection of the Grb2 antibody. The rabbit polyclonal antibody was effective at detecting and immunoprecipitating the Grb2 protein.

The results of the *in vivo* interaction assay neither confirm nor reject the putative dopamine D4 receptor interaction with either the Nck or Grb2 SH3 domain adapter proteins in our mammalian expression systems. Problems with the antibodies, purification process and receptor concentration may make it difficult to detect this interaction. In addition, it is possible that the dopamine D4 receptor actually interacts with other SH3 domain containing proteins that exhibit higher affinity interactions than that seen with either Nck or Grb2. This interaction would compete with the Nck and Grb2 interaction and preclude seeing these proteins bind. In order to establish such an interaction, different SH3 domain containing proteins need to be screened.

The results from the yeast two-hybrid system confirm the interaction established using the *in vitro* assay, however, we were unable to co-immunoprecipitate the proteins. The success in the yeast two-hybrid assay may be the result of several reasons. The yeast two-hybrid assay has been shown to be more sensitive than the co-immunoprecipitation technique (for discussion see Fields and Sternglanz, 1994). Expression of just the third intracellular loop confirmed that it is this portion of the receptor that mediates the SH3 binding. The expression of just the hydrophilic portion of the receptor may allow for proper folding of this portion of the receptor, which may not occur when the

full length receptor was purified from the membrane in the mammalian cells.

The best strategy to demonstrate dopamine D4 receptor interaction with SH3 domain containing proteins appears to be one that immunoprecipitates the D4 receptor and then immunoblots for possible interacting SH3 domain containing proteins. Although, the dopamine D4 receptor antibodies employed in our experiments failed to purify or immunoblot the D4 receptor in any appreciable amount, a number of labs have developed high affinity antibodies that soon will be available. Dopamine D4 receptor expression has been shown to be the highest using adenoviral expression techniques. Dopamine D4 mutants have been shown to have functional effects in HEK-293 cells in terms of internalization. A number of immunoprecipitation techniques have been developed that effectively co-immunoprecipitate membrane-bound GPCRs with cytosolic proteins, while maintaining their interaction (Karoor *et al.*, 1998; Klein *et al.*, 1997). It is also important to consider the receptor activation and phosphorylation state, since receptor phosphorylation of serine and threonines near the SH3 binding domain PXXP motifs may disrupt SH3 interactions (Chen *et al.*, 1996a).

5.3.3 Screen of Potential SH3 Domain Proteins

To date over 60 SH3 domain containing proteins have been identified. Many of these, including Grb2 and Nck, have been shown to be important in signal transduction mechanisms for both RTK and GPCR signalling. In addition, there are a number of SH3 domain containing proteins, including amphiphysin and Grb2, that have been shown to be critical in both GPCR signalling and endocytosis (Wigge and McMahon, 1998). In the *in vitro* assay, a number of SH3 domain containing proteins were screened for their ability to interact with the dopamine D4 receptor. Of these, Grb2 and Nck displayed the highest affinity for the receptor. The list of SH3 domains tested for their ability to interact with the dopamine D4 receptor using the *in vitro* assay, was not exhaustive. It is our intention

to screen a number of likely candidate SH3 domain containing proteins for their interaction potential with the dopamine D4 receptor using the *in vivo* system. These include proteins that have already been shown to be important in signal transduction mechanisms for both RTKs and GPCRs. These also include proteins that have been shown to be important in endocytosis. In addition, SH3 domain containing proteins that are likely to be co-localized should be screened. It is possible that the dopamine D4 receptor does not interact with any of the SH3 domain containing proteins identified to date. It may also be that the dopamine D4 receptor interacts with multiple SH3 domain containing proteins with varying affinities. To this end, efforts have been underway to identify potential novel D4-SH3 interaction using the yeast two-hybrid system.

The number of SH3 domain containing proteins that have been identified include over 60 proteins (Table 5.2). The functions of many of these proteins remain unknown. The function of other SH3 domain containing proteins have been well characterized. Many of these proteins, however, appear to be involved in multiple interactions and functions which are still being identified. Listed below are many of the identified SH3 domains, and some of their identified functions. Possible candidate proteins for D4 interaction are those that have already been identified to be involved in signalling. Those proteins involved in endocytosis are of particular interest, since removal of all the PXXP motifs as demonstrated in the internalization studies (Chapter 4) affects internalization of the receptor.

As discussed in the introduction, a number of important SH3 domain containing proteins are involved in both RTK and GPCR signalling. These include Grb2, Src and the Src-like kinases (Yes, Fyn, Lyn), p85 and PLC γ proteins. Their possible involvement in the dopamine D4 receptor function and possible direct interaction need to be accessed. Furthermore, removal of the region of the dopamine D4 receptor shown to be involved in SH3 binding results in a distinct constitutive internalization phenotype. A number of proteins that are involved in SH3-mediated interactions are

	IMPLICATED IN		DESCRIPTION
	SH2	SH3	GPCR FUNCTION ENDO-CYTOSIS
✓ <i>Abp1p</i>		1	✓ SH3 domain required for endocytotic function of Abp1p
✓ <i>Amphiphysin I</i>		1 (C)	✓ SH3 domain mediated interactions with dynamin and synaptojanin
✓ <i>Amphiphysin II</i>		1 (C)	✓ SH3 domain mediated interactions with dynamin and synaptojanin
✓ <i>Bcr-Abl (Abl or cAbl)</i>	1	1	✓ A tyrosine kinase, Ctk I is a major substrate for Bcr-Abl
<i>Btk</i>	1 (-C)	1 (N-)	A Src-like protein tyrosine kinase related to Lyn
<i>Bmx</i>	1	1	Member of Bcr/Btk/Tec family of protein tyrosine kinases
<i>Brk</i>	1	1	A non-receptor protein tyrosine kinase involved in signal transduction
✓ <i>Btk (also known as Atk, BPK, CAP)</i>	1	3 (-C)	✓ Binds with G alpha subunit of heterotrimeric G protein that belongs to the Tec family
<i>Cas (p130Cas, CasL)</i>		1 (N-)	Associated with Cbl, involved in the organization of the cytoskeleton
✓ <i>c-Crk-I</i>	1 (N-)	1 (-C)	✓ Related to the v-Crk
✓ <i>c-Crk-II (Crk)</i>	1 (N-)	2 (-C)	✓ Tyrosine phosphorylated after IGF-1 stimulation, involved in NGF internalization
✓ <i>v-Crk</i>	1	1	✓ Activates the Rho-signalling pathway, induces cell transformation
✓ <i>CrkL</i>	1 (N-)	2 (-C)	✓ Interacts with Bcr-Abl through its SH3 domain
<i>CHK (MATK)</i>	1 (-C)	1 (N-)	Homologous to Csk, expressed in breast tissue, involved in cell spreading
<i>Coractin</i>		1 (-C)	An actin filament binding protein involved in the assembly of actin at the neuromuscular junction
✓ <i>Csk</i>	1	1	✓ A protein tyrosine kinase that can inactivate Src family kinases
<i>DdRacGAP</i>		1	Homologous to moGAP, contains a GAP domain
<i>DOCK</i>	1	3	Drosophila homologue of Nck, transduces signals from tyrosine kinases through the Crk Adapter protein
✓ <i>Drk</i>	1	2 (N- and -C)	✓ Drosophila homologue of Grb2, involved in Ras-MAPK signalling
✓ <i>endophytin</i>		1	✓ Interact with amphiphysin, II and synaptojanin
✓ <i>Eps8</i>		1	A substrate for RTKs, involved in mitogenic signalling, possibly acts as a docking site
✓ <i>Ese1</i>		5 (-C)	✓ Links dynamin and other endocytotic pathway components
✓ <i>Ese2</i>		5 (-C)	✓ Links dynamin and other endocytotic pathway components
<i>FAP52</i>		1 (-C)	A focal adhesion protein
✓ <i>c-Fgr</i>	1 (-C)	1 (N-)	✓ Binds to the Wiskott-Aldrich syndrome protein, member of Src kinase family
✓ <i>Fyn</i>	1 (-C)	1 (N-)	✓ A Src-like kinase involved in signalling
<i>Gads</i>	1 (-M-)	2 (N- and -C)	Similar to Grb2 and Grap, binds to tyrosine phosphorylated Shc
<i>Grap</i>	1 (M)	2 (N- and -C)	Grb2 related protein kinase; interacts with Shc, Sos, dynamin and Sam68
<i>Grap-2</i>	1 (-M-)	2 (N- and -C)	Related to Grb2 and Grap
✓ <i>Grb2 (Sem-5)</i>	1 (M)	2 (N- and -C)	✓ Important adapter protein in signalling and endocytosis
<i>Grb3-3</i>		2 (N- and -C)	Grb2 homologue that lacks a functional SH2 domain
✓ <i>Hck</i>	1 (-C)	1 (N-)	✓ Member of Src kinase family, expressed in lymphoid and myeloid cells
<i>Itk (Itk/Ernt)</i>	1	1	Forms a complex with CD28 mediated by an SH3 domain
<i>Lck</i>	1 (-C)	1 (N-)	✓ Member of Src kinase family, associates with the immunoglobulin, CD4
✓ <i>Lyn</i>	1 (-C)	1 (N-)	✓ Member of Src kinase family, activates MAPK by Gi and Gq
<i>MAGUK (hdg)</i>		1	Assemble multiprotein complex on inner surface of plasma membrane
<i>MLK 1</i>		1	Serine/threonine kinase, related to map kinase kinase
<i>MLK 2</i>		1	Interacts with activated (GTP bound) forms of Rac and Cdc2
<i>MLK 3</i>		1	Interacts with activated (GTP bound) forms of Rac and Cdc2
<i>Myosin I</i>		1 (-C)	✓ Involved in controlling actin-based cell movements
<i>Nck</i>	1 (-C)	3 (N-)	Associated with the cytoskeleton and PDGF receptor
<i>Nck-2</i>	1 (-C)	3 (N-)	Interacts with PINCH and components of the growth factor RTK pathway
<i>p40phox</i>		1	Involved in phagocyte NADPH oxidase complex, associated with p67phox
<i>p47phox</i>		2	Interact with p67phox and p40phox
<i>p67phox</i>		2	Involved in the phagocyte NADPH oxidase complex
<i>p53BP2</i>		1	p53 binding protein, interact with protein phosphatase 1
✓ <i>p85 (of PI3K)</i>	2 (N- and -C)	1	✓ Mediate GPCR activated mitogenesis
<i>PLCgamma 1</i>	2 (A-)	1 (-C)	✓ Is phosphorylated by GPCR or RTK activation, associates with Grb2
<i>PLCgamma 2</i>	2 (A-)	1 (-C)	✓ Involved in RTK and GPCR signalling
<i>POSH</i>		4	A scaffolding protein involved in Rac-induced signalling
✓ <i>PSD95</i>		1	Involved in protein clustering and organizing signalling molecules at synaptic sites
✓ <i>Ras (RasGAP)</i>	2 (A- and -C)	1 (-M-)	✓ Associates with Sos, involved in RTK and GPCR function
<i>SH3BP2</i>	1	1	Negative regulator of abl oncogene
<i>SH3D2B</i>		1	A mouse SH3 domain containing protein
<i>SH3P7</i>		1	Cytoskeleton adapter protein, a substrate for Syk
<i>Slap</i>	1	1	Src-like adapter protein, without kinase function, a negative regulator of mitogenesis
✓ <i>c-Src</i>	1 (-C)	1 (N-)	✓ Associates with activated RTKs and activated by GPCRs
<i>n-Src</i>		1	Neural form of Src
<i>STAM</i>		1	Involved in cytosine-mediated signal transduction
<i>Syk</i>	1 (-C)	1 (N-)	✓ A Src family kinase, interacts with cbl
✓ <i>Tec</i>	1	1	✓ Involved in GPCR signalling
<i>Tsk</i>	1	1	Involved in signalling
<i>Vav</i>	1 (Center)	2 (N- and -C)	Interacts with cbl and Grb2, involved in the activation of Ras
<i>c-Yes</i>	1	1	A src-family kinase
<i>Yrk</i>	1 (-C)	1 (N-)	Member of Src protein-kinase family, yes related kinase

TABLE 5.2 Candidates for SH3 interaction with the dopamine D4 receptor. The identified SH3 domain containing proteins are listed. Those that are involved in GPCR function or endocytosis are indicated with checkmarks. Proteins that may interact with the dopamine D4 receptor are denoted in italics.

required for this internalization, including the SH3 binding domain containing proteins dynamin and arrestin and the SH3 domain containing protein amphiphysin. These SH3 domain containing proteins represent the prime candidates for potential D4 interactions.

The screening of these proteins is contingent on an effective co-immunoprecipitation protocol, the availability of antibodies directed against the SH3 domain containing proteins, high expression levels of the dopamine D4 receptor and a high affinity dopamine D4 antibody. A number of co-immunoprecipitation techniques have been developed that effectively demonstrate co-immunoprecipitation between GPCRs and cytosolic proteins. Furthermore, antibodies directed against most of the SH3 domains containing proteins are commercially available. A high affinity and specific antibody directed against the dopamine D4 receptor, however, is not readily available. An antibody that effectively purifies the D4 receptor is a necessary prerequisite to the demonstration of co-purification.

5.3.4 Summary

The yeast two-hybrid system confirmed the D4-SH3 interactions demonstrated using the *in vitro* system. This interaction required the presence of multiple SH3 domains for high affinity interaction. The demonstration of the D4-SH3 interaction in the yeast two hybrid system confirmed this interaction in a living cell system with a full complement of proteins. Our efforts to demonstrate the interaction *in vivo* were hampered by a number of technical difficulties, including D4 expression levels, solubilization techniques and the lack of a high affinity and specific antibody to the dopamine D4 receptor. Schemes to deal with these limitations were discussed and present possibilities to try in the future.

Chapter 6

Peptide Binding and Mutagenesis of the Dopamine D4 Receptor

Andrew Ray (M.Sc. thesis, Department of Pharmacology, University of Toronto) was instrumental in helping perform some of the mutagenesis experiments.

6.1 Introduction

Using the *in vitro* assay, as described in Chapter 3, we established that the dopamine D4 receptor could interact with SH3 domain containing proteins. This interaction is mediated by the third intracellular loop of the receptor, since the loop was sufficient and necessary for this interaction. Expression of just the third intracellular loop or removal of the portion of the loop containing the potential SH3 binding motifs (PXXP) from the full length dopamine D4 receptor confirmed these results. By the use of a number of deletion mutants of the D4 receptor, we established that the interaction is mediated by at least two sites in the third intracellular loop that act in a cooperative fashion for maximal affinity. These sites appear to be amino- and carboxyl-terminal to the variable repeat region of the D4 receptor.

Although the dopamine D4 deletion mutants indicate the areas of the receptor, and specifically the regions in the third intracellular loop, that are involved in SH3 binding, the exact binding domains and the minimal amino acid sequence required for the SH3 interaction have not been identified. We attempted to employ peptide binding and point mutations techniques to determine the specific sites of SH3 interaction in the D4 receptor. Short peptides matching the amino acid sequence of the third intracellular loop of the D4 receptor were designed to contain the putative SH3 binding sites. These were used to bind to the SH3 domains of Nck and Grb2 in order to compete with the full length receptor and prevent its binding. Specific point mutations targeting the prolines in the multiple PXXP

motifs in the third intracellular loop of the dopamine D4 receptor were created in order to eliminate the critical SH3 binding sites responsible for SH3 binding and thus disrupt the established interaction.

Short peptide sequences have been successfully used to characterize SH3 binding sites (Cheadle *et al.*, 1994; Ren *et al.*, 1993; Sparks *et al.*, 1994; Sparks *et al.*, 1996), determine SH3 binding affinity (Knudsen *et al.*, 1995; Lee *et al.*, 1995; Pisabarro and Serrano, 1996; Smithgall, 1995) and to disrupt established functional SH3 interactions (Shupliakov *et al.*, 1997; Vidal *et al.*, 1998). Early studies used short peptide sequences to determine SH3 binding domain characteristics. These studies indicated that SH3 domains contain PXXP motifs and that the amino acid residues immediately surrounding this motif are important for SH3 specificity (Cheadle *et al.*, 1994; Ren *et al.*, 1993; Sparks *et al.*, 1994; Sparks *et al.*, 1996). General rules for the binding of functionally well characterized SH3 binding domains have been established using phage-displayed libraries of peptides to determine the sequences that shows the strongest interaction (Sparks *et al.*, 1996).

Short proline based peptide sequences (about nine amino acid residues) have also been used to biochemically and pharmacologically characterize SH3 binding (Chen *et al.*, 1993; Knudsen *et al.*, 1995; Lee *et al.*, 1995; Pisabarro and Serrano, 1996; Yu *et al.*, 1994). Peptide sequences matching known SH3 binding motifs have been used to bind to specific SH3 domain containing proteins and compete with the endogenous interaction protein. The addition of an excess of peptide should disrupt the specific SH3 interaction that the peptide is directed against and can help to determine the affinity of the particular interaction.

A number of groups have used peptides to disrupt functional SH3 interactions within the cell (Shupliakov *et al.*, 1997; Vidal *et al.*, 1998; Wang and Moran, 1996; Yoon *et al.*, 1997). Microinjection of a peptide sequence containing the dynamin SH3 binding site successfully inhibited synaptic vesicle endocytosis (Shupliakov *et al.*, 1997). The *in vivo* interaction between Grb2 and dynamin II was abrogated by the addition of peptide corresponding to the SH3 binding motif (Yoon

et al., 1997). The use of peptides allows for the determination of the specific SH3 binding sites and characterization of the binding. For the dopamine D4 receptor, the use of peptides can be used to confirm the established binding and the peptides may aid in establishing a functional role for the interaction.

Peptides sequences have also been used to determine binding affinity and characteristics of SH3 interaction by means of the fluorescent properties of SH3 domains. Tryptophans within proteins have the ability to fluoresce under the proper excitation conditions. Stimulation of tryptophans with a wavelength of ~295 nm results in an emission of wavelengths greater than 320 nm. This property of tryptophans along with the fluorescence of tyrosines is commonly used to calculate the concentration of purified proteins in solution. SH3 domains contain a conserved tryptophan residue in its sequence required for its proper functioning. A property of the SH3 binding is that the tryptophan within the SH3 domain is obscured upon complex formation, which, under the proper wavelength of excitation, results in a shift in the intensity and wavelength of the fluorescence emission spectrum as observed using fluorescence spectroscopy (Chen *et al.*, 1993; Phizicky and Fields, 1995). Increasing amounts of peptide results in larger emission spectrum shifts until the binding is saturated. In this way specific peptide binding to an SH3 domain can be confirmed and the affinity of binding can be determined (Chen *et al.*, 1993; Knudsen *et al.*, 1995; Lee *et al.*, 1995; Pisabarro and Serrano, 1996; Smithgall, 1995; Yu *et al.*, 1994). Such peptide binding typically result in affinities between 0.2 and 300 μ M (Booker *et al.*, 1993; Chen *et al.*, 1993; Pisabarro and Serrano, 1996; Yu *et al.*, 1994). The technique of fluorescence spectroscopy has been successfully used to characterize the interaction between the SH3 domain of Abl and its binding motif by the use of peptides (Pisabarro *et al.*, 1994; Pisabarro and Serrano, 1996) and has been used to characterize the SH3 binding domains of Hck (Lee *et al.*, 1995) and Crk (Knudsen *et al.*, 1995).

Point mutations can be used to target one of the two prolines required for SH3 binding in the

PXXP motif. Most SH3 binding requires the presence of two prolines (PXXP) to form a left-handed polyproline type II helix (Feng *et al.*, 1994; Pawson and Scott, 1997). There are a few exceptions to the requirement of two prolines for SH3 binding (Gorina and Pavletich, 1996; Sicheri *et al.*, 1997; Xu *et al.*, 1997), but these interactions are generally much weaker and tend to be intramolecular (Nguyen *et al.*, 1998). Mutation of either of the prolines, therefore, substantially reduces or abolishes SH3 binding. Point mutations can be used to determine the specific PXXP motifs that are required for SH3 binding, can be used to help determine the functional importance of the SH3 interaction and in the case of the dopamine D4 receptor can confirm the involvement of SH3 binding in receptor internalization, as suggested by the deletion mutant studies. If disruption of SH3 binding displays the same functional characteristics as the deletion mutants of the dopamine D4 receptor, then this would provide strong evidence that this phenotype is mediated by specific SH3 interactions. To date, we have been unable to demonstrate *in vivo* interaction of the dopamine D4 receptor with SH3 domain containing proteins, but the demonstration of a functional impairment of receptor function due to a point mutation in a PXXP motif would provide strong support for such an SH3 interaction and function *in vivo*.

The objectives of this chapter were to develop point mutations and peptides that would interfere with the established D4-SH3 interactions. These would then be used to functionally characterize the SH3 interaction.

6.2 Methods

6.2.1 Peptide Studies

6.2.1.1 Peptides

The following peptides were synthesized (Quality Controlled Biochemicals Inc., MA) corresponding to the amino acid sequence of the dopamine D4.4 receptor: P(232-245)

(GRAPRRPSGPGPPS), P(240-248) (GPGPPSPTP), P(245-253) (SPTPPAPRL), P(248-256) (PPAPRLPQD) and P(320-328) (ALPPQTPPQTRR). All peptides were 90% purified and the ends were blocked by an acetyl and amide group on the amino- and carboxyl-terminal ends respectively. Peptides were dissolved in water to yield a 100 mM solution.

6.2.1.2 *in vitro* Peptide Competition Assay

An excess of peptides were added to the *in vitro* interaction assay incubation mixture (see Materials and Methods, Chapter 2, page 78) to determine if these peptides could interfere with the D4-SH3 binding. Each of the D4 peptides (~300 μ M) was added to the *in vitro* interaction assay containing labeled [35 Cys] D4.4 (2 nM) and Nck-GST or Grb2-GST (30 μ M) in lysis buffer. The mixture was incubated, at 4 °C for 2 h. The beads were washed, size separated using SDS-PAGE and visualized as previously described (page 78).

6.2.1.3 Fluorescence measurements

The changes in the fluorescence emission spectra of the tryptophans in the Nck 3SH3-GST or Grb2-GST proteins were monitored in the presence of the different D4 peptides. The GST fusion proteins were purified from bacterial lysates as described in chapter 2 (page 78) and were eluted from the glutathione beads by adding glutathione elution buffer (10 mM Glutathione, 10 mM Tris-HCl pH 8.0, 0.2 mM EDTA and 250 mM KCl) to the aspirated beads containing the GST fusion protein. All experiments were completed in the glutathione buffer. Changes in emission of the GST fusion protein in response to the addition of peptide was measured.

Measurements were made either with an AVIV Circular Dichroism Spectrometer (Model 62A DS, Lakewood, New Jersey) at an excitation wavelength of 295 nm (slit width of 2 nm) and measuring total fluorescence >320 nm (slit width of 2 nm) over a 30 s period at either 700 or 800 V

or with a Perkin-Elmer Luminescence Spectrometer (Model LS 50B) at an excitation wavelength of 295 nm, (slit width 2.5 nm) and measuring total fluorescence >330 nm (slit width of 15 nm) over a 30 s period. A mini magnetic stirrer was used to mix the solution in a 1 cm² quartz fluorescence cell. A circulating water bath was used to maintain the sample temperature at 22 or 25 °C.

6.2.2 Point Mutation Studies

6.2.2.1 Point Mutations

The following prolines were targeted for mutation to leucines in the dopamine D4.4 receptor: D4(P235L), D4(P238L), D4(P241L), D4(P243L), D4(P246L), D4(P249L), D4(P251L), D4(P323L), and D4(P326L). For the described targets the following deprotected, desalted and 5' phosphorylated oligonucleotides were ordered (Gibco BRL, the mutated nucleotide is capitalized): For D4(P235L); (692F) ac ggc cgc gcg cTc cgc cga ccc ag, for D4(P238L); (701F) cg ccc cgc cga cTc agc ggc cct gg, (708F) c cga ctc agc ggc cct ggc ccg cct tc, and (684F) c aag ctg cac ggc cgc gcg ccc cgc cga cTc agc ggc cct g, for D4(P241L); (710F) ga ccc agc ggc cTt ggc ccg cct tc, for D4(P243L); (715F) agc ggc cct ggc cTg cct tcc ccc a, (739R) t ggg gga agg cAg gcc agg gcc gct, for D4(P246L); (725F) gc ccg cct tcc cTc acg cca ccc gc, (722F) ct ggc ccg cct tcc cTc acg cca c, (722F') ct ggc ccg cct tcc cTc acg cca ccc gcg ccc cgc etc, for D4(P249L); (734F) cc ccc acg cca cTc gcg ccc cgc ct, (722F'') ct ggc ccg cct tcc ccc acg cca cTc gc, and (731F) ct tcc ccc acg cca cTc gcg ccc cgc etc ccc cag gac, for D(P251L); (740F) cg cca ccc gcg cTc cgc etc ccc ca, (744F) a ccc gcg cTc cgc etc ccc cag gac ccc tg, and (731F') ct tcc ccc acg cca ccc gcg cTc cgc etc ccc cag gac, for D4(P323L); (955F) cc gcg etc cca cTc cag act cca cc, and (961F) etc cca cTc cag act cca ccg cag ac, and for D4(P326L); (961F') etc cca ccc cag act cTa ccg cag acc cgc ag.

The D4.4 dopamine receptor nucleotide sequence was subcloned into the EcoRI site of pALTER®-Ex1 vector (Promega) to create D4.4(pAlt). Mutants were checked by sequencing the

D4.4(pAlt) sequence. Successful D4.4 mutant nucleotide sequences were subcloned into the EcoRI site of pBluescript vector under T7 promoter for higher levels of *in vitro* expression.

6.2.2.2 Mutagenesis

The Altered Sites® II *in vitro* Mutagenesis Systems (Promega) and the Chameleon™ Double-Stranded Site-Directed Mutagenesis Kit (Stratagene) were used to create the desired mutant receptors. The manufacturer's instructions were carefully followed and changes in protocol are noted. For the Altered Sites® II *in vitro* Mutagenesis Systems ES1301 mutS and JM109 competent cells were prepared as described by the manufacturer (RbCl method) or obtained directly from the manufacturer. Either double stranded or single stranded DNA, prepared using the described phagemid procedure, was used. The mutagenic oligonucleotide, the Amp repair oligonucleotide and the Tet knockout oligonucleotide were annealed to the D4.4(pAlt) vector at an annealing temperature of 75 °C or 90 °C. Transformation of ES1301 mutS cells were performed either using the double-stranded DNA or phagemid single-stranded DNA (manufacturer's directions) by the described heat shock method or by electroporation using the manufacturer's recommended conditions (using the BTX 600 electroporater (Genetronics), settings: KV 2.5, R5-R6 (129-186 ohms), 10-50 µF, and a time constant 4.5-7.0 ms).

For the Chameleon™ Double Stranded Site Directed Mutagenesis Kit the oligonucleotides were annealed to the D4.4 receptor and KpnI site in the D4.4(1-419) construct (D4.4 in pBluescript SKII(-)) using the rapid annealing technique described (5 min at 100 °C, 5 min. on ice) or using a slow annealing process (5 min. at 100 °C, cool to RT over a 2 h period).

For those sites that were resistant to mutation by the oligonucleotides, the oligonucleotides were used as sequencing primers to determine if they were able to anneal to the D4.4 sequence. Successful sequencing was taken as evidence that the oligonucleotide could successfully anneal to

the receptor sequence and used for subsequent round of mutagenesis.

In addition, two dopamine D4.4 receptor mutants were created: The D4(M345A) mutant and the D4(PQTTP) which has an additional amino acid residue inserted between the two prolines in the carboxyl-terminal PXXP motif of the D4.4 receptor, changing the sequence from ³²¹LPPQTPPGTRR to ³²¹LPPQTTTPGTRR. Both mutants were subcloned into the pBluescript expression vector under T7 control.

Successful mutant receptors were tested for their ability to interact with the established SH3 domain interactions using the *in vitro* assay (Materials and Methods, Chapter 2, page 80). Equivalent amounts of wild type D4.4(1-419) receptor and that of the mutant receptors were incubated with Nck-GST or Grb2-GST to determine if the interaction was reduced for the created mutants.

6.3 Results and Discussion

6.3.1 Peptide Interaction Study

Peptides were designed to target the amino- and carboxyl-terminal regions to the repeat region of the dopamine D4 receptor (Figure 6.1). Four overlapping peptides were created that included all the PXXP motifs amino-terminal to the repeat region. One peptide was created to contain the PXXP

FIGURE 6.1 Peptides directed against the PXXP motifs of the dopamine D4.4 receptor are illustrated. The amino acid sequence of the various peptides tested are shown in relation to the third intracellular loop sequence of the dopamine D4 receptor. The PXXP sequences are indicated and the various deletion mutants previously tested are illustrated.

motif and surrounding sequence carboxyl-terminal to the repeat region. The peptides were designed so that all of the PXXP motifs in the dopamine D4 receptor were targeted and that each of the PXXP sites in the dopamine D4 receptor had a corresponding peptide that placed the PXXP site near the center of the peptide sequence in order to ensure that there was sufficient sequence surrounding the core motif for peptide binding. Peptide studies have indicated that peptides of nine amino acids in length are capable of binding to SH3 domains (Sparks *et al.*, 1994). These peptides typically contain two or three amino acids on either side of the PXXP motif.

6.3.1.1 *In vitro* Peptide Competition Assay

Various peptides were added to the *in vitro* interaction assay using conditions which previously demonstrated strong D4/SH3 binding (Chapter 3) in order to determine the ability of the specific peptides to disrupt the established interaction. None of the peptides tested (P(232-245), P(240-248), P(245-253) and P(248-256)) altered the binding of D4.4(1-419) to Nck (3SH3) or Grb2 when added in excess to the incubation mixture (Figure 6.2 A and B). The P(232-245) peptide was unable to alter binding of D4.4(1-419) with Nck (3SH3) at concentrations of the peptide up to 100-fold higher than that of the D4 receptor (Figure 6.2 A). Furthermore, a 150 000-fold greater concentration of the 9-mer peptides than the receptor did not result in an appreciable reduction in the D4/SH3 interaction for Nck or Grb2 for any of the peptides tested. (Figure 6.2 B). Under similar conditions, a 10-fold higher concentration of unlabeled *in vitro* D4.4(1-419) was able to reduce labeled [³⁵Cys] D4.4(1-419) binding significantly to both Nck (3SH3)-GST and Grb2-GST (Chapter 3, page 93). These results suggest that the peptides containing the amino-terminal PXXP motifs of the dopamine D4 receptor could not bind to the SH3 domains of Nck or Grb2.

FIGURE 6.2 *In vitro* interaction of dopamine D4 receptor with Nck and Grb2 in the presence of an excess of D4 peptide. (A) The P(232-245) peptide was added at various concentrations (0.01 - 100X the concentration of D4.4) to an *in vitro* incubation mixture containing control (luciferase) or ~2 nM [³⁵Cys] D4.4(220-336) and ~30 μM Nck-GST. (B) The 9-mer peptides were added to a final concentration of ~300 μM to an *in vitro* incubation mixture containing control (luciferase) or ~2 nM [³⁵Cys] D4.4(220-336) and ~30 μM GST, Nck (3SH3)-GST or Grb2-GST. The mixture was incubated for 2 h at 4 °C. The immobilized *in vitro* product was size separated on a protein gel and exposed to film (24 h exposure). Molecular masses are indicated in kilodaltons.

6.3.1.2 Fluorescence Spectroscopy

Fluorescence spectroscopy was used to determine the binding potential of the D4 peptides to the adapter proteins Nck and Grb2. Peptide binding to the SH3 domain typically results in a change in the fluorescence of a conserved tryptophan found in the SH3 domain upon complex formation. Various peptides were added to solutions containing GST, Nck (3SH3)-GST and Grb2-GST in order to determine if the addition of peptide can alter the emission characteristics of the Nck and Grb2 proteins. The glutathione elution buffer and the glutathione elution buffer with peptides had minimal emission properties (there are no tryptophans in the peptides). GST, Nck (3SH3)-GST or Grb2-GST

(1 μ M) dissolved in the glutathione elution buffer all demonstrated a robust measurable fluorescence when excited at a wavelength of 295 nm (AVIV Circular Dichroism Spectrometer (Model 62A DS. Lakewood, New Jersey), 700 V, 25 °C (slit width 2 nm)) and measured at an emission of greater than 320 nm (signal strength between 0.31 and 0.34). Addition of the different peptides (P(232-245), P(240-248), P(245-253), or P(248-256)) to a final concentration of 10, 20 40 or 50 μ M did not result in a change in fluorescence intensity when allowed to equilibrate over time. Furthermore, reducing the amount of GST fusion protein in the glutathione buffer (30, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.1563 μ M) while maintaining a constant concentration of peptide did not result in changes in emission at the different concentrations of Nck or Grb2 when measured in the absence or presence of peptide. We were unable to observe any change in fluorescence intensity using the peptides directed against the amino-terminal PXXP motifs at the various concentration of the peptide and of the GST fusion protein.

We utilized two different spectrometers (AVIV Circular Dichroism Spectrometer (Model 62A DS. Lakewood, New Jersey) and a Perkin-Elmer Luminescence Spectrometer (Model LS 50B)) to measure the shift in emission. Both machines demonstrated a measurable emission pattern for the adapter proteins, but this was unaffected by the addition of peptides. We attempted to maximize the emission properties of the Nck and Grb2 proteins by altering the excitation wavelength and emission wavelength measured. Furthermore, we varied the power and slit width to maximize the sensitivity and resolution in measuring the fluorescence of the proteins. We were unable to detect any shift in emission induced by peptide binding with any of the conditions tested.

The use of fluorescence spectrometry to determine protein interactions and affinity is limited by the number of tryptophans in the two interacting proteins (for review see Phizicky and Fields, 1995). The more tryptophans, the more difficult it is to detect a change in fluorescence since the fluorescence is dependent on the sum of all the tryptophans in the protein. Furthermore, the sensitivity

is limited to the inherent fluorescence of the protein and the change in fluorescence that the interaction produces (Phizicky and Fields, 1995). Typical interactions contain one and no more than six tryptophans in the interacting proteins (Phizicky and Fields, 1995). As a result of these limitations, many protein interactions are excluded as candidates for fluorescence spectrometry (Phizicky and Fields, 1995). The Nck (3SH3)-GST and Grb2-GST fusion proteins contain 13 and 9 tryptophans respectively, which likely results in only a small percentage change in fluorescence, too small to detect using our conditions. Removal of the GST portion of the GST fusion protein, reduces the number of tryptophans by 4. This still leaves 9 and 5 tryptophans in the remainder of the Nck (3SH3) and Grb2 proteins respectively, which is likely too many to detect changes in emission. Further reductions in the number of tryptophans, by the expression of individual SH3 domains will likely lead to a level that may be able to detect differences in fluorescence.

These results do not necessarily demonstrate that the amino-terminal PXXP motifs of the dopamine D4 receptor are not involved in SH3 binding. The results from the *in vitro* deletion studies demonstrate that this region is required for binding. A reduction in D4/SH3 binding may not have been seen because of a number of factors. Peptides often demonstrate a much lower affinity for SH3 domains than the full length binding sequences (Feng *et al.*, 1995). The affinity of the peptides may have been too low to demonstrate a reduction in binding. This would be especially true for the P(232-245) peptide that was only tested at a 100-fold higher concentration than D4. Peptides containing the SH3 binding motif may not bind to the SH3 binding domain due to physical properties of the peptide. Improper folding of the peptide may interfere with its binding to the SH3 domain. The large number of prolines in the peptide sequence induces sharp turns that may result in the obstruction of the SH3 binding site which may not occur with the expression of the entire sequence because of additional constraints on the protein structure by flanking amino acid sequences. Varying the length and position of the peptide may overcome this problem. From our *in vitro* studies, we demonstrated that full

strength interaction of the dopamine D4 receptor with the adapter protein Grb2 required at least two sites. Disruption of one of the sites resulted in a significant drop in affinity. The peptides are targeted to only one SH3 domain, and thus cannot display the cooperative interaction that leads to high affinity binding seen with the entire third intracellular loop. The strength of interaction of the peptides may be too low so that it cannot effectively compete off the full length D4 receptor that binds with higher affinity. A longer peptide directed to both SH3 binding sites may display a significantly higher affinity for the SH3 domain and be more effective at competing for the D4 receptor but will give less information about the specific binding sites.

6.3.2 Point Mutation Studies

From the deletions studies (Chapter 3) it appears that SH3 binding occurs to regions flanking the repeat region of the dopamine D4 receptor and may also involve the first five amino acids of the first repeat, since removal of most of the repeat region did not reduce SH3 binding, and both the amino- and carboxyl-terminal regions to the repeat were required for maximal binding. The interaction of the dopamine D4 receptor with the SH3 domain is most likely mediated by PXXP motifs. Therefore, we targeted all the PXXP motifs amino- and carboxyl-terminal to the repeat region (Figure 6.4, p. 157). Due to the overlapping nature of the PXXP motifs, the mutation of one proline could target more than one PXXP sequence. We attempted to create eight point mutations, targeting every possible PXXP motif by converting prolines to leucines in the full length dopamine D4.4 receptor.

Three of the eight targeted sites in the dopamine D4.4 receptors, D4(P235L), D4(P241L) and D4(P243L) were successfully mutated from prolines to leucines. Using conditions that showed strong interactions of Nck and Grb2 with the wild type dopamine D4.4 receptor (Chapter 3), the three mutated receptors were tested for their ability to interact with Nck and Grb2. At the conditions tested,

no reduction in binding of the three different mutated [³⁵Cys] D4.4(1-419) receptors were seen compared to that of the wild type receptor to both Nck (3SH3)-GST and Grb2-GST (Figure 6.3).

FIGURE 6.3 Determination of the effect of point mutations on the *in vitro* interaction of the dopamine D4 receptor with the SH3 domain containing proteins Nck and Grb2. [³⁵Cys] *in vitro* labeled dopamine D4.4 receptor (D4.4) or D4.4 point mutants (~1.5 nM) or control (luciferase) (top panel) were incubated with the GST, GST-Nck 3SH3 or GST-Grb2 fusion proteins (30 μM) (lower panels). The immobilized *in vitro* product was size separated on a protein gel and exposed to film (24 h exposure). Molecular masses are indicated in kDa.

The carboxyl-terminal PXXP motif was targeted by a mutation that added an additional amino acid residue between the two prolines creating a PXXXP motif. This additional amino acid residue prevents the two prolines from being presented on the same plane in the left-handed polyproline type II helical (PPII) conformation and presumably precludes SH3 interactions. The receptor that contained this mutation (D4(PQTTP)), did not appear to reduce binding when assayed using the same conditions that demonstrated the D4/SH3 interaction using the *in vitro* assay described above (data not shown). In addition to targeting the prolines of the PXXP motifs, a mutation of methionine to an alanine

D4(M345A) was tested using the *in vitro* assay. D4(M345A) is situated at the third intracellular loop/TM6 junction and in functional studies shows disruption of dopamine binding and a distinct localization of the dopamine D4 receptor in clusters at or near the plasma membrane (unpublished results). The D4(M345A) receptor did not appear to reduce SH3 binding (data not shown).

The various mutations successfully targeted the first four putative PXXP motifs amino-terminal to the repeat and the single carboxyl-terminal PXXP motif, in the third intracellular loop of the dopamine D4 receptor. These results suggest that the prolines mutated do not participate in SH3 binding and do not make up the SH3 binding domain in the dopamine D4 receptor. The results of the point mutations in combination with those of the deletion mutants would tend to indicate that at least one of the three remaining amino-terminal PXXP motifs, straddling the first repeat, may be responsible for SH3 binding (Figure 6.4) if the D4-SH3 interaction is mediated by a PXXP motif.

FIGURE 6.4 Prolines targeted for mutation in the third intracellular loop of the dopamine D4 receptor. Asterisks indicate the prolines targeted for mutation to leucines. The prolines that have been successfully mutated to leucines are indicated by arrows. The PXXP motifs successfully targeted by mutation are indicated by bold lines. The insertion of a threonine (T) into the final PXXP motif is shown. The PXXP motifs whose involvement in SH3 binding has been eliminated either by the deletion mutants or point mutations are shown.

In contrast to these findings, comparison of the human dopamine D4.4 receptor to the rat and mouse sequence would tend to suggest, based on the conserved amino acid sequence between species and the ability of the rat D4 sequence to interact with Nck and Grb2, that the mutated PXXP motifs that did not result in a reduction in binding, are the likely candidates for SH3 binding. Furthermore, the deletion results suggest that weak SH3 binding is mediated by a carboxyl-terminal SH3 binding site implicating the single carboxyl-terminal PXXP site. It is necessary to mutate all the remaining

prolines to leucines in order to determine if these other sites are responsible for binding or if binding is contingent on other factors.

The remaining prolines, that were targeted for mutation, proved to be difficult to mutate to leucines. The dopamine D4 receptor has been shown to be difficult to manipulate at both the nucleotide and amino acid level (Cheong *et al.*, 1996). The high GC content of the third intracellular loop makes it difficult to perform mutagenesis. Furthermore, the repetitive and homologous nature of the third intracellular loop makes it difficult to design a specific oligonucleotide for the D4 receptor that shows specific annealing. Two different commercially available mutagenesis kits (Promega and Stratagene) were used in an attempt to mutate the targeted prolines. In addition at least three distinct mutating primers were designed for the sites that were resistant to mutation. Various annealing temperatures (75, 90 and 100 °C) and conditions (rapid (5 min) versus slow annealing (2h)) were used to maximize the annealing conditions.

The oligonucleotides were evaluated for their ability to anneal to the dopamine D4 receptor nucleotide sequence by using the oligonucleotides as primers in DNA sequencing. It was found that a number of these primers effectively annealed to the D4 sequence as evidenced by unambiguous sequencing results. It was shown that the following oligonucleotides could successfully produce the expected sequence (clear, unambiguous, single primed sequence). The oligonucleotide (692F) that had successfully mutated D4(P235L) gave a weak, but correct sequence. The oligonucleotides targeted to D4(P238L) 684F, D4(P246L) 722F', D4(P249L) 731F, D4(P251L) 744F and D4(P323L) 955F were all able to produce medium to strong sequencing signals, that demonstrated the ability of the oligonucleotides to single prime and to return the expected sequence. These oligonucleotides were used in subsequent mutagenesis reactions using both the Altered Sites and Chameleon mutagenesis systems. The other oligonucleotides targeted to the same prolines as above either showed reduced or produced no sequencing signal and were not used in further rounds of mutagenesis. The remaining

prolines remained resistant to mutagenesis using all the different conditions described and the oligonucleotides that had been shown to successfully anneal to the D4 sequence.

Mutagenesis of the prolines in the third intracellular loop of the dopamine D4 receptor has proven to be difficult. Future attempts will utilize synthetic dopamine D4 receptors whose GC content in the nucleotide sequence has been reduced, yet still encodes the same amino acid sequence, and that have introduced multiple convenient restriction sites to allow for easy manipulation of the DNA (Dr. T. Sakmar; Howard Hughes Medical Institute, Rockefeller University, New York).

The inability to create all the desired point mutations to target the different PXXP motifs believed to be essential for SH3 binding to the dopamine D4 receptor has not allowed us to fully characterize and identify the SH3 binding sites. From the mutations that we were able to create, it appears that the first four putative PXXP motifs and the last PXXP motif are not essential for full SH3 binding. These results appear to contradict the results obtained using the D4 deletion mutant studies.

Examples exist for non-PXXP-mediated SH3 interactions (Gorina and Pavletich, 1996; Sicheri *et al.*, 1997; Xu *et al.*, 1997). These interactions have been shown to be intramolecular and of relatively low affinity (Nguyen *et al.*, 1998). At least one proline appears to be involved in the SH3 binding site and it appears the sequence still conforms to the left-handed polyproline type II helix. The possibility exists that two such weak interactions mediate the D4-Grb2 binding, in a cooperative manner, to produce a higher affinity interaction between the two proteins. In such a scenario, all the prolines in the third intracellular loop would need to be targeted in order to identify the possible SH3 binding sites.

Our studies have not excluded the possibility that multiple PXXP sites in the dopamine D4 receptor may be able to bind to the SH3 domains. The large number of PXXP sequences in the third intracellular loop may mean that multiple SH3 binding sites exist. Our *in vitro* deletion studies suggest that at least two sites are required for maximal, high affinity binding. It may be necessary to

mutate multiple PXXP sites simultaneously to prevent SH3 binding. The possibility exists that Grb2 and Nck can bind to any number of PXXP sites in the D4 receptor with about equal affinity. Binding to one site may obscure other SH3 binding sites. The removal of one site (ie. by mutagenesis) may shift binding to the other site without altering the binding or affinity of adapter proteins significantly. The deletion studies suggested that SH3 binding could occur amino- and carboxyl-terminal to the repeat, but did not necessarily eliminate the involvement of the repeat sequence as seen in the dopamine D4.4 receptor. Point mutations of the deletion mutant D4(Δ 254-315), which removed most of the repeat sequence yet still showed high affinity binding, may allow for the identification of the prolines involved in SH3 binding, without the confounding effect of the repeat and additional PXXP motifs that are not strictly required for SH3 binding.

The identification of the exact SH3 binding sites in the dopamine D4 receptor are required to characterize this interaction. In addition disruption of this interaction by the use of point mutations will allow for functional characterization of this interaction.

Chapter 7

General Conclusions and Recommendations For Future Research

7.1 Dopamine D4 and G Protein-Coupled Receptor Interactions with SH3 Domains

Much of the research on the superfamily of GPCRs has focused on prototypical receptors, including the adrenergic, rhodopsin and LPA receptors. These receptors have been instrumental in learning about receptor binding, G protein-coupling, and in determining the second messenger systems activated by this family of receptors. What has been learned from these receptors is thought to be generally applicable to other members of the GPCRs that share the same structural and functional characteristics. For the dopamine family of receptors, the dopamine D1 and D2 receptors have been most extensively studied and are best characterized with regard to pharmacology, biochemistry and function. These receptors have been shown to activate second messenger pathways in classical GPCR fashion for both G_s and G_i -coupled receptors, and recently the D2 receptor has also been shown to activate the Ras-MAPK pathway traditionally associated with RTK signalling (Luo *et al.*, 1998).

The dopamine D4 receptor is classified as a D2-like receptor that activates G_i -coupled pathways. A great deal of the research on the dopamine D4 receptor has focused on two areas: determining the functional role of the receptor in the whole organism and identifying the biochemical role of the unique repeat polymorphism and how this relates to disease states. Although studies have indicated that the dopamine D4 receptor exhibits high affinity binding to the atypical neuroleptic clozapine and that the repeat sequence of the receptor may affect certain personality characteristics such as novelty seeking and attention deficit hyperactivity disorder (Benjamin *et al.*, 1996; Ebstein

et al., 1996; Ebstein *et al.*, 1997; LaHoste *et al.*, 1996), the mechanisms by which the dopamine D4 receptor may mediate some of these effects on the biochemical level have not been delineated.

Early research on the dopamine D4 receptor had focused on the possible involvement of the receptor in schizophrenia and in determining the pharmacological and biochemical differences of the various D4 variants. The role of the dopamine D4 receptor in schizophrenia has been questioned (Wilson *et al.*, 1998) and only small differences in receptor binding and activation of adenylyl cyclase have been found for the different D4 variants (Asghari *et al.*, 1995; Asghari *et al.*, 1994). Although the receptor variants appear to be associated with specific personality and behavioural characteristics, no major differences in function of the different receptor variants have been discovered at the molecular level.

The focus of the research in this thesis has been on the polymorphic repeat sequence and the immediate surrounding sequence in the third intracellular loop in the dopamine D4 receptor. This region is proline rich and contains a large number of PXXP sequences which may function as binding sites for the SH3 modular binding domain (Dalgarno *et al.*, 1997; Sparks *et al.*, 1994; Sparks *et al.*, 1996). No other identified GPCR exhibits such a large number of prolines and PXXP sequences as observed in the D4. The aim of our research was to determine if the D4 receptor could serve as a binding partner for SH3 domain containing proteins, establish the function of this interaction within the cell, and determine if other GPCRs may also serve as direct SH3 binding partners. Furthermore, we attempted to determine the role of the repeat in mediating this potential SH3 interaction.

7.1.1 SH3 Interactions With GPCRs

We have shown that all of the GPCRs that we tested in this study, including the dopamine D3 and D4 receptors, the β_1 -adrenergic receptor and the m4 muscarinic receptor, showed moderate to strong interaction *in vitro* with at least one of the tested SH3 domain containing proteins. The D4

receptor interacts *in vitro* with the SH3 domains of Grb2, Nck, Abl, Crk, p85 α and c-Src, but not with PLC γ and spectrin. An interaction with Grb2 and Nck was also observed with the dopamine D3 receptor. The interaction of the β_1 -adrenergic and m4 muscarinic receptors with these proteins appears to be restricted to the adapter proteins Grb2 and Nck. These results demonstrate that receptor interactions with SH3/SH2 adaptor proteins are not restricted to the dopaminergic receptor family and may serve a more general role in GPCR function.

An *in vitro* saturation analysis was used to determine the affinity of the various dopamine D4 constructs with the SH3 domain containing adapter proteins Nck and Grb2. The SH3 interaction with the dopamine D4 receptor appears to be both specific and saturable. The interaction of the full length D4.2, D4.4 and D4.7 dopamine receptors was estimated to have a K_d between 50 and 200 μ M for Grb2 and Nck. Similar values have been observed for established SH3 interactions (Dalgarno *et al.*, 1997; Pisabarro and Serrano, 1996; Smithgall, 1995; Yu *et al.*, 1994). It is possible that other SH3 domain containing proteins, that were not tested, interact with the D4 receptor with a higher affinity than those observed for Nck and Grb2. Furthermore, the interaction conditions, including the lysis buffer, the presence of a full complement of proteins and the receptor state, may also affect the binding affinity of the dopamine D4 receptor for SH3 domains. Expression of just the third intracellular loop of the dopamine D4.4 receptor resulted in an interaction of higher affinity (10-40 μ M) than that of the full length D4 receptors. The lower binding affinity observed with the full length receptors may be the result of conformational interference due to folding of the receptor in the solubilized state. The interaction strength of the membrane-bound full length receptor could not be determined because of the difficulty in expressing the D4 receptor in the presence of microsomal membranes using the *in vitro* transcription translation system (data not shown).

The interaction of the dopamine D4 receptor with SH3 domains is mediated by the third intracellular loop, but does not appear to require the repeat region. Complete removal of all the PXXP

sequences in the third intracellular loop of the full length human dopamine D4 receptor eliminates all SH3 interactions. Expression of just the third intracellular loop of the human and rat D4 receptor is sufficient for SH3 binding with Nck and Grb2. The rat sequence, however, does not contain the polymorphic repeat region, but does contain conserved PXXP sites that are found to flank the repeat region in the human dopamine D4 receptor, indicating that the repeat is not absolutely required for GPCR-SH3 interactions and that the sequences containing the PXXP motifs surrounding the repeat region are likely involved in SH3 binding.

Deletion analysis of the dopamine D4 receptor indicated that Grb2 and the fragment of Nck containing the three SH3 domains interact with SH3 binding domains immediately upstream and downstream from the repeat sequence in the D4 receptor, but not necessarily with the repeat sequence itself. These deletion mutant studies suggest that the interaction is cooperative between these two regions in the D4 receptor with the adapter proteins since D4.4(Δ 254-315), which lacks the repeat, displays a strong interaction (K_d of $\sim$ 30-50 μ M) while D4.(Δ 221-315), which lacks the repeat and amino PXXP motifs, has a significantly reduced interaction ($K_d > 600 \mu$ M), which is further diminished by the consecutive deletion of the fragment containing the PXXP motif carboxyl-terminal to the repeat in D4.(Δ 221-337) (no binding). The higher affinity of the deletion mutant D4.4(Δ 254-315) for Nck and Grb2 than the full length dopamine D4 receptor with the repeat region may be the result of a more favorable conformation of the receptor that does not contain the repeat. Normally, the repeat may have to undergo appropriate folding in order to present the two SH3 binding sites without the repeat itself interfering with these binding sites. Removal of the repeat may eliminate the folding requirement of the repeat and allow for easy access of the adapter proteins to the receptor's binding sites.

By examining the interaction of the dopamine D4 receptor with the amino-terminal SH3 domain compared to the carboxyl-terminal domain of Grb2, it was found that the carboxyl-terminal

SH3 domain fragment is the primary site of interaction, although the whole Grb2 protein shows a significantly enhanced interaction with D4. The amino SH3 domain by itself showed no interaction with D4, but mutagenesis of Pro⁴⁹ to Leu⁴⁹ in the amino-terminal SH3 domain, shown to disrupt established SH3 binding (Motto *et al.*, 1994), significantly reduced the interaction of the whole protein with the D4 receptor, indicating that the interaction of Grb2 with the D4 receptor is of a cooperative nature between the amino-terminal and the carboxyl-terminal SH3 domains. Since only the full-length Grb2 protein appears to interact with the muscarinic m4 and β_1 -adrenergic receptor, Grb2 may also interact in a cooperative manner with these receptors. This type of cooperative interaction has been previously demonstrated for Grb2 binding to HPK1 (Anafi *et al.*, 1997). Our D4 deletion studies, together with the observed differential interactions between the D4 and the different Grb2 fragments and Grb2 point mutant, strongly indicate that the different SH3 domains of Grb2 interact in a cooperative manner with the two SH3 binding domains up- and downstream from the repeat sequence in the D4 receptor. These data indicate that the absence of SH3 domain mediated interaction using single SH3 domains should be interpreted with caution with respect to their involvement in the binding of the holoprotein.

Three of the prolines amino-terminal to the repeat region were successfully mutated to alanines creating three separate constructs that targeted the first four putative PXXP motifs in the human dopamine D4 receptor. These mutants, however, did not decrease the SH3-mediated binding of either Nck or Grb2 with the D4 receptor. Results from the deletion studies suggest that Nck and Grb2 binding requires at least two SH3 binding domains located amino- and carboxyl-terminal to the repeat region of the D4 receptor. Combined, the results from the deletion and point mutation studies seem to indicate that one of the two remaining PXXP motifs that straddles the junction of the first repeat and amino-terminal flanking sequence may be responsible for the amino-terminal SH3 binding of the dopamine D4 receptor. Sequence alignment of the human dopamine D4 receptor sequence to

that of the mouse and rat sequence, however, shows that the amino acid sequence that includes the first four PXXP motifs is conserved among species and is likely responsible for the observed SH3 binding in rat. Based on this sequence homology, one would expect that these sites mediate the conserved SH3 interaction both in the rodent and human receptors. Point mutations of the rat sequence will help to identify the exact binding sites in this species. One other mutant receptor was created by inserting an amino acid within the only carboxyl-terminal PXXP motif of the human D4 sequence, disrupting the PXXP motif. This mutant did not eliminate or reduce the observed SH3 interaction, even though the deletion studies implicated this site in cooperative binding.

Although it appears that the results from the deletion studies contradict those observed with the point mutation studies, several possible reasons may account for these apparently conflicting results. 1) Examples exist of SH3 mediated interactions that do not require a PXXP sequence but are mediated by a binding site that only contains one proline, yet still forms a left-handed polyproline type II helix conformation sufficient for SH3 binding (Gorina and Pavletich, 1996; Sicheri *et al.*, 1997; Xu *et al.*, 1997). The point mutation of just one proline in the various PXXP sequences of the dopamine D4 receptor may, therefore, not necessarily destroy the SH3 binding motif. Examples of SH3 binding to SH3 binding motifs containing only one proline have only been observed for the intramolecular interactions as seen for Src and Hck and are in general much weaker than PXXP-mediated interactions (Gorina and Pavletich, 1996; Nguyen *et al.*, 1998; Sicheri *et al.*, 1997; Xu *et al.*, 1997). It may be necessary to target both prolines of a PXXP sequence or also target prolines that do not form part of a PXXP sequence in the D4 receptor in order to identify the specific SH3 binding sites. 2) Although, from our studies, the repeat region does not appear to be necessary for SH3 binding and the elimination of this region does not reduce SH3 binding, it is possible that the SH3 domains of Nck and Grb2 are able to bind to multiple PXXP sequences within the dopamine D4 receptor with approximately equal affinities. Binding of an SH3 domain to one site may preclude by,

steric hindrance, binding of another SH3 domain to alternate binding sites. Elimination of only one amino-terminal PXXP site at a time may drive SH3 binding to other amino-terminal or repeat situated PXXP sites. Elimination of the repeat region may force SH3 interactions with the amino-terminal PXXP sequences, which may display an equivalent binding affinity and thus cannot be distinguished. Point mutations of single and multiple amino-terminal PXXP sequences in combination with the D4 deletion mutant (D4.(Δ 254-315)), that demonstrated full strength SH3 interaction in the human dopamine D4 receptor, may be required to definitively determine the site(s) of SH3 binding. In addition creating point mutations in the rat sequence, which does not contain the repeat sequence, may help to identify specific conserved sites of SH3 interaction for the D4 receptor.

Expressing just the 32 amino acid fragment of the $\alpha\zeta$ repeat sequence of the dopamine D4 receptor using the *in vitro* transcription/translation system did not result in SH3 interactions with either the Nck or Grb2 adapter proteins. No interaction with this fragment may have been observed because the PXXP sequences within this sequence do not act as SH3 binding domains. Alternatively, no interaction with this fragment may have been observed because the PXXP motif(s) involved in SH3 binding are found at the extreme amino-terminal end of the repeat fragment and may require additional amino acids that were not expressed for binding. Binding of peptides appears to require at least three amino acids on either side of the PXXP sequence for binding (Sparks *et al.*, 1994). In addition, the deletion and point mutation studies indicate that two PXXP sites that straddle the intersection of the first repeat with the amino-terminal sequence are likely candidates for SH3 interaction. These PXXP sites are not expressed in the $\alpha\zeta$ fragment. It is also possible that SH3 binding is mediated only by specific repeat variants, but not by the α and ζ repeat units. A more systematic targeting of prolines in the third intracellular loop and repeat region will be required to determine the exact binding sites.

Short peptides sequences, corresponding to regions of several GPCRs, including the third

intracellular loop, have been successfully used to characterize GPCR function and intracellular protein interactions (Hébert *et al.*, 1996; Malek *et al.*, 1993; Ng *et al.*, 1996). The dopamine D4 receptor peptides designed to disrupt SH3 interactions in this study were unable to prevent or alter SH3 binding. The short peptides may not duplicate the conformation of the sequence as found in the D4 receptor sequence. Not all peptides with known SH3 binding sequences are able to demonstrate the interaction that can be observed with the full length protein encoding the entire SH3 binding domain. Possibly the shorter peptides have a lower affinity for Grb2 than the receptor and therefore cannot effectively compete.

An interaction in cells was demonstrated by expressing the interacting fragment of the dopamine D4 receptor and the three SH3 domains of Nck as fusion proteins with the *GAL4* activator and DNA binding proteins in yeast. The yeast two-hybrid system has been successfully used to determine several SH3 interactions with good predictive capabilities that subsequently have been confirmed *in vivo* (Anafi *et al.*, 1996; Lussier and Larose, 1997). Although Nck showed an interaction in this system, the amino- or carboxyl-terminal fragments of Grb2 failed to interact with the D4 receptor. The failure of Grb2 to interact is not due to a lack of expression, since we could successfully demonstrate an interaction in yeast between Grb2 and the Sos tail or the Crk SH2 domain. Furthermore, western analysis demonstrated approximately equivalent levels of expression of Grb2 and Nck fusion proteins (data not shown). We cannot exclude that the conformation of the Grb2-DNA binding and D4.4(219-336)-activator complex may have interfered with the ability of this complex to bind to, or activate, the *GAL4* promoter of the reporter construct. Alternatively, it is possible that Grb2 failed to show an interaction because we only expressed either the amino- or carboxyl-terminal SH3 domain of Grb2 as a fusion construct, which were shown *in vitro* to have a significantly reduced interaction with the D4 receptor. Attempts at expressing the entire Grb2 protein in yeast resulted in self activation thus preventing us from observing an interaction with D4. The data are consistent with

the interpretation that the D4-SH3 interaction is of a cooperative nature between two distinct SH3 domains and their binding sites in the D4 receptor.

Attempts at demonstrating a dopamine D4/SH3 interaction *in vivo* was hampered by the lack of a specific dopamine D4 receptor antibody, low cellular expression levels of the dopamine D4 receptor and difficulties in purifying the D4 receptor from the membrane. In addition, it appears that the Nck and Grb2 antibodies employed are directed against the SH3 domain regions of these proteins and may, therefore, interfere with the D4/SH3 interaction in co-immunoprecipitation experiments. Besides these technical difficulties that may account for the inability to demonstrate co-immunoprecipitation of these proteins, several other possibilities exist for failing to detect an interaction between D4 and either Nck or Grb2. The dopamine receptor may have an endogenous SH3 domain containing binding partner which is neither Nck nor Grb2. The interaction with the endogenous binding partner may have a much higher affinity for the D4 receptor than that observed with Nck and Grb2 and thus prevent the interaction of D4 with these proteins *in vivo* in the presence of a full complement of cellular proteins. Alternatively, subcellular distribution may not support Grb2 or Nck binding to D4, although Grb2 does associate with receptors at the membrane and can for those reasons, a priori, not be excluded as an endogenous binding partner. A complete screen of all identified SH3 domain containing proteins and the search for novel D4/SH3 interactions using a library expression system (ie. screen a yeast two hybrid or phage display library) may be required to identify the D4 binding partner(s) and confirm the SH3 interaction *in vivo*. Failing to detect the SH3 interaction *in vivo* may also be the result of differences in the receptor state *in vivo* compared to that of the receptor in the *in vitro* system. SH3 interactions have been shown to be modulated by phosphorylated serines and threonines which are in close proximity to the PXXP motif (Chen *et al.*, 1996a). For the dopamine D4 receptor, several serines and threonines are located near some of the identified PXXP sequences which may modulate SH3 binding. These amino acids may also serve as

second messenger-dependent phosphorylation sites and thus may affect the strength of the SH3 interactions. The phosphorylation status of the dopamine D4 receptor *in vivo* and *in vitro* is unknown. Although the cells were not treated with drugs and were cultured in serum free media to prevent second messenger-dependent kinases from phosphorylating the receptor, it cannot be ruled out that the phosphorylation of serines and threonines near the PXXP sites may have prevented the observation of a D4/SH3 interaction *in vivo*. The results from our *in vivo* interaction assays neither confirmed nor rejected the putative dopamine D4 receptor interaction with Nck or Grb2.

The functional importance of the identified SH3 binding domains in the dopamine D4 receptor was explored by the disruption of these domains in the amino-terminal epitope-tagged receptor. Functionally and pharmacologically the wild type D4 receptor was indistinguishable from both the HA and Flag epitope tagged versions of the receptor. With respect to receptor binding we demonstrated that both spiperone binding and the binding of dopamine is unaffected by the deletion of the putative SH3 binding domains. Inclusion of the non-hydrolysable GTP analogue Gpp(NH)p in the binding assays resulted in a decreased binding affinity for dopamine. In addition, dopamine can stimulate GTP for GDP exchange for both the wild type and mutant D4 receptors as measured by a GTP γ ³⁵S binding assay. This indicated that the receptor has retained the ability to interact functionally with G proteins. Nevertheless, the deletion of the putative SH3 binding domains resulted in disruption of functional coupling of the activated receptor to adenylyl cyclase and MAPK.

Disruption of the SH3 binding domain region of the dopamine D4 receptor affected receptor internalization. Internalization was studied by an immunofluorescence detection protocol that allowed the detection of receptors pre-labeled with a primary antibody on the extracellular surface of the cell (Chu *et al.*, 1997). Following a 30 min incubation period, in the absence of receptor stimulation, the receptors were detected with a secondary immunofluorescent antibody. This way it was shown that the SH3 binding domain deleted receptors were predominantly localized in endosomal vesicles,

suggesting a strong constitutively internalized phenotype of these receptors. The constitutively internalized phenotype may explain the inability of the receptor to show significant coupling to the second messenger pathways. This is further supported by the deletion mutant that still contains the putative carboxyl-terminal SH3 binding domain, which shows an intermediate phenotype for internalization and also shows a moderate ability to couple to adenylyl cyclase. The deletion mutant D4.(Δ 254-315), which does not express the repeat, is still functionally active (Asghari *et al.*, 1995; Asghari *et al.*, 1994) and displays no internalization deficit (not shown) suggesting that the repeat sequence itself is not involved in the interaction deficit.

Removal of the repeat region from the dopamine D4 receptor has been shown in this study and previous studies (Asghari *et al.*, 1995; Asghari *et al.*, 1994) not to have significant effects on receptor binding, G protein-coupling and the ability to inhibit adenylyl cyclase activity. Additional deletion of both amino- and carboxyl-terminal flanking regions to the repeat in the third intracellular loop (D4.4(Δ 221-337)) also did not affect receptor binding and G protein-coupling. This deletion mutant, however, was impaired in its ability to inhibit cAMP levels and its ability to activate the MAPK pathway, but it is unknown whether this is the result of impaired function of the receptor, or if this is the result of the internalized phenotype that may prevent the receptor from being stimulated because it is not exposed to dopamine at the surface of the cell. The present results indicate that even the removal of 116 amino acids from within the central portion of the third intracellular loop resulted in a receptor that showed normal receptor binding and still demonstrated G protein-coupling.

While the transmembrane domains of GPCRs appear to be the most important in determining receptor binding (Hibert *et al.*, 1992; Trumpp-Kallmeyer, 1992 #126; Hibert *et al.*, 1993; Strader *et al.*, 1989; Strader *et al.*, 1988), the second and third intracellular loop as well as the carboxyl-terminal tail appear to mediate G coupling (Dohlman *et al.*, 1991; Hedin *et al.*, 1993; Savarese and Fraser, 1992; Wess, 1997; Wess, 1998). The relative contribution of these regions in G protein-coupling of

GPCRs varies between receptors. From chimeric D1/D2 studies both the second and third intracellular loop appear to be required for maximal G protein activation (Kozell *et al.*, 1994). Removal of large portions of the third intracellular loop, as seen with the deletion mutants studied in this study, at first blush, would be expected to affect G protein-coupling, however, there are a number of studies that suggest that G protein-coupling is mediated by the extreme amino- and carboxyl-terminal ends of the third intracellular loop. The third intracellular loop may be between 15-240 amino acids in length, but only 8-15 amino acids at the amino- and carboxyl-terminal-end appear to be involved in G coupling (Strader *et al.*, 1994). Synthetic peptides directed against the amino- and carboxyl-terminal portion of the third intracellular loop in the dopamine D2 receptor disrupt functional G protein-mediated coupling to adenylyl cyclase while also activating GTPase activity (Malek *et al.*, 1993). The low homology seen in the central portion of the third intracellular loop in combination with other studies that have shown that removal of large portions of the third intracellular loop of the m1 muscarinic receptor does not impair G protein-coupling (Shapiro and Nathanson, 1989) suggests that the region deleted in the dopamine D4 receptor may indeed not be involved in G protein-coupling.

Although the deletion mutant dopamine D4 receptor still demonstrated G coupling, our studies did not investigate whether G protein specificity was affected. A recent study (Daaka *et al.*, 1997) demonstrated that phosphorylation of the β_2 -adrenergic receptor changed G protein specificity from G_s to G_i . Similarly, it has been shown that complete replacement of the third intracellular loop between related, but functionally distinct receptors as seen with the m2 and m3 muscarinic acetylcholine and the α_1 - and β_2 -adrenergic receptors can alter GPCRs from G_i to G_s linked receptors (Cotecchia *et al.*, 1992; Liggett *et al.*, 1991; Wess *et al.*, 1990a; Wess *et al.*, 1990b). It is unknown whether the dopamine D4 receptor deletion mutants studied here maintained their specificity for the G_i -coupled proteins. Furthermore, it is unknown whether the dopamine D4 receptor lost the ability

to couple to the complete repertoire of G proteins including G_i and G_t or whether the receptor can now only bind to specific subset of these G proteins.

The ability of the dopamine D4 receptor deletion mutants to still bind and couple to G proteins suggests that the constitutive internalized phenotype seen with the deletion mutant that had all the PXXP sequences removed is not mediated by an impairment in G protein-coupling. Rather, removal of this sequence appears to lead to receptor internalization by another mechanism. These results strengthen the argument that the modified function of the dopamine D4 receptor observed are likely mediated by the established SH3 interactions. The mechanism by which this region affects internalization requires further investigation.

7.1.2 Implications of SH3 Interactions with the Dopamine D4 Receptor and GPCRs

The demonstration of a direct SH3 interaction with the dopamine D4 receptor is the first such example for the superfamily of GPCRs (Oldenhof *et al.*, 1998). The results suggest that this type of interaction is not just limited to the dopamine D4 receptor, but is likely a more general phenomenon seen with other members of the dopamine receptor family and GPCRs as well. Certain aspects of GPCR function are likely mediated by these SH3 interactions. The constitutive internalization phenotype and the inability of the dopamine D4 receptor that lacks all SH3 binding sites to activate the MAPK pathway suggests a possible involvement for direct SH3 interactions in these D4 receptor functions. It is unclear from the present results, however, how SH3 interactions may be involved in these functions. Although there are no other examples of GPCR-SH3 interactions, the identified binding partners from this study and examples of SH3 involvement in GPCR function, as seen in MAPK activation and endocytosis, may help to identify a possible role and mechanism for this type of SH3 interaction.

The functional importance of the observed interactions remains speculative, primarily because

Grb2 and Nck are SH2-SH3 adapter proteins that can complex with a variety of proteins. The relatively strong interaction of the D3 and D4 receptors with Grb2 may point to a common activity mediated by these receptors. Grb2 plays a crucial role in the Ras-mediated mitogenesis pathway and Nck is also thought to play a dominant role in RTK signalling and mitogenesis (Hu *et al.*, 1995; Lowenstein *et al.*, 1992). A variety of GPCRs, including the dopamine D2, D3 and D4 receptor, are known to stimulate mitogenesis (Lajiness *et al.*, 1995). The Ras-dependent pathways can be activated by GPCRs, like the D2-like receptors, that couple through pertussis toxin-sensitive G proteins (Koch *et al.*, 1994; Luo *et al.*, 1998). This is achieved through the ability of the G protein to promote Shc tyrosine phosphorylation, possibly through the activation of a non-RTK, thus stimulating the Shc/Grb2/Sos/Ras complex formation (Luttrell *et al.*, 1997c). However, it is unclear how the receptor specificity in the G protein-mediated activation of mitogenesis is achieved. With the observed interaction between the D4 receptor and the Grb2 and Nck adapter proteins we have identified a possible mechanism by which G proteins can be brought in close proximity to the Ras activating complex in a receptor-specific manner. This may occur either by a direct interaction with Grb2, or through an adapter-protein-mediated coupling of the RTK and/or non-RTK to the GPCR by means of their respective SH2 and SH3 binding domains. This type of protein interaction may also provide a means for mediating the reported activation of G_{q-11} by a tyrosine phosphorylation which will require the recruitment of a tyrosine kinase to the receptor complex (Umemori *et al.*, 1997).

Many of the established SH2 interactions are between cytosolic SH2 domain containing proteins and phosphorylated membrane-bound receptors. For example, the adapter protein Grb2 has been shown to bind to a phosphorylated tyrosine site on some RTKs by means of its SH2 domain. Recent evidence has extended this type of interaction to the membrane-bound β_2 -adrenergic GPCR, which has been shown to bind directly to the SH2 domain of Grb2 (Karoor *et al.*, 1998). Most of the identified SH3 interactions, however, occur between two cytoplasmic proteins as exemplified by the

Grb2 and Sos protein interaction that is involved in both GPCR and RTK activation of the MAPK pathway. Typically, in these interactions, receptor activation leads to the phosphorylation of tyrosine sites which then allows for the binding of the SH2 domain of the interacting protein. SH3 domains on the same protein interact simultaneously with cytosolic effector proteins, recruiting these both to the receptor and other membrane associated proteins such as Ras. The general pattern is that SH2 domains are involved in binding to upstream molecules, while SH3 domains on the same protein interact with downstream targets (Schwartzberg, 1998). Few examples exist of proteins targeted to membrane-bound receptors mediated by SH3 domain interactions. One such example, however, is the SH3 domain of the Src-like Fyn protein that has been shown to target a proline-rich sequence in the cytoplasmic domain of CD43, an abundant transmembrane protein in T lymphocytes (Pedraza-Alva *et al.*, 1998). The non-receptor tyrosine kinase protein Src has also been shown to bind to potassium channels by means of its SH3 domain (Holmes *et al.*, 1996).

The various identified mechanisms of cell proliferation by GPCRs all appear to require heterotrimeric G proteins as intermediaries (Dhanasekaran *et al.*, 1998). In one such major pathway, the G $\beta\gamma$ subunit appears to activate Src by means of PI3-K after receptor activation, which then leads to the subsequent interaction of Shc with Src, Grb2 with Shc, Sos with Grb2 and then Ras-MAPK activation (Della Rocca *et al.*, 1997; Dhanasekaran *et al.*, 1998; Hawes *et al.*, 1996). The activation of the MAPK pathway by the dopamine D4 receptor by means of a direct SH3 interaction with the receptor could circumvent various intermediary steps including that of G coupling. A direct SH3 interaction of a non-receptor tyrosine Src-like kinase such as Fyn with the dopamine D4 receptor may lead to the activation of this protein tyrosine kinase and result in the subsequent binding by the Shc protein in a similar fashion as that seen with the transmembrane protein CD43 and Fyn (Li *et al.*, 1996; Pedraza-Alva *et al.*, 1998). This interaction has been shown to lead to both Fyn and Shc phosphorylation and the activation of the MAPK pathway by the formation of the Grb2-Sos-Ras

complex (Pedraza-Alva *et al.*, 1998). The phosphorylation of a tyrosine site in Fyn presumably serves as an SH2 domain binding site for Shc. Shc is also phosphorylated on a tyrosine site and becomes the binding partner for the SH2 domain of Grb2. In a recent study by Luttrell *et al.* (Luttrell *et al.*, 1999), it was found that c-Src binds by means of its SH3 domain to arrestin, which is bound to the receptor in response to GPCR phosphorylation. The phosphorylated Src interacts with Shc and leads to MAPK activation. In the proposed direct SH3 interaction mechanism, arrestin binding to the receptor would not be necessary, but rather Src could bind directly to the SH3 binding site of the receptor and this could lead to the MAPK cascade in a manner similar to that mediated by arrestin. Such SH3 interactions may serve as just one possible mechanism of how the dopamine D4 receptor and other GPCRs could initiate MAPK pathway activation. The activated protein tyrosine kinase would serve the role normally associated with RTKs and become a substrate for Shc. Activation of MAPK signalling in the proposed mechanism may be G protein independent, or G protein-coupling may modulate this interaction. Results indicate, however, that the activation of the MAPK pathway by the dopamine D4 receptor in CHO-K1 cells is PTX sensitive (unpublished results), implicating a G_i-mediated mechanism. Removal of the G protein-coupling site in the dopamine D4 receptor, while maintaining the SH3 domain binding sites may help to determine exact involvement or requirement of G protein-coupling in the activation of such a proposed pathway.

In the proposed mechanism discussed above, the interaction of Src-like kinases is mediated by only one SH3 domain. Evidence from our studies suggest that both SH3 domains of Grb2 are required for maximal binding. Either there exist Src-like kinases not tested in the present study that show significantly higher strength of interaction with the dopamine D4 receptor, or SH3-mediated interactions of the D4 receptor is mainly associated with proteins that contain multiple SH3 domains, as found in the adapter proteins such as Grb2, Nck and Crk. How these proteins may mediate activation of the MAPK pathway is unclear since they only possess the ability to interact with other

proteins by their free SH2 domain. The activation of the MAPK pathway by some GPCRs has been shown to occur by the transactivation of RTKs (Daub *et al.*, 1996). Grb2 could act as the adapter molecule that could link these two receptors by an SH2 mediated binding to the phosphorylated RTK receptor. In such a scenario, however, the Grb2 protein would occupy the phosphorylated tyrosine SH2 binding site of the RTK but be unable to recruit the Sos protein by its occupied SH3 domains and the RTK would be prevented from interacting with effector molecules because of the occupied SH2 binding domain.

GPCR endocytosis is mediated by a variety of cellular mechanisms that is receptor and cell type dependent. Modular binding domains, including SH3 domains, are critical in this function (Grady *et al.*, 1997; Scaife and Margolis, 1997; Wigge and McMahon, 1998). As described in more detail in the introduction (p. 57) a major pathway of GPCR internalization requires phosphorylation of the receptor, binding of an arrestin protein, the involvement of the mechanoprotein dynamin and clathrin-mediated endocytosis. Important SH3 interactions occur between the SH3 binding site of dynamin and the Grb2 adaptor protein in RTK endocytosis, but in GPCR endocytosis, dynamin interaction appears to be mediated by a PH domain to the G β γ protein dimer (Scaife and Margolis, 1997). Amphiphysin appears to recruit dynamin, by means of its SH3 domain, during endocytosis (Wigge and McMahon, 1998). Src has been shown to bind to dynamin (Foster-Barber and Bishop, 1998) and the arrestin protein, which is required for both GPCR endocytosis and MAPK activation, by means of an SH3 interaction (Luttrell *et al.*, 1999). Not all GPCRs require arrestin binding to exhibit endocytosis as seen with the m2 muscarinic receptor (Pals-Rylaarsdam *et al.*, 1997). A direct interaction of the Src protein with the GPCR, as demonstrated to be possible with the dopamine D4 receptor in this study, may negate the requirement for arrestin binding yet still lead to internalization.

Although these may be possible mechanism for endocytosis of the dopamine D4 receptor, the phosphorylation status, ability to interact with such proteins as arrestin, as well as the ability to be

sequestered has not yet been established for the dopamine D4 receptor. Furthermore, results from the internalization study suggest that the SH3 interactions do not lead to internalization since removal of all SH3 binding domains results in a receptor that is localized in endosomal vesicles, while the wild type receptor is localized on the cell membrane. SH3 domains have also been shown to mediate protein localization and function as cytoskeletal proteins (Lu *et al.*, 1997; Weng *et al.*, 1993). The SH3 interaction may be important in anchoring the dopamine D4 receptor to the cell membrane, by means of an interaction with a membrane associated protein. In support of such a role for SH3 interactions, it is known that the adapter protein Nck has been shown to help localize PAK at the membrane (Lu *et al.*, 1997). Furthermore, a recent study has shown that for the Na⁺/H⁺ exchanger (NHE2) removal of both carboxyl-terminal SH3 binding sites in the protein maintained normal function with regard to Na⁺/H⁺ exchange, sensitivity to intracellular ATP, and activation by hyperosmolarity (Chow *et al.*, 1999). The removal of these SH3 binding sites, however, altered the localization of the exchanger from the apical membrane to that of the basolateral surface, but also resulted in intracellular accumulation, suggesting that these SH3 binding regions are important for subcellular targeting of NHE2 (Chow *et al.*, 1999). It is worth pointing out that the sequence termed Pro-1 in NHE2 (⁷⁴³PPSVTPAP⁷⁵⁰), required for the normal localization of the exchanger (Chow *et al.*, 1999), shows but a one amino acid difference with the dopamine D4 receptor sequence (²⁴⁶PPSPTPPAP²⁵⁴) found just amino-terminal to the repeat region that is likely involved in SH3 binding, and may bind the same SH3 domain containing protein that is responsible for normal NHE2 localization. These results demonstrate that removal of SH3 binding sites may lead to alterations in protein targeting, but may not affect protein function, as might happen with the dopamine D4 receptor.

The postsynaptic density protein, PSD-95, is an SH3 domain containing protein that has been implicated in organizing signalling molecules at pre- and postsynaptic structures. The D4 receptor

and other GPCRs may interact with PSD-95 by an SH3 interaction. The PSD-95 protein can interact with other cellular components by means of an N-terminal palmitoylation site, three PDZ domains and a guanylate kinase (GK) domain. The PDZ domains appears to be necessary and sufficient to localize PSD-95 to caveolar-like domains (Perez and Brecht, 1998). PSD-95 and related proteins may be of importance in the targeting of the dopamine D4 receptor and other GPCRs to the synaptic membrane mediated by SH3 domain interactions. The importance of such proteins in D4 function needs to be investigated.

The function of the SH3 binding domains of the dopamine D4 receptor remain speculative until further evidence of the endogenous binding partner and function of this interaction are found. Especially curious is the effect of removing all SH3 binding domains that results in a constitutive internalized phenotype. These results suggest that SH3 binding is responsible for the receptor localization at the cell membrane or that this interaction may play a role in recirculating the receptor to the membrane after endocytosis that occurs in a similar fashion to other GPCRs.

7.2 Future Research

As discussed, technical limitations have hindered the characterization of the D4/SH3 interaction in determining the sites of binding in the dopamine D4 receptor, identifying the endogenous interaction *in vivo*, and the mechanism and function of this interaction. During the preparation of this thesis, several “tools” have become available that may help to address these unresolved issues. For the *in vivo* interaction studies, specific, high affinity antibodies suitable for immunoprecipitation and directed against the dopamine D4 receptor have been developed (Hoffman-LaRoche and Allelix/Hoechst). These antibodies in combination with the adenoviral expression techniques developed in our lab, to express high levels of the dopamine D4 receptor, in addition to recently developed immunoprecipitation protocols that have been successfully used to demonstrate

GPCR interaction with intracellular proteins (Karoor *et al.*, 1998; Klein *et al.*, 1997) should help to demonstrate the D4/SH3 interaction *in vivo*. Immunopurified dopamine D4 receptor can be used to either confirm the Nck and Grb2 interactions and/or screen for other possible SH3 interactions using commercially and privately developed antibodies. To date over 60 SH3 domain containing proteins have been identified, many of which have commercially or privately available antibodies. SH3 domain containing proteins that are likely candidates for D4 interaction because of cellular localization and/or possible functional involvement will be screened to identify the endogenous binding partners. Furthermore, efforts will need to focus on the identification of other and possibly novel SH3 domain containing proteins that may serve as binding partners for the D4, as well as screening for proteins that may not contain SH3 domains, but can still interact with the dopamine D4 receptor, in particular with the repeat region. This may be achieved by expressing the third intracellular loop or just the repeat region of the dopamine D4 receptor as GST fusion proteins and screening against a phage display library that expresses large protein fragments containing the binding domains of proteins.

In trying to demonstrate the D4/SH3 interaction *in vitro*, we tried to express a GST fusion protein of the full length D4 receptor and a fragment encoding the third intracellular loop. The D4 receptor and the third intracellular loop fragment were resistant to expression in the GST-fusion protein system when expressed in bacterial cells. Similar problems for the D4 receptor have been reported by others (Cheong *et al.*, 1996). In human, the amino acid sequence that includes the GRAP residues, encoded in the third intracellular loop preceding the repeat region, appears to prevent bacterial expression due to toxicity to the cell (Dr. T. Sakmar, Howard Hughes Med. Inst., Rockefeller University, NY, personal communication). A dopamine D4 receptor with a modified GRAP sequence has been shown to express in bacteria. Expression of the third intracellular loop fragment of the dopamine D4 receptor using the GST fusion system can be used in conjunction with the phage display

system to identify possible new SH3 interactions with the D4 receptor.

Our attempts at mutating specific prolines in the dopamine D4 receptor was for the most part unsuccessful likely due to the high GC content and the repetitive nature of the nucleotide sequence. A number of laboratories have developed a modified or “synthetic” dopamine D4 receptor sequence (Dr. R. Huff, UpJohn/Pharmacia and Dr. T. Sakmar, Howard Hughes Med. Inst., Rockefeller University, NY). These nucleotide sequences encode the wild type amino acid sequence of the dopamine D4 receptor, but the nucleotide sequence has been modified to reduce the GC content and to introduce unique and convenient restriction endonuclease sites. These nucleotide sequences are easily and precisely manipulated using standard molecular biology techniques. Exact deletion mutations can be made to remove the repeat sequence and the carboxyl-terminal PXXP sequence. More importantly, creating the desired point mutations targeting specific prolines using this synthetic sequence should be easier. Both single and multiple point mutations can be made to determine the sequences involved in SH3 interactions. A combination of deletion mutants and point mutations targeting the prolines believed to be involved in SH3 binding in the dopamine D4 receptor will help to identify the important SH3 binding sites.

As discussed in more detail in Chapter 5 (page 139), the identification of binding partners for the dopamine D4 receptor by the use of a yeast two-hybrid library screen may help to identify endogenous interactions. A more directed screen of a subset of SH3 domain containing proteins, that are involved in MAPK activation, internalization or localization using co-immunoprecipitation techniques may help to identify important D4 interactions. It may prove fruitful to focus on proteins implicated in internalization as observed in HEK293 cells, since the D4 deletion mutants containing no potential SH3 binding sites, exhibited a constitutive internalized state in these cells. Another strategy that may prove useful in identifying D4 interacting proteins is an overlay experiment. In such an experiment either whole cell extract or immunoprecipitated D4 receptor are size separated by SDS-

PAGE and then transferred to a PVDF membrane. The membrane is then probed (overlayed) with various *in vitro* labeled [³⁵Cys]D4 constructs containing the full length D4 receptor, just the third intracellular loop, or the deletion mutant that exhibits no SH3 interaction. D4 binding to the PVDF membrane identifies proteins that were originally copurified with the D4 receptor. Such an experiment may show that the D4 can interact with multiple proteins and help to identify their sizes. Differences in binding of the various D4 constructs to the membranes may be attributed to the region of the D4 receptor implicated in SH3 binding.

Demonstration of a specific binding partner for the dopamine D4 receptor *in vivo* in combination with the specific point mutations that result in the disruption of the SH3 interaction will lead to a set of experiments that can characterize the function of this interaction. The point mutations that lead to the successful disruption of the established SH3 interaction *in vitro* and *in vivo* can be used to see if they can duplicate the constitutively internalized phenotype of the dopamine D4 receptor that was observed with the deletion mutant (D4.4(Δ 221-337)). Furthermore, it can be determined whether receptor binding and function with respect to G protein-coupling, cAMP level inhibition and MAPK activation is also affected. These deletion mutants will aid in identifying other possible functions of D4-SH3 interactions that may be involved in localization, endocytosis and signal transduction. These studies will help to determine the precise function and mechanism of this interaction.

The demonstration of an SH3 interaction *in vivo* and the identification of the specific SH3 domain containing proteins that interact *in vivo* with the dopamine D4 receptor will be required to confirm the importance of this type of interaction for GPCRs and will help to determine the functional consequences of such an interaction. These same dopamine D4 receptor mutants that fail to demonstrate SH3 interactions can also be used to study the effects of disrupting this interaction in the whole organism. Transgenic expression of the mutant D4 receptor may help to establish a

physiological function for the dopamine D4 receptor.

7.3 Final Conclusions

In conclusion, we have shown that the dopamine D4 receptor, and possibly several other GPCRs, can interact *in vitro* with SH3 domain containing proteins, in particular with the adapter proteins Nck and Grb2. This interaction appears to be mediated through a high affinity cooperative binding between two distinct SH3 domains and their binding sites. Deletion of the putative SH3 binding domains results in a receptor that still can bind dopamine, couple to G-protein, but has lost its ability to stimulate MAPK and inhibit forskolin-stimulated cAMP levels. Furthermore, the deletion mutant receptor is constitutively internalized. These results suggest that GPCRs, in particular the dopamine D4 receptor, may interact with SH3 binding domains and that these domains fall within the region of the receptor that is involved in receptor internalization. These studies have not identified a role for the polymorphic repeat region in the human dopamine D4 receptor and further research will be required to identify its function. Although SH3 binding in this region cannot be ruled out, further studies will be required to ascertain this.

Finally, the identification of SH3 domain mediated interactions with GPCRs represents an important new finding that may help to identify new roles of GPCRs and may help in the understanding of the complex integration of all these signalling molecules in regulating cellular as well as physiological functions.

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