Identification and Characterization of a Homophilic Binding and Neuritogenic Site in the Cell Adhesion Molecule L1

by

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Abstract

The neural cell adhesion molecule L1 is an integral membrane glycoprotein which mediates cell-cell adhesion and promotes neurite outgrowth from neuronal cells. Mutations in the L1 gene have been implicated in several neurological diseases. To investigate the mechanism and the relationship of these two intrinsic functions of L1, studies were carried out to identify specific domain(s) and sequences involved in homophilic binding and the promotion of neurite outgrowth.

Recombinant L1 domain proteins were used to identify the domain involved in L1 homophilic binding. Results based on Covasphere binding assays indicate that the second Ig-like (Ig2) domain of L1 is sufficient and necessary for homophilic interaction. Results from neurite outgrowth assays further demonstrate that the Ig2 domain of L1 is a potent neuritogenic substrate. The colocalization of these two intrinsic activities of L1 suggests a close relationship between homophilic binding and the promotion of neurite outgrowth.

Several HSAS/MASA (Hydrocephalus as a result of stenosis of the aqueduct of Sylvius/ mental retardation, aphasia, shuffling gait and adducted thumbs) mutations have been localized to L1 Ig2. The effects of two HSAS/MASA mutations on the homophilic binding and neuritogenic activities of recombinant L1 Ig2 were assessed. The HSAS mutation R184Q abolished both Ig2-associated activities, while the MASA mutation H210Q had only modest effects. The deleterious effects of these two mutations thus correlate very well with the severity of their respective clinical phenotype. The results also implicate a role for Arg-184 in L1 homophilic binding

The synthetic oligopeptide approach was used to identify the homophilic binding site in L1 Ig2. The peptide containing sequences flanking Arg-184 (HIKQDERVTMGQNG) inhibited both L1-dependent cell aggregation and neurite outgrowth, whereas the peptide consisting of flanking sequences of His-210 had only minor effects. Further analysis using peptide analogues indicates that the charged residues as well as the hydrophobic residues immediately adjacent to Arg-184 may also play an important role in L1 homophilic binding. Peptides that inhibited L1 homophilic binding also inhibited L1-dependent neurite outgrowth, suggesting a direct link between these two intrinsic activities of L1.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABGP	Ankyrin-binding glycoprotein
APC	Adenomatous Polyposis Coli
CAR	Cell adhesion recognition
CEA	Carcinoembryonic antigen
CP	Cytoplasmic domain
DCC	Deleted in colorectal carcinoma
DTRK	Drosophila receptor tyrosine kinase
EC	Extracellular Cadherin Repeats
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELAM 1	Endothelial leukocyte adhesion molecule 1
ESL 1	E selectin ligand 1
FAK	Focal adhesion kinase
FGFR	Fibroblast Growth Factor Receptor
GlyCAM 1	Glycosylation dependent adhesion molecule 1
GPI	Glycophosphatidylinostol
GRASP	Growth-associated cell surface protein
GST	Glutathione S-transferase
HSAS	Hydrocephalus as a result of stenosis of the aqueduct of Sylvius
IgSF	Immunoglobulin super family
LAD	leukocyte adhesion deficiency
LBP	Laminin binding protein
LFA-1	Leukocyte function associated molecule-1
LTP	Long-term potentiation
MadCAM 1	Mucosal addressin cell adhesion molecule 1
MAG	Myelin-associated glycoprotein
MASA	Mental retardation, aphasia, shuffling gait and adducted thumbs

NCAM	Neural cell adhesion molecule
NgCAM	Neuron-glia cell adhesion molecule
NILE	Nerve growth factor inducible large external glycoprotein
NrCAM	NgCAM related cell adhesion molecule
OP-1	Osteogenic protein-1
PADGEM	Platelet activation dependent granule external membrane protein
PCR	Polymerase chain reaction
PLC	Phospholipase C
PSA	Polysialic acid
PSGL	P selectin glysoprotein ligand 1
SPG1	Spastic paraplegia type I
TAG-1	Transient axonal glycoprotein-1

CHAPTER ONE

INTRODUCTION

I. CELL ADHESION

Cell adhesion is a process important for virtually all forms of life. It is a form of cellular communication, and represents the way a cell senses its environment through contact. In the bacterial world, adhesion is required for colonization of host tissues (Beachey. 1980); in animals, it is required for the development of multicellular organisms and specific tissues within those organisms (McClay and Ettensohn, 1987). Cell adhesion plays an important role in processes such as homing of blood forming cells (Matsuoka and Tavassoli., 1989), platelet aggregation (Jennings et al., 1982; Plow and Ginsberg. 1989), tumor cell metastasis (Hart et al., 1989), neuronal cell migration (Miura et al., 1992; Takeda et al., 1996), and axon fasciculation (Tessier-Lavigne and Goodman, 1996).

Cells are constantly involved in both long range and short range interactions. The long range interactions usually involve cell motility and secreted molecules, while the short-range interactions involve intermolecular interactions, such as van der Waals force, electrostatic force and hydrodynamic forces. To facilitate these kinds of interactions, morphological changes of cells, such as ruffles and pseudopodia, are developed to provide parts that have small radii of curvature and allow a smaller distance of approach to be achieved (Bangham and Pethica, 1960; Angarska et al., 1991).

The fact that cell adhesion is cell-type specific suggests that cell adhesion is mediated primarily by cell adhesion molecules rather than general "stickiness" of the cell surface. When different types of cells, such as cartilage and kidney cells, are mixed in suspension, they always sort out according to type to form solid blocks of kidney tissue or cartilage tissue but not a random mixture of both (Moscona, 1957). Another example

comes from studies on the cellular slime mold *Dictyostelium discoidieum*. The sorting of prespore and prestalk cells in a randomly mixed cell aggregate is blocked by the addition of Fab directed against the cell adhesion molecule gp150 (Siu et al., 1983), demonstrating the involvement of specific cell surface components.

As the cDNAs encoding more and more cell adhesion molecules have been cloned and characterized, evidence has accumulated that the specificity of cell adhesion is based on interactions among specific cell adhesion molecules expressed on the cell surface. During development, cell-cell adhesion is often regulated by the expression level of cell adhesion molecules as well as the modulation of their binding affinity. For instance, NCAM expression is higher during embryogenesis but lower in the adult stage, and its PSA content, which can modulate NCAM adhesion, is also regulated during development (Edelman et al., 1983; Sunshine et al., 1987; Rutishauser et al., 1988).

Given the important role of cell adhesion molecules in development, it is not surprising that loss of function mutations of cell adhesion molecules generally result in disease. For instance, a defect in the integrin molecule, GPIIb/IIIa, on platelets has been shown to be responsible for Glanzmann's thrombasthenia (Phillips and Agin, 1977). Therefore, research on the molecular mechanisms of cell-cell interaction not only will enrich our knowledge of fundamental cell biology, but also will open up a new vista of opportunities for the development of anti-disease therapies.

II. CELL ADHESION MOLECULES

Cell adhesion molecules are divided into four major categories according to their structure and function. They are: integrins, selectins, cadherins and members of the immunoglobulin superfamily (IgSF). Cell adhesion molecules mediate cell-cell and cell-substratum adhesion by undergoing either homophilic or heterophilic interactions. Homophilic interaction refers to the binding between two identical molecules, such as NCAM-NCAM interaction (Rao et al., 1992), whereas heterophilic interaction refers to the binding between two different molecules, such as LFA1-ICAM interaction (Simmons et al., 1988; Huang et al., 1995). Through interacting with one another, regulating their distribution pattern, and modulating their affinities with ligands, cells organize cell adhesion molecules from all four groups in concert to accomplish complicated processes, such as blood cell homing, axon guidance, and tumor cell metastasis (Dunon et al., 1996; Tessier-Lavigne and Goodman, 1996).

A. INTEGRINS

Integrins are named as such because of their integral role in mediating cell adhesion (Tamkun et al., 1986). They were first identified ~15 years ago as extracellular matrix protein receptors (Neff et al., 1982; Pytela et al., 1985). Recent research has demonstrated that certain integrins can also bind to soluble ligands (such as fibrinogen) or counter receptors (such as ICAM) on adjacent cells, leading to homotypic or heterotypic cell aggregation. Integrins are heterodimers composed of two transmembrane subunits α and ß. To date, 15 α and 8 ß subunits have been identified and there are over twenty

different αß heterodimeric combinations expressed on the cell surface (Varner and Cheresh, 1996).

Integrins can be divided into three major subfamilies which contain heterodimers comprised of a single ß subunit paired with a unique set of α subunits (Haas and Plow, 1994; Edelman et al., 1995; Hynes, 1992). The largest subfamily consists of the "ß1" integrins and includes most of the cell-substratum adhesion receptors. The ß2 subfamily consists of most of the leukocyte integrins that are involved in cell-cell interactions. The final subfamily is comprised of those receptors that contain the ß3 subunit, such as the platelet integrin GPIIb/IIIa and the vitronectin receptor αvß3 (Figure I.1).

Integrins bind to extracellular matrix proteins or members of the Ig-super family on the cell surface through short peptide sequences. Although some integrins selectively recognize a single extracellular matrix protein, others bind two or more ligands (Hynes, 1992; Cheresh, 1993). Several integrins recognize the tripeptide RGD (Ruoslahti and Pierschbacher, 1986), whereas others recognize alternative short peptide sequences (Hynes, 1992). Though most integrins are involved in heterophilic interaction, a homophilic mechanism has been proposed for α4ß1 integrin in α4 chain binding (Altevogt et al., 1995). This interaction was proposed to play an role in the adhesion of leukocytes to brain endothelium.

Combinations of different integrins on the cell surface allow cells to recognize and respond to a variety of extracellular environments. Integrins mediate cellular adhesion to, and migration on, extracellular matrix proteins found in intercellular spaces and basement membranes (Edelman et al., 1995). They also regulate cellular entry into, and withdrawal from, the cell cycle (Guadagno et al., 1993; Varner et al., 1995). Integrins play important roles in a number of cellular processes and in the development of tumors,

including regulation of cell proliferation and apoptosis, cellular motility and invasion, cell surface localization of metalloproteinases, and angiogenesis (Varner and Cheresh, 1996). In neuronal systems, integrin-extracelluar matrix interactions have also been implicated in axon guidance (McKerracher et al., 1996).

(a) Structure and Function Relationships of Integrins

An integrin is a non-covalently linked heterodimer, comprised of a larger α subunit and a smaller ß subunit. Each subunit is anchored in the plasma membrane by a single short transmembrane region, with its C-terminal domain in the cytoplasm of the cell (Figure I.2). Chemical and photoaffinity cross-linking experiments have suggested that both subunits contain extracellular ligand-binding regions (D'Souza et al., 1988; Smith et al., 1988), while the cytoplasmic domains are involved in cytoskeleton interaction and signal transduction (Schaller et al., 1995; Clark and Brugge, 1995).

The extracellular domain of each known α subunit contains three to four consensus regions for calcium binding. All known α subunits are synthesized as a single polypeptide chain; however, one group of them is completely cleaved at a region in the extracellular domain near the membrane, resulting in two chains joined by a single disulfide bond (Humphries, 1990). For those integrins which do not have cleaved α subunits, each of them contains an additional sequence inserted between the N-terminus and the first calcium binding site (Hemler, 1990). This inserted or I domain (also known as "A" domain) contains "type A" repeats similar to those found in collagen and other adhesive proteins, and is believed to contain the ligand binding activity of its parent integrin (Xie et al., 1995; Kamata and Takada, 1994). The crystal structures of I-domains from several α subunits show that they contain an alternating α helix/ß sheet type

structure resembling the Rossmann (or dinucleotide-binding) fold (Lee et al., 1995a; Lee et al., 1995b; Qu and Leahy, 1995). Although the binding of cations to I-domain has been demonstrated (Stewart et al., 1995), the studies of cations on ligand binding to I-domains produce divergent results, ranging from complete dependence on cation to complete independence (for a review, see Humphries, 1996).

The known integrin ß subunits are remarkably similar, with their amino acid sequences displaying 40 to 48% similarity (Hemler, 1990). There are 56 cysteine residues found in the extracellular domain and they are conserved in most ß subunits. Most of these cysteines are organized into four repeating units located in the C-terminal half (Cheresh, 1993). The amino terminal portion of ß subunit is folded into a loop that is stabilized by intramolecular disulfide bonds formed between the N-terminal cysteine and cysteine within the first cysteine-rich repeat (Cheresh, 1993), lending credence to the electron microscopic evidence of a globular head (Calvete et al., 1989).

It appears that high mannose glycosylation occurs on both subunits, but the addition of complex carbohydrates occurs only after the heterodimer has been formed (Hynes et al., 1989). The glycosylation pattern depends on the specific heterodimer. For example, when paired with αIIb, ß3 subunit is glycosylated differently from that paired with αv (Troesch et al., 1990).

Although the short cytoplasmic domains of the α and ß integrin subunits do not have any intrinsic enzymatic activity, they appear to function by coupling with cytoplasmic proteins that nucleate the formation of large protein complexes containing both cytoskeletal and catalytic signaling proteins (Sastry et al., 1993). For instance, the cytoplasmic domain of ß subunits is able to bind directly to the cytoskeletal protein α-actinin (Otey et al., 1990), and talin (Horwitz et al., 1986; Tapley et al., 1989).

(b) Integrin-mediated Signaling

Integrins mediate not only cell adhesion and migration, but also bi-directional transfer of information across the plasma membrane (Dedhar and Hannigan, 1996). Ligation of integrins with ECM molecules or with their receptors on counter cells would generate "outside-in" signals, such as an increase in intracellular pH (Schwartz et al., 1989, Schwartz et al., 1990), intracellular calcium, (Schwartz, 1993), phosphoinositide (PI) hydrolysis (McNamee et al., 1993), and GTP-bound $p21^{Ha\text{-}Ras}$ levels (Kapron-Bras et al., 1993). This flow of information within the cell initiated by integrin ligation is essential for cell survival (Boudreau et al., 1995; Frisch and Francis, 1994), entering or exiting cell cycle (Guadagno et al., 1993; Fang et al., 1996; Clarke et al., 1995), and cellular differentiation (Streuli et al., 1995).

Clustering integrin receptors leads to the formation of focal adhesions where integrins link to intracellular cytoskeletal complexes and actin filaments (Gumbiner 1993). FAK (focal adhesion kinase) is the most studied kinase found in the focal contact. It is tyrosine-phosphorylated, and its tyrosine kinase activity is enhanced upon integrin engagement (Schaller and Parsons, 1994). Though not yet demonstrated in vivo, FAK has been shown to associate with the cytoplasmic domains of ß1 and ß3 through its N-terminal sequence (Schaller et al., 1995). In addition, clustering of ß cytoplasmic domain is essential and sufficient to induce FAK phosphorylation (Schaller and Parsons, 1994; Lukashev et al., 1994). Phosphorylated FAK thus serves as a binding site to couple FAK with cellular proteins that contain SH2 domains. Through these linkages, FAK is capable of integrating multiple signals triggered by integrins (Clark and Brugge, 1995).

Other than "outside-in" signaling, association of intracellular components to the cytoplasmic domain of integrin would generate "inside-out" signals to modulate the affinity of integrin to its ligand, thus to regulate the adhesive properties of the cell (Williams et al., 1994; O'Toole et al., 1994). A calcium-binding protein, calreticulin, is able to bind to the cytoplasmic domain of α subunit This interaction switches the integrin to, and stabilizes it in, an active form (Coppolino et al., 1995). The other example comes from members of the small GTP-binding proteins. R-Ras, highly homologous to Ha-ras but with activities distinct from those of the transforming Ras proteins, has been shown to activate integrins. Over expressing R-Ras results in converting integrins from a low-affinity to a high-affinity state, whereas a dominant-negative form of R-Ras reduced the adhesiveness of cells (Zhang et al., 1996).

(c) Integrin Related Diseases

The role of integrins in inflammatory response and in coagulation can be dramatically illustrated by several rare inherited deficiencies of integrin ß subunits. Patients with nonfunctional ß2 subunit manifest a clinical syndrome termed "leukocyte adhesion deficiency" (LAD), which is characterized by recurrent bacterial infections, delayed separation of the umbilical cord, leukocytosis, and abnormal leukocyte function. These phenotypes are due to the inability of neutrophils to reach sites of extravascular inflammation (Anderson et al., 1987). Replacement of the defective ß2 subunit in LAD lymphocytes restores their ability to bind to endothelial cells (Wilson et al., 1990).

Absence of the ß3 or αIIb in patients results in disordered thrombosis called Glanzmann's thrombasthenia (Newman, 1991). Patients suffer from a bleeding disorder which is characterized by the inability of platelets to aggregate after activation (Newman,

1991). A similar phenotype has also been reported in one patient whose platelets lack the α2β1 integrin. The patient's platelets were unable to aggregate in response to collagen (Edelman et al., 1995).

The level of integrin expression is also altered in cancer cells (Varner and Cheresh, 1996). Integrins play roles in the regulation of tumor cell survival, proliferation and invasion. Importantly, tumor progression is accompanied by an elevated level of $\alpha_v\beta_3$ integrin, which is known to play crucial roles in tumor metastasis and angiogenesis (Brooks et al., 1994a).

B. SELECTINS

The selectins are a family of three proteins that mediate leukocyte-endothelium adhesion and leukocyte-platelet adhesion in the blood vascular compartments. They are L- (leukocyte), P- (platelet), and E- (endothelial) selectins, which are expressed on leukocytes, platelet and endothelial cells, respectively (Rosen and Bertozzi, 1994).

Selectins mediate specific cell-cell adhesion in a carbohydrate-dependent manner (Lasky, 1995). Recent papers have demonstrated that the sialyl Lewis X or sLe^X antigen constitutes the minimal oligosaccharide epitope for selectin-mediated adhesion (Foxall et al., 1992). The sLe^X antigen is a carbohydrate epitope of myeloid cell-surface lactosaminoglycan, which comprises of sialic acid, galactose, fucose and N-acteyl glucosamine (NeuNacα2-3Galß1-4(Fuc1-3)GlcNac). It has been shown that both the sialic acid and the fucose residues are absolutely required for carbohydrate recognition by selectins (Tyrrell et al., 1991). In the case of L-selectin, the large quantity of inorganic

sulfate associated with carbohydrate has also been demonstrated to be involved in high-avidity binding of selectin with its ligand (Imai et al., 1993).

Several biological selectin ligands with high binding affinity and avidity have been identified (Varki, 1994) (Figure I.3). Most, but not all, of these ligands are mucin-type glycoproteins, which contain serine/threonine rich regions, and are heavily O-glycosylated (Shimizu and Shaw, 1993). Mucin domains of these ligands present a densely clustered array of oligosaccharides on a polypeptide backbone, enabling avid recognition by the cognate selectin on the apposing cell.

The binding of selectin to their mucin ligand is involved in leukocyte-endothelium interaction, which is the first step of the recruitment of leukocytes to an inflammatory tissue site (Dunon et al., 1996). The fast "on" and "off" rate of selectin-ligand association can rapidly arrest freely flowing leukocytes and mediate rolling of the cells along the endothelium of blood vessels (Springer, 1994). The localization of L-selectin at the tip of the villi of leukocytes further facilitates selectin-mediated cell rolling (Von Andrian et al., 1995).

(a) Structure and Function Relationships of Selectins

All three selectins are similar in structure. They contain an amino-terminal C-type lectin domain or carbohydrate-recognition domain (CRD) followed by an epidermal growth factor (EGF)-like motif, short consensus repeats (SCRs) similar to those found in complement-regulatory proteins, a transmembrane domain, and a short cytoplasmic tail (Figure I.3).

The C-type lectin domain of selectins is the region interacting with their carbohydrate ligand. Several monoclonal antibodies directed against the lectin domain of

selectin are capable of inhibiting selectin binding to either carbohydrates or cells (Bowen et al., 1990). Mutagenesis (Erbe et al., 1992), together with structural analysis of the lectin domain of E-selectin (Graves et al., 1994; Weis et al., 1991, 1992), have identified a hydrophilic pocket on the face of the lectin domain, which contains a calcium binding site involved in carbohydrate recognition and cell adhesion.

The EGF domain, though not directly involved in carbohydrate binding, plays an important role in selectin activity. Deletion constructs of both L and E selectin have revealed that removal of the adjacent EGF domain abolishes monoclonal antibody recognition of the lectin domain, suggesting that the conformation of the lectin domain is dependent on interactions with the EGF domain (Bowen et al., 1990; Walz et al., 1990). This hypothesis has been partially confirmed by crystal structure of these two domains of E-selectin (Graves et al., 1994). Furthermore, the EGF domain is capable of modulating the carbohydrate recognition specificity of selectins. Replacement of the L-selectin EGF domain with that of P selectin confers P selectin cell binding specificity on the chimeric protein (Kansas et al., 1994).

The region containing short consensus repeats, though less well characterized, has been suggested to play a supportive role in the high-avidity binding of selectin (Li et al., 1994; Bargatze et al., 1994). However, the mechanism is currently unknown.

The cytoplasmic domain of L-selectin is associated with the cytoskeleton structure through binding with α actinin, vinculin and talin (Pavalko et al., 1995). This association is important in the microvilli localization of L-selectin on leukocytes, since truncated L-selectin distributes over the entire cell surface and is, consequently, unable to mediate leukocyte rolling in vivo (Kansas et al., 1993).

Also, L-selectin function is transiently enhanced upon activation of leukocytes (Spertini et al., 1991). Upon leukocyte activation, L-selectin is shed rapidly from the cell surface by cleavage at a distinct proteolytic site (Chen et al., 1995; Walcheck et al., 1996). The shedding of L-selectin controls the velocity of leukocyte rolling, hence controlling the duration of interaction of a leukocyte with the endothelium.

(b) Signaling of Selectins

The second step of the leukocyte-endothelium interaction is the rapid activation of integrins on leukocytes. Selectins plays an important role in this signaling pathway (McEver et al., 1995). As shown in neutrophils, ligand interaction with L-selectin or antibody crosslinking leads to partial activation of leukocyte β2 integrins, thus enhancing their ligand binding affinity (Simon et al., 1995). Serial signaling events occur upon ligation or crosslinking of L-selectin, such as increased cytosolic Ca^{2+} levels, potentiation or activation of the oxidative burst (Waddell et al., 1994), enhanced tumor necrosis factor, and IL-8 gene expression (Laudanna et al., 1994). Most importantly, L-selectin ligation enhances tyrosine phosphorylation and activation of MAP kinase (Waddell et al., 1995).

(c) Selectin Related Disease

To date, only one human disease, termed leukocyte adhesion deficiency 2 (LAD2), has been correlated with deficient selectin-carbohydrate interactions (Etzioni et al., 1992). In these patients, a metabolic defect leads to a complete loss of fucose production, resulting in the lack of functional myeloid ligands for E- and P- selectins. They also show an inability to recruit neutrophils to sites of inflammation (Von Andrian

et al., 1993). This disease underlines the importance of the adhesive interactions mediated by selectins during the inflammatory response.

C. CADHERINS

Cadherins refer to a superfamily of Ca^{2+}-dependent cell adhesion molecules. All solid tissues in vertebrates express some members of cadherin family. The different cadherin subtypes show distinct developmental and tissue-specific patterns of expression and binding specificities. Cadherins are thought to influence cell sorting, morphogenesis and the maintenance of adult tissues (Takeichi, 1991).

The classic cadherins are transmembrane proteins composed of a highly conserved cytoplasmic region and an ectodomain with five structural domains containing calcium-binding motifs (Figure I.4). More than 15 members have been identified for this subfamily, which includes E- (epithelial), N- (neural), and P- (placental) cadherin (Takeichi, 1995). Non-classic members differ in domain structure from the classic cadherins in many ways, such as additional extracellular repeats, no cytoplasmic region, and modified or distinctly different cytoplasmic domains. They include the fat tumor suppresser gene product, T- (truncated) cadherin, molecules of the desmosomal desmocollin and desmoglein subfamily and the ret proto-oncogene (Takeichi, 1993; Koch and Franke, 1994) (Table I.1).

In principle, classic cadherins mediate cell-cell adhesion by homophilic interaction (Takeichi, 1995). However, heterophilic interactions of cadherin with other molecules have been reported. In chick, B-cadherin has been demonstrated to bind to L-CAM (Murphy-Erdosh et al., 1995). Also, the binding of lymphocytes to epithelial cells

is mediated by E-cadherin and $\alpha_E\beta_7/\alpha_{M290}\beta_7$ heterophilic interactions (Cepek et al., 1994; Karecla et al., 1995). Also, not all non-classic cadherins contain adhesive properties. Despite the presence of cadherin-like repeats, some molecules in the cadherin superfamily are not considered cell adhesion molecules.

(a) Structure Function Relationships of Cadherins

Cadherins are integral membrane glycoproteins. They are composed of multiple cadherin repeats (EC domains) in the extracellular region, a single transmembrane domain, and a cytoplasmic domain (Figure I.4). The EC domains characteristically contain ~110 amino acids with the motifs DXNDN and DXD in conserved positions. These motifs are involved in binding Ca^{2+} (Ozawa et al., 1990). Structural studies of the extracellular domains of both E- and N-cadherins have demonstrated that Ca^{2+} binding at these sites stabilizes the functional architecture of the extracellular region of cadherins (Shapiro et al., 1995; Overduin et al., 1995; Nagar et al., 1996). When Ca^{2+} is depleted, cadherins can no longer mediate cell-cell adhesion (Jones, 1996).

The first extracellular domain of each classic cadherin harbors the cell adhesion recognition (CAR) sequence HAV (Blaschuk et al., 1990; Munro and Blaschuk, 1996). This sequence is believed to mediate cadherin homophilic interaction, since both peptide and antibodies against this sequence are potent inhibitors of cadherin functions (Chuah et al., 1991; Doherty et al., 1991b). Despite the conservation of the HAV site in all classic cadherins, the adhesive affinity of heterotypic cadherin complex is much lower than that of homotypic cadherin complexes. It is believed that the non-conserved amino acid residues immediately adjacent to the HAV site are involved in modulating the specificity of cadherin homophilic interaction (Nose et al., 1990).

The cytoplasmic portion of classic cadherins is the most highly conserved region. It is composed of two cytoplasmic (CP) domains. The second CP domain is involved in interacting with cytosolic components, known as α, ß, and γ catenins (Ozawa et al., 1989; Hinck et al., 1994). The catenins mediate the interaction between cadherins and the actin based cytoskeleton, which is crucial to the adhesion function of cadherins (Ranscht, 1994; Huber et al., 1996). The association of β-catenin and the cytoplasmic domains of cadherins is regulated by tyrosine phosphorylation on both components (Grunwald, 1993).

Non-classical cadherins usually have less conserved cytoplasmic domains which are varied both in size and numbers of functional domains (Table I.1). For instance, the non-classical cadherin c-ret contains a tyrosine kinase domain in its cytoplasmic domain (Schneider, 1992), while T-cadherin, anchored on the cell surface through a GPI linker, has no cytoplasmic domain (Ranscht et al., 1991). Interestingly, T-cadherin still retains the Ca^{2+} -dependent homophilic binding activity (Vestal and Ranscht, 1992).

(b) Cadherin-mediated Signaling

The study of cadherin signaling is more focused on ß-catenin due to the fact that ß-catenin has been found to be involved in mutiple signal transduction pathways (Gumbiner, 1995). ß-catenin is highly homologous to the *Drosophila* segment polarity gene product Armadillo, with 70% amino acid sequence identity (McCrea et al., 1991). Activation of the ß-catenin signaling pathway induces axis duplication in *Xenopus* similar to the effect of Wnt growth factors, thus suggesting the participation of ß-catenin in Wnt signaling, embryonic patterning, and cell fate determination (Funayama et al., 1995). On the other hand, ß-catenin has been found to interact with the adenomatous polyposis coli

(APC) tumor suppresser protein (Su et al., 1993; Rubinfeld et al., 1993). This interaction implicates a role for β-catenin in both cell growth control and tumorigenesis (Hülsken et al., 1994a). Although ß-catenin signaling activity may be independent of cell adhesion (Funayama, 1995), cadherins can modulate ß-catenin signaling activity by regulating the availability of cytoplasmic ß-catenin (Gumbiner 1995, Hülsken et al., 1994b).

Cadherins are also suggested to transduce signals by *cis*-interaction with growth factor receptors. N-cadherin *cis*-interaction with FGFR has been proposed to underlie the signaling pathway stimulating neurite outgrowth from primary neuronal cells (Williams et al., 1994a). Disruption of this interaction by synthetic peptides could inhibit neurite outgrowth on N-cadherin substrates. (Williams et al., 1994a).

(c) Cadherin-Related Diseases

Regulation of cadherin expression on tumor cells have been correlated with tumor cell invasion and metastasis, suggesting important roles for cadherins in cancer development (Takeichi, 1993). Two non-classic cadherins have been found to be antigens in severe human autoimmune blistering skin disease (Stanley, 1995). Desmoglein 1 (PF-antigen) has been identified as a target for autoantibodies in *Pemphigus foliaceus*, and desmoglein 3 (PV-antigen) is recognized by autoantibodies in patients suffering from *Pemphigus vulgaris*. These antibodies are all pathogenic (Amagai et al., 1992).

D. IMMUNOGLOBULIN SUPERFAMILY MEMBERS

The immunoglobulin-superfamily (IgSF) is currently the largest group of recognition molecules. Members of the IgSF contain one or more Ig domains. Their

characteristic Ig-fold contains two conserved cysteines separated by ~70 amino acids and is folded into a ß-barrel structure (Amzel et al., 1979; Williams and Barclay, 1988). Members of the IgSF are predominantly found on the surface of cells and implicated in cell-cell interactions (Buck, 1992; Yoshihara et al., 1991; Brümmenderf and Rathjen, 1994).

After the identification of the first cell adhesion molecule NCAM in the nervous system, a large number of cell adhesion molecules belonging to the IgSF have been characterized. They include L1, axonin-1/TAG-1, F3/F11, DCC, MAG, and P_0. They are broadly implicated in various aspects of brain development, such as neurite extension, neuronal cell migration, synapse formation, and memory (Cunningham, 1995; Walsh and Doherty, 1996). Some of them are potent inducers of neurite outgrowth and they may serve as short-range cues in axon guidance during neural development (Tessier-Lavigne and Goodman, 1996). The recent finding of DCC being the netrin receptor further suggests the involvement of IgSF molecules in long-range guidance of axon targeting (Kein-Masu et al., 1996; Leonardo et al., 1997). In addition to the nervous system, IgSF members are involved in various biological processes, such as cancer cell metastasis, blood cell homing, inflammation, and antigen presentation in the immune system. (Brümmendorf and Rathjen, 1994).

Members of IgSF mediate cell-cell adhesion through Ca^{2+}-independent homophilic interactions as well as heterophilic interactions with their ligands (Table I.2). Many molecules bind multiple ligands. For instance, other than L1 itself, NCAM has been found to be a binding partner of the cell adhesion molecule L1 (Kadmon et al., 1990a). In addition to heterophilic interactions between two members of IgSF, several Ig-CAMs are capable of interacting heterophilicly with other groups of cell adhesion molecules, such

as integrins and selectins. V-CAM and I-CAM are well-known integrin receptors that belong to IgSF (Fawcet et al., 1992; Staunton, et al., 1990; Osborn et al., 1994), while MadCAM is a ligand of L-selectin (Berg et al., 1993). Ig-CAMs are also receptors of extracellular matrix proteins. The axonal glycoprotein may bind tenascin-R (Rathjen et al., 1991), while CEA and MAG may be involved in cell attachment to collagen (Pignatelli et al., 1990; Probstmeier et al., 1992). Recently, MAG has been shown to be a sialic acid binding protein (Tang et al., 1997).

(a) Structure-Function Relationships of IgSF

Ig-CAMs are primarily type I transmembrane proteins. The extracellular portion is usually composed of one or more Ig-like domains in the N-terminus, followed by zero to several fibronectin-type III-like repeats. The molecules are anchored on the cell surface either through a single transmembrane domain followed by a cytoplasmic C-terminal region or through a GPI anchor (Table I.2).

Each Ig domain is composed of 70 to 110 amino acid residues, which are organized into two antiparallel ß pleated sheets packed face to face. The two ß sheets are composed of three to five antiparallel ß strands. These strands are 5-10 amino acids long and are connected by loops of variable length. The side chains of amino acids in ß sheets pointing inwards to the apposing ß sheet tend to be hydrophobic, which stabilize the interaction between both sheets, while those pointing outwards are usually hydrophilic and may mediate interactions with ligands. In most Ig-like domains, the two cysteines on strands B and F form a disulfide bridge between the two ß sheets providing further stability to the Ig-fold (Figure I.5) (Jones et al., 1992; Dirscoll et al., 1991).

Ig folds can be categorized into either the variable (V) type which contains nine ß-strands, or the constant (C) type, which contains seven ß strands (Figure I.5) (Williams and Barclay, 1988). The C-type sequences can be further subdivided into the C1-set and C2-set, which differ by the ß-sheet with which the D strand associates through hydrogen bonds. In the C1-set, the D strand is bonded with the ß-sheet of ABE strands, while in the C2-set, the D strand is flipped over to the CFG sheet, and is referred to as the C' strand (Figure I.5). Other than these two types, an intermediate type (I-set) between V-type and C1-type of Ig fold was proposed recently (Harpaz and Chothia, 1994). The β strand arrangement is similar to that of V-type, except for a missing of C" strand.

The Ig-like domains of several Ig-CAMs are found to harbor adhesion activities. The V domain of P_0 has been demonstrated to be involved in P_0 homophilic interaction (Zhang et al., 1996). The first C2 type Ig-like domain of ICAM-1 and the first two C2 domains of ICAM-3 are involved in heterophilic interaction with LFA-1 integrin (Staunton, 1990; Fawcett et al., 1992). In chick NCAM, the third Ig-like C2 type domain is involved in homophilic binding, and the C' strand together with the C'-E loop constitute the homophilic binding site (Rao and Siu, 1992, 1993).

The fibronectin type III-like repeat (FNIII) was originally identified as a 90-residue module repeated 16 times in the ECM glycoprotein fibronectin (Kornblihtt et al., 1985). The FNIII domains are characterized by conserved tryptophan and tyrosine residues in their N- and C- terminal regions, respectively. However, they do not contain conserved cysteine residues. Structural analysis of FNIII domains derived from fibronectin and tenascin (Main et al., 1992; Leahy et al., 1992) has revealed strong similarities in their secondary structure to the Ig fold (Figure I.5). Like the C-type domains, the FNIII repeats are composed of two ß-sheets with three and four antiparallel

strands. However, the two ß-sheets of FNIII repeats are not stabilized by intersheet disulfide bonds, but rather by hydrophobic amino acid residues exclusively. Although the involvement of FNIII in NrCAM adhesion is suggested (Mauro et al., 1992), the exact structure-function relationships of FNIII repeat in most molecules require further investigation.

Most of the Ig-CAMs are glycosylated, and the glycosylation is important to their adhesion function (Krog and Bock, 1992). An O-linked sugar moiety of MadCAM contains the selectin binding moiety (Berg et al., 1993). A high mannose moiety in L1 is involved in L1-NCAM *cis*-interaction (Kadmon et al., 1990b). The most important and well-studied carbohydrate moiety is the polysialic acid (PSA) on NCAM. PSA is known to regulate both the adhesive properties and the neuritogenic activity of NCAM, and it may also play an important role in NCAM mediated synapse formation and plasticity and in memory (Rutishauser 1996).

Some of the Ig-CAMs are anchored to the plasma membrane through a single transmembrane domain, while others are anchored through a GPI moiety (Table I.2). The higher mobility and easy cleavage of the GPI anchor of Ig-CAMs may facilitate the fast on and off switch of the coupling of binding and signaling (Brümmendorf and Rathjen, 1994). The GPI anchor may also be involved in the specific distribution of certain molecules on axons (Powell et al., 1991).

The cytoplasmic domain of several Ig-CAMs are highly conserved among species. Association of cytoplasmic domains with various signal transduction components and cytoskeletal elements have been reported (Brümmendorf and Rathjen, 1994). In contrast to cadherins and integrins, deletion of the cytoplasmic domain usually does not affect the adhesion properties of Ig-CAMs (Hortsch et al., 1995).

(b) Signaling of Ig-CAMs

Evidence has accumulated to indicate that Ig-CAMs can modulate the intracellular second messenger pathways (Doherty and Walsh, 1992). It is conceivable that Ig-like proteins with adhesive functions directly activate second messenger system via their cytoplasmic segments or by *cis*-interactions with neighboring cell surface proteins. In the nervous system, the *Drosophila* IgSF molecule DTRK contains a tyrosine protein kinase module (Pulido et al., 1992), which is activated upon DTRK homophilic adhesion. These observations suggest that DTRK is directly involved in signal transduction.

Although most Ig-CAMs do not have enzymatic activities associated with their cytoplasmic domain, their role in signaling has been reported. Antibody binding of NCAM and L1 has been shown to reduce the intracellular levels of IP_2 and IP_3, and elevate the levels of free intracellular calcium by opening the L- and N- type calcium channels (Schuch et al., 1989; Walsh and Doherty, 1996).

In addition to the nervous system, Ig-CAMs are involved in T cell and B cell activation (Brümmendorf and Rathjen, 1994). For instance, binding of CD28 to B7 or B7-2 cells would induce the proliferation of resting T cells and stimulates the expression of lymphokines, such as IL-2, IL-3, and TNF-α. (Fraser et al., 1992; Damle et al., 1992; Linsley et al., 1991).

(c) Diseases-associated with IgSF Membranes

Two Ig-CAMs have been implicated in human diseases. Mutations in the P_0 gene are implicated in certain forms of hereditary motor and sensory neuropathies. P_0 point mutations are linked to type 1B of Charcot-Marie-Tooth neuropathy (Hayasaka et al., 1993a; Kulkens, et al., 1993). Other point mutations are responsible for some sporadic

cases of Dejerne-Sottas disease, a severe demyelinating disorder with extremely low nerve conduction velocities (Hayasaka et al., 1993b).

Mutations in the L1 gene have been implicated in X-linked hydrocephaly, the most common form of hydrocephalus. It has also been related to two other neurological diseases, MASA syndrome and SPG1 disease. They are characterized by mental retardation, adducted thumbs, spastic paraplegia, and enlarged brain ventricles (Jouet et al., 1993; Wong et al., 1995).

III. THE CELL ADHESION MOLECULE L1

The neural cell adhesion molecule L1, a glycoprotein with an apparent molecular mass of 200 kDa, is known to be involved in neuron-neuron adhesion (Keilhauer et al., 1985), neurite outgrowth (Chang et al., 1987), fasciculation (Fischer et al., 1986) and migration of neuronal cells (Lindner et al., 1983). L1 homologues have been described and cloned from several species. Representative examples include L1 in mouse (Moos et al., 1988), NILE (nerve growth factor (NGF) inducible large external glycoprotein) in rat (Miura et al., 1991), Ng-CAM/8D9/G4 in chick (Burgoon et al., 1991), L1 in human (Hlavin and Lemmon, 1991; Kobayashi et al., 1991), and neuroglian in *Drosophila* (Bieber et al., 1989) (Table I.3). Complementary DNA sequences have revealed that L1 belongs to the IgSF (Moos et al., 1988).

L1 is expressed primarily, but not exclusively, in the central nervous system. L1 is enriched on fasciculating axons in hippocampus, in fetal brain, on the developing cerebellum granule cells and on differentiating sensory neurons (Faissner et al., 1984; Brümmendorf and Rathjen, 1994). It is expressed transiently in mouse embryos from E13 to E16 on neuronal cell bodies in the cortical plate (Fushiki and Schachner, 1986; Persohn and Schachner, 1987, 1990). L1 is also expressed on Schwann cells before the onset of myelination, but the expression is lost after myelination on both Schwann cells and axons (Martini et al., 1988). In addition to the nervous system, L1 expression is also found in bone marrow, spleen and thymus, on haematopoietic tumor cell lines, on melanoma and neuroblastoma cell lines, and on proliferating epithelial progenitor cells of crypts of the intestine (Mujoo et al., 1986; Thor et al., 1987; Kowitz et al., 1992; Kowitz et al., 1993; Reid and Hemperly, 1992).

A. STRUCTURAL CHARACTERISTICS OF L1

(a) Domain Structure of L1

Predicted from its cDNA sequence, the L1 protein contains six Ig-like domains localized in the N-terminal region of the molecule, followed by five FNIII domains, a single transmembrane domain, and a short cytoplasmic C-terminal tail (Figure I.6). Each of its six Ig-like domain contains approximately 110 amino acids and there are ~70 amino acids between the two cysteines. Sequence alignment suggests that they may belong to the C2-set of Ig domains (Moos et al., 1988).

(b) Polypeptide Components of L1

Immunoblots with several anti-L1 monoclonal antibodies showed three major bands with *M*r 200,000, 135,000, and ~80,000. Pulse-chase studies have shown that the *M*r 200,000 protein is synthesized first, while the other two lower molecular weight forms appear later. These results suggest that the *M*r 200,000 form is the precursor, which is cleaved to yield the two smaller polypeptides (Grumet, 1992). By N-terminal sequencing and peptide mapping, the *M*r 135,000 component of chick NgCAM contains most of the extracellular region including all of the Ig-like domains and two and a half FNIII repeats. Although it does not contain a transmembrane segment, the 135 kDa component is still tightly associated with the plasma membrane through an unknown mechanism (Grumet and Edelman, 1988). The *M*r 80,000 species contains the remaining C-terminal portion of L1 (Grumet, 1992).

(c) Primary Structure of L1

L1 has been cloned and sequenced from various species and they show different degree of sequence similarity. Human L1 is most closely related to mouse L1, with 92% amino acid identity (Hlavin and Lemmon, 1991). In the case of rat L1, there is 95.7% cDNA similarity and 96.7% amino acid identity to mouse L1 (Miura et al., 1991). The cytoplasmic domain of L1 is completely conserved among human, mouse and rat, with 100% sequence identity. Although all six Ig-like domains of L1 show structural similarities, their internal sequence similarity is lower than that of similar domains among different species. Figure I.7 shows the alignment of the 6 Ig-like domains of human L1. The internal amino acid sequence identity ranges from 25 to 35%, whereas the percentage identity of each Ig-like domains among species ranges from 83 to 93% in mouse L1 vs. human L1, and 39 to 66% with chick NgCAM vs. human L1 (Hlavin and Lemmon, 1991). Apart from the cytoplasmic domain, the amino acid sequence of the second Ig-like domain shows the highest degree of sequence conservation among species (Hlavin and Lemmon, 1991).

In mouse L1 Ig6, there are two RGD sequences, which are potential integrin recognition sites (Moos et al., 1988). Only one of these two sites is conserved in human L1 (Hlavin and Lemmon, 1991). In NgCAM, however, an RGD sequence is not found in Ig6, but in the third FNIII repeat (Burgoon et al., 1991). The shifting of the location of the RGD sequence in NgCAM as well as the relatively low sequence identity with other vertebrate L1 raises another concern whether the chick NgCAM is the true homologue of L1 in mammals (Burgoon et al., 1991).

The cytoplasmic domain is the highest conserved region in L1. It contains ~113 amino acid residues (Hlavin and Lemmon, 1991). Several consensus phosphorylation

sites exist within the cytoplasmic domain. There are one protein kinase C recognition motif (Ser/Thr-X-Arg/Lys) and several casein kinase II recognition motifs between residues 1154 and 1232 (Miura et al., 1991). These potential phosphorylation sites may play important roles in L1 signaling.

(d) Gene Structure and Transcription

Southern blot analysis has revealed that L1 is encoded by a single gene in mouse, rat and human (Tacke et al., 1987; Djabali et al., 1990; Miura et al., 1991). The L1 gene has been mapped to the X chromosome in both human and mouse (Chapman et al., 1990; Djabali et al., 1990). Human L1 is located at the Xq28 locus and has been found to co-segregate with the X-linked hydrocephalus disease (Wong et al., 1995). Analysis of the mouse L1 gene structure by Kohl et al., (1992) demonstrates that the Ig-like domains are encoded by exon pairs, similar to the arrangement of the NCAM gene (Owens et al., 1987). The 5' upstream sequence of the L1 gene does not contain a TATA box, but there is a CAAT-box-like motif. Again, similar features have been observed in NCAM gene (Hirsch et al., 1990).

Although Northern blot analysis of L1-expressing tissues shows a single band, an alternatively spliced form of L1 has been reported. A slightly shorter form of L1, called L1cs, with 4 amino acids (RSLE) deleted in the cytoplasmic domain has been reported in both rat and human (Miura et al., 1991; Harper et al., 1991). This isoform lacks one of the putative casein kinase II phosphorylation sites (Miura et al., 1991). L1 and L1cs show different tissue distribution. L1 is expressed predominantly in brain, while L1cs is detected in non-neuronal cells, such as sciatic nerve tissue, Schwann cells, and melanoma cells (Miura et al., 1991; Harper et al., 1991).

(e) Posttranslational Modification of L1

L1 is a heavily glycosylated molecule. There are many potential N-glycosylation sites present on the extracellular domains of L1 (Moos et al., 1988). Treatment of L1 with endoglycosidase F to remove N-linked oligosaccharides reduces the apparent *Mr* of NgCAM components from 135,000 to 115,000 and from 80,000 to 60,000 (Wolff et al., 1987). In contrast, O-glycosylation of L1 has not been reported. Some of the N-linked carbohydrate epitopes of L1 are shared by other cell adhesion molecules (e.g. NCAM) and substrate adhesion molecules, such as NC-1 (Vincent et al., 1983) and HNK-1 (Abo and Balch, 1981). Different from NCAM, L1 contains only a small amount of sialic acid (Grumet et al., 1984b).

L1 is phosphorylated in its cytoplasmic domain, which is demonstrated by the incorporation of radioactive phosphate (Grumet et al., 1984a). Kinase activities also co-purify with L1 immunoprecipitates (Sadoul et al., 1989), among them are $p90^{rsk}$ and casein kinase II (Wong et al., 1996a,b). Phosphorylation is found to occur in some serine residues (Wong et al., 1996a,b). On the other hand, Klinz et al. (1995) have demonstrated that purified L1 or L1 antibodies can activate a phosphatase activity in growth-cone enriched membranes. These results supported the idea that L1 signaling events depend on the tight regulation of phosphorylation and dephosphorylation (Cervello et al., 1991).

B. LIGAND BINDING PROPERTIES OF L1

L1 was first described in the central nervous system of mouse as a 200-kD integral membrane glycoprotein (Rathjen and Schachner, 1984). Cerebellar cells and N2A neuroblastoma cells express L1 on the cell surface and form aggregates in

suspension cultures in a Ca^{2+}-independent manner. This aggregation process can be blocked by polyclonal anti-L1 Fab fragments. These results provide the evidence that L1 is a cell adhesion molecule (Rathjen and Schachner, 1984). Further experiments have demonstrated that L1 undergoes not only homophilic interaction but also heterophilic interactions with several other cell adhesion molecules, such as NCAM, axonin-1/TAG-1, and integrins.

(a) L1-L1 Homophilic Interaction

To investigate the mechanism by which L1 mediates cell-cell adhesion, membrane vesicles, protein-coated Covaspheres, and cells expressing L1 have been applied. When chick NgCAM is conjugated to 0.5μm Covaspheres or incorporated into liposomes, both Covaspheres and liposomes are able to undergo self-aggregation. Aggregate formation is specifically inhibited by anti-L1 Fab (Grumet and Edelman, 1988). These results provide the first evidence that L1 is able to undergo homophilic interaction.

Fibroblast L cells acquire cell-cell adhesiveness when transfected with rat L1 cDNA. These cells are able to undergo self-aggregation. More importantly, they are able to sort out from untransfected parental cells, demonstrating that cell-cell adhesion is mediated by L1 homophilic interaction (Miura et al., 1992). On the other hand, mouse L1-coated substrates induce cerebellar neuron adhesion, which can be inhibited by precoating neuronal cells with anti-L1 Fab fragments (Kadmon et al., 1990a).

L1 homophilic interaction has also been found to occur among different species. Chick Ng-CAM-conjugated Covaspheres are able to bind to neuronal cells derived from either chick, mouse or rat (Grumet and Edelman, 1988). Chick neurons are able to adhere

and send out neurites on substrate-coated L1 isolated from either chick or mouse. The neuronal cell adhesion and neurite outgrowth on substrate L1 is inhibited by antibodies against either mouse L1 or chick Ng-CAM. Similar results have been obtained with mouse neurons (Lemmon et al., 1989). These results suggest that L1-mediated axon outgrowth is dependent on L1 homophilic binding.

The homophilic binding activity is localized on the extracellular region of L1, since L1-mediated adhesion is abolished by absorbing polyclonal anti-L1 Fab fragments to the cell surface. Unlike cadherins and integrins, the cytoplasmic domain of L1 is not required for homophilic binding. When mutant L1 with its cytoplasmic domain deleted is expressed on the cell surface, it retains the cell binding activity and mediates cell-cell aggregation (Hortsch et al., 1995). A secreted chimeric form of L1 has been produced by fusing the extracellular portion of L1 to the Fc domain of immunoglobulin. This L1-Fc chimera is able to bind to L1 on the neuronal cell surface and stimulate neurite outgrowth (Doherty et al., 1995).

L1 homophilic interactions promote many critical events in neural development, such as cell recognition, adhesion, neurite elongation, and cell migration (Lemmon et al., 1989; Miura et al., 1992). Mapping of the homophilic binding site of L1 would provide a better understanding of L1 function, and this is the focus of my thesis research.

(b) L1-NCAM Interaction

The neural cell adhesion molecule NCAM is the first cell adhesion molecule identified in the nervous system which belongs to IgSF (Edelman, 1985). NCAM mediates cell-cell aggregation through a Ca^{2+}-independent homophilic manner (Edelman,

1985). The homophilic binding site of chick NCAM has been mapped to a decapeptide sequence localized within its third Ig-like domain (Rao et al., 1992, 1993, and 1994).

Both NCAM and L1 are expressed in the nervous system, and they display extensive overlap in distribution (reviewed by Brümmendorf and Rathjen, 1994). The first evidence suggesting L1-NCAM heterophilic interaction comes from their co-purification by immunoaffinity chromatography (Grumet et al., 1984b; Pollerberg et al., 1987). In addition, when L1 is clustered on the cell surface by anti-L1 antibody, NCAM is found to co-patch with L1 (Pollerberg et al., 1987).

In 1990, Kadmon et al. proposed the "assisted homophilic binding" model, suggesting that the *cis*-binding of L1 and NCAM on one cell forms a more potent receptor complex for L1 on an apposing cell. In other words, *cis*-interaction between NCAM and L1 enhances L1-L1 *trans*-interaction. Cells expressing both NCAM and L1 coaggregate with L1-expressing cells more rapidly than cells expressing L1 alone. Also, the aggregation of L1-expressing cells can be enhanced by the addition of soluble NCAM (Kadmon et al., 1990a). Further studies have suggested that the NCAM-L1 interaction is carbohydrate-dependent (Kadmon et al., 1990b). A castanospermine-sensitive, and swainsonine-resistant carbohydrate moiety expressed on L1 molecule has been suggested to interact with the fourth Ig-like domain of NCAM, which harbors a C-type lectin consensus sequence (Horstkorte et al., 1993). A peptide corresponding to part of this sequence in NCAM Ig4 is able to inhibit the NCAM-L1 *cis*-interaction (Horstkorte et al., 1993).

(c) L1-Axonin-1/TAG-1 Interaction

TAG-1 and axonin-1 are presumptive species homologues of rat and chick, respectively. They are cell surface glycoproteins concentrated primarily on axons (Furley et al., 1990). TAG-1 has been implicated in neurite extension in the vertebrate nervous system. cDNA cloning of these molecules have shown that they belong to the IgSF. TAG-1/axonin-1 contains six Ig-like domains and four FNIII repeats (Zuelig et al., 1992; Hasler et al., 1993; Furley et al., 1990). Two isoforms of TAG-1/axonin-1 are expressed; one is associated with the axonal membrane by a glycophosphatidylinositol (GPI) anchor (Ruegg et al., 1989), while the other one lacks the GPI moiety and is secreted by neuronal cells (Stoeckli et al., 1989; Karagogeos et al., 1991).

In chick, axonin-1 and Ng-CAM coexpress in several nerve fiber tracks in tissue sections (Ruegg et al., 1989). At the cellular level, they colocalize on cell somas and neurites of dorsal root ganglia (DRG) neurons (Kuhn et al., 1991). Covaspheres conjugated with axonin-1 coaggregate with those conjugated with NgCAM, thus providing evidence for axonin-1-NgCAM heterophilic interaction. Axonin-1-conjugated Covaspheres are also able to bind to neuronal cells via interaction with NgCAM. Furthermore, both substrate-coated TAG-1 and axonin-1 can promote neurite outgrowth through TAG-1-L1 or axonin-1-NgCAM heterophilic interaction (Kuhn et al., 1991; Felsenfeld et al., 1994).

(d) L1-Integrin Interaction

In mouse and rat L1, two integrin recognition motifs (RGD) are present within the sixth Ig-like domain. One of them is conserved in human. In chick, one RGD sequence is

present in the third FNIII repeat of NgCAM. The presence of the RGD sequence suggests that L1 is capable of undergoing heterophilic interaction with integrins.

The RGD receptor VLA-5 is the first integrin identified to interact with L1 (Ruppert et al., 1995). L1-coated polystyrene beads bind to VLA-5 expressing platelet cells. This binding is inhibited by monoclonal antibodies against either L1 or VLA-5. Soluble L1 and L1-RGD peptides are also able to inhibit their co-aggregation in a dose-dependent manner. These results are further supported by direct binding of L1 to VLA-5 in an ELISA-based assay. Biotinylated soluble L1 interacts with immobilized VLA-5 in the presence of divalent cations, and this binding is inhibited by ~80% in the presence of L1-RGD peptide at 500 μg/ml.

$\alpha_V\beta_3$ is another integrin known to interact with L1 through binding with the RGD sequence of L1. Montgomery et al. (1996) have demonstrated that the melanoma cell line M21 displays Ca^{2+}-dependent adhesion and spreading on substrate-coated rat or human L1. The attachment of M21 cells is dependent on the expression of the α_V-integrin subunit, and can be inhibited by anti-$\alpha_V\beta_3$ antibodies. This adhesive interaction involves the RGD sequence of L1, since mutation of this sequence abolishes M21 cell adhesion and spreading. These results indicate that integrins interact with L1 and that these heterophilic interactions may be important in integrin-mediated cell-cell interactions.

(e) L1 Interaction with Chondroitin Sulfate Proteoglycan

There is increasing evidence that chondroitin sulfate proteoglycans play important roles in modulating cell adhesion and migration. Two of them have been found to interact with NgCAM in brain. They are phosphacan (Maurel et al., 1994) and neurocan (Rauch et al., 1992).

Phosphacan is a chondroitin sulfate proteoglycan produced by glial cells in the central nervous system, and represents the extracellular domain of a receptor-type protein tyrosine phosphatase (Maurel et al., 1994). Soluble phosphacan binds to NgCAM via a single high affinity site with a Kd of ~0.1 nM in a chondroitinase-sensitive manner. Phosphacan binding can inhibit the self aggregation of NgCAM coated microbeads, as well as NgCAM mediated neuronal adhesion and neurite outgrowth (Milev et al., 1994).

The other chondroitin sulfate proteoglycan known to bind to L1 is neurocan (Rauch et al., 1992). Soluble neurocan was able to inhibit the aggregation of Ng-CAM-coated microspheres (Grumet et al., 1993). Further studies show that radioactively labeled neurocan binds to purified NgCAM in a chondroitinase-sensitive manner, and that this binding is inhibited by free chondroitin sulfate. Protein deletion experiments demonstrate that the binding activities is localized in the C-terminal half of neurocan (Friedlander et al., 1994). The binding of neurocan to NgCAM inhibits neuronal adhesion and neurite extension on NgCAM substrate, suggesting a role for neurocan in neuronal development (Fridlander et al., 1994).

(f) L1 Heterophilic Interaction with DM-GRASP

DM-GRASP is a cell adhesion molecule in the IgSF, which is expressed in the developing nervous system as well as the immune system (Burns et al., 1991). Specific populations of neurons respond to GRASP by extending neurites which require homophilic interactions between GRASP molecules (DeBernardo and Chang, 1995).

NgCAM, which is often referred as chick L1, is able to interact with DM-GRASP and bind to a GRASP-Sepharose affinity column. This interaction may have a role in promoting neurite extension from sympathetic neurons on the NgCAM substratum, since

blocking antibodies against GRASP decrease their mean neurite length on NgCAM-coated substrate (DeBernardo and Chang, 1996).

(g) L1 cis-interaction with CD24

CD24, formerly named heat-stable antigen (HSA) and nectadrin, is a highly heterogeneously glycosylated GPI-linked surface protein of haematopoietic and neural cells. It has been implicated in mediating B lymphoblast aggregation (Kadmon et al., 1994). It co-expresses with L1 in murine cerebellar granule cells and neuroblastoma N2A cells. Purified CD24 binds to L1 with a 5:1 ratio at saturation. In addition, CD24 co-caps with L1 and NCAM (Kadmon et al., 1995a). Antibodies against CD24 cooperate with those against L1 in stimulating strong intracellular Ca^{2+} signals in N2A cells and cerebellar neurons (Kadmon et al., 1995b). These results suggest that CD24 participates in cell adhesion by cooperating with L1.

(h) L1-F11 Interaction

F11 is a GPI-anchored axonal surface glycoprotein that belongs to the IgSF (Brümmendorf et al., 1989). F11-conjugated beads can co-aggregate with those conjugated with NgCAM, a L1 homologue. However, they fail to undergo self-aggregation. This result indicates that F11 binds to NgCAM heterophilically. Using mutant forms of F11 expressed in COS cells and epitope mapping by monoclonal antibodies, they show that the first two Ig-like domains of F11 are involved in NgCAM binding. However, this heterophilic interaction mechanism is not involved in F11-mediated neurite outgrowth (Brümmendorf et al., 1993).

C. FUNCTIONS OF L1

L1 is involved in neural cell adhesion and migration, neurite elongation, axonal fasciculation, and memory formation. In addition, L1 is also expressed in some non-neuronal tissues and has been implicated in leukocyte invasion and cancer cell metastasis.

(a) Neuronal Cell Adhesion and Migration

L1 is known to undergo homophilic interaction as well as heterophilic interactions with other cell adhesion molecules. Antibodies against L1 inhibit the adhesion and migration of various primary neurons on L1 substrate or monolayers of L1 expressing cells (Miura et al., 1992; Asou et al., 1992b; Takeda et al., 1996). However, a heterophilic binding mechanism is responsible for L1-mediated neuron-glia adhesion (Grumet et al., 1984a). Fab fragments against NgCAM, a L1 homologue, lower the rate of binding by >40% of chick brain neurons to chick brain astroglia, in which NgCAM is not expressed (Grumet et al., 1984a).

To examine the function of L1 under more physiological conditions, the effects of antibodies on cell behavior have been examined in explants of neural tissues. Developing cerebellum explants are used to examine neuronal cell migration. During cerebellar development, granule cells in the external granule layer become postmitotic, emit parallel fibers that fasciculate in the molecular layer, and then migrate radially to form the internal granule layer (Mugnaini, 1970). When radioactively-labeled cerebellum explants of chick are treated with anti-NgCAM antibodies, the rate and extent of granule cell migration are reduced. Most granule cells remain in the external granule layer and do not enter the molecular layer (Hoffman et al., 1986; Barami et al., 1994).

(b) Neurite Extension and Regeneration

L1, either coated on a substratum or presented on the cell surface, has been found to provide a permissive substrate for primary neurons to extend neurites (Lemmon et al., 1992). *In vitro* studies have shown that substrate-coated L1 promotes neurite outgrowth (Lemmon et al., 1989; Bixby and Jhabvala, 1990; Williams et al., 1994; Seilheimer and Schachner, 1988). This neurite outgrowth promotion activity is believed to be mediated by a homophilic binding mechanism (Lemmon et al., 1989). Meanwhile, L1 on neuronal cells is known to interact heterophilically with substrate bound TAG-1/axonin-1, and play an important role in TAG-1/axonin-1 promoted neurite outgrowth (Kuhn et al., 1991; Felsenfeld et al., 1994).

The neurite outgrowth promotion activity of L1 is also involved in axon regeneration. When a sciatic nerve autograft is anastomosed to the proximal stump of the transected rat optic nerve, L1 is confined to the interface of axon-astrocyte and of axon-Schwann cell, and resulted in the regeneration of the rat optic nerve (Dezawa and Nagano, 1996). Kobayashi et al. (1995) also show that when L1 expressing fibroblast L cells are grafted to a lesion of rat spinal cord immediately after hemisection, they drastically promote regeneration of the axons in the injured spinal cord 2 weeks after the surgery. Some of these regenerating axons also penetrate the glial scar along the host-graft interface and extend into the L1-expressing L cell graft.

(c) Neurite Fasciculation

The preferential localization of L1 on axon suggests that it may be involved in neurite fasciculation. Polyclonal Fab fragments against fish E587 glycoprotein, a member

of the L1 family of cell adhesion molecule, disrupt the orderly fascicle pattern of ganglia cell axons in the retina when injected into the eye of the goldfish (Bastmeyer et al., 1995). Similarly, in explants of chick dorsal root ganglia, retina and tectum, antibodies against NgCAM reduce the fascicle diameter and increase fascicle length and number (Hoffman et al., 1986; Kröger et al., 1990).

(d) Axon Guidance

Cell adhesion molecules, being permissive substrate for neurite outgrowth, are considered as short range cues for axon guidance, and play an important role in axon guidance (Tesser-Lagvin and Goodman, 1996). L1 expression during development is stage-dependent and in discrete regions. Perturbation of L1 function by antibodies has deleterious effects on axon projection and pathway finding. When anti-L1 Fab is administered to the growth cones of retinal ganglion cell axons in the mammalian retina, the growth cones would stall for a while, and then change their direction (Brittis et al., 1995). When anti-L1 antibodies are injected into the developing chick hindlimb, the pathfinding of sensory axons is affected (Honig and Rutishauser, 1996). Fewer sensory axons cross the anterior-posterior axis of the plexus, and both cutaneous and muscle sensory projections are affected. Taken together, these results suggest an important role for L1 in axon guidance.

(e) Growth Cone Morphology

The growth cone of developing and regenerating axon is specialized for motility and guidance functions. Growth cones extend and retract both lamellipodia, which advance the cell margin, and filopodia, which are thought to direct axon elongation. The

morphology of retinal ganglion cell growth cones has been analyzed on L1 substrate (Payne et al., 1992). Elaborated lamellipodial structure is found in response to the L1 substrate and more filopodia are present than other substrates. The substrate influence on growth cone morphology cannot be attributed to the strength of adhesion *per se*. It has been suggested that substrate modulation of growth cone behavior is a complex process, with cell adhesion molecules exerting their effect via interactions with the cytoskeleton and other cytoplasmic components.

(f) Memory Formation

L1 expression is found in developing postnatal mouse hippocampus (Persohn and Schachner, 1990), in which morphologically detectable changes in synaptic connectivity have been observed during learning. Following learning, changes in the efficacy of synaptic activity leading to the phenomenon of long-term potentiation (LTP) have been observed. LTP, therefore, is used as an indication marker for memory in higher vertebrates (Bliss and Collingridge, 1993). In rat, LTP of CA1 neurons in hippocampal slices is reduced by anti-L1 antibodies (Lüthi et al., 1994). The inhibition of LTP by L1 Ig-like domains but not FNIII repeats emphasizes the role of L1 Ig-like domains and further supports the involvement of L1 in LTP generation.

(g) L1 Function in Organs other than the Nervous System

L1 is also expressed in cells and tissues outside the nervous system, such as the epithelium of small intestine (Thor et al., 1987), melanoma cells (Linnemann et al., 1989), bone marrow, spleen and thymus (Kowitz et al., 1992). The cell-cell adhesion mechanisms mediated by L1 are also evident in these systems. For example, L1

homophilic interaction mediates the aggregation of L1-expressing lymphoma cells (Kowitz et al., 1992). On the other hand, L1-$\alpha_v\beta_3$ heterophilic interaction is involved in mediating melanoma cell adhesion, spreading and migration (Montgomery et al., 1996). L1 is further implicated in tumor metastasis (Kowitz et al., 1993). When the lymphoma cell line Esb-MP is subcloned according to the level of L1 expression and then injected subcutaneously into mice, clones with low L1 expression show faster primary tumor growth, develop visceral metastasis significantly faster than those with high L1 expression. In addition, expression of L1 on the tumor variants correlate directly with their homotypic aggregation, but negatively with their metastatic capacity. These results suggest that L1 may decrease the malignant potential of tumor cells by interfering with cell-cell interactions.

D. REGULATION OF L1 FUNCTION

L1 expression is spatially restricted and developmental stage dependent. Regulation of L1 expression, therefore, is important in modulating L1 function. L1 function can also be regulated by direct modulation of its adhesiveness and neuritogenic activity.

(a) Regulation of L1 Expression

The fact that rat L1 is identical to NILE (nerve growth factor inducible large external glycoprotein) provides the first hint that growth factors can regulate L1 expression (Bock et al., 1985; McGuire et al., 1978). Other than NGF, several transforming growth factor ßs (TGF-ß) are expressed in the nervous system and they are

able to up-regulate L1 expression *in vitro*. Examples are TGF-ß2, TGF-ß3, and osteogenic protein-1 (OP-1) (Perides et al., 1993; Perides et al., 1994; Stewart et al., 1995).

Contrary to growth factors, glucocorticoids are found to lower the level of L1 in terms of both mRNA and protein (Grant et al., 1996). This regulation may cause the differential expression of L1 in chromaffin cells, and the segregation of chromaffin cells into homotypic catecholaminergic groups in the adrenal gland (Léon et al., 1992a,b)

In addition to growth factors and hormones, several other factors are known to regulate L1 expression. For instance, cell-cell contact is believed to enhance L1 expression in PC12 cells (Kobayashi et al., 1992), while repeated low-frequency electrical pulses down-regulates L1 expression in mouse sensory neurons (Itoh et al., 1995). The latter is also found to result in decreased cell adhesion and neurite fasciculation. As the low-frequency electrical pulses are within the normal physiological range, this regulatory mechanism is particularly attractive for the control of cellular interactions during development and regeneration of the nervous system.

(b) Modulation of L1 Function

L1 is a multidomain protein, and is found to *cis*-interact with several other cell adhesion molecules. Some of these *cis*-interactions are known to modulate L1 adhesion and neuritogenic activity. For instance, L1-NCAM *cis*-interaction enhances L1-L1 homophilic interaction (Kadmon et al., 1990a). On the contrary, L1-phosphacan or L1-neurocan heterophilic interactions inhibit L1-mediated cell adhesion as well as L1-promoted neurite outgrowth (Maurel et al., 1994; Rauch et al., 1992).

Alcohol, through an unknown mechanism, has been demonstrated to inhibit specifically L1 mediated cell-cell adhesion but not NCAM function (Ramanathan et al., 1996). Half-maximal inhibition occurs at 7 mM ethanol, a concentration achieved in blood and brain after ingesting one alcoholic beverage. This effect might contribute to the fetal alcohol syndrome and ethanol-associated memory disorders (Ramanathan et al., 1996).

E. L1-MEDIATED SIGNAL TRANSDUCTION

There is increasing evidence that L1 not only plays important roles in cell-cell interactions during development of the nervous system, but also influences second messenger systems, such as intracellular pH and phosphoinositide turnover (Schuch et al., 1989; Bohlen et al., 1992). Clustering of L1 by antibodies induces the rise in intracellular Ca^{2+} concentration by influx of extracellular Ca^{2+} through N- and L- type Ca^{2+} channels (Asou, 1992a), and the influx of Ca^{2+} is believed to solely account for the L1-dependent neurite outgrowth (Williams et al., 1992). Neurite outgrowth promoted by cell adhesion molecules appears to go through a pathway different from that of ECM-promoted neurite outgrowth (Bixby and Jhabvala, 1990; Williams et al., 1994b). In contrast to laminin, fibronectin, and collagen, which promote neurite outgrowth through integrin activation, L1 and N-cadherin promote neurite outgrowth in a protein kinase C insensitive, but pertussis toxin sensitive mechanism (Bixby and Jhabvala, 1990; Saffell et al., 1992; Doherty et al., 1991a).

However, it is still unclear how cell-cell adhesion mediated by L1 is transduced into a complex morphological response, such as neurite outgrowth. Extensive studies are

being carried out in several laboratories and several components have been identified to be downstream components of the L1 signaling pathway and they include FGFR (Williams et al., 1994), c-*src* (Ignelzi et al., 1994), $p90^{rsk}$ (Wong et al., 1996b), casein kinase II (Wong et al., 1996a), and the cytoskeleton component ankyrin (Davis and Bennett, 1994).

(a) L1 Signaling through FGFR

FGFR is a member of receptor protein tyrosine kinases, and can stimulate neurite outgrowth in response to basic FGF (Schlessinger and Ullrich, 1992). It has been demonstrated that basic FGF stimulates neurite outgrowth by activating a second messenger pathway similar to the one activated by L1, NCAM, and N-cadherin (Williams et al., 1994a). Further investigation has identified a CAM homology domain composed of 20 amino acids and localized in the extracellular region of the FGFR. This CAM homology domain contains an L1-like sequence. Antibodies against this region, as well as a synthetic peptide containing the L1-like sequence, can inhibit specifically L1-promoted neurite outgrowth (Williams et al., 1994). In addition, an L1-Fc chimera has been shown to induce neurite outgrowth (Doherty et al., 1995), and to increase tyrosine phosphorylation of the same set of neuronal proteins as FGF (Williams et al., 1994). Therefore, it has been suggested that L1 promotes neurite outgrowth through *cis*-interaction with FGFR, which in turn, activates the tyrosine kinase activity of FGFR, and initiates the signaling cascade, which involves an increase of AA (Arachidonic acid) and PLC (Phospholipase C) activity and intracellular Ca^{2+} level.

(b) Role of pp60$^{c\text{-}src}$ in L1 Signaling

The nonreceptor tyrosine protein kinases pp60$^{c\text{-}src}$ has been localized to the growth cone and is expressed at high levels during periods of axonal growth and regeneration (Maness et al., 1988). Demonstration of c-*src* being a component of the intracellular signaling pathway in L1-mediated axonal growth has been achieved by performing a neurite outgrowth assay using cerebellar neurons from *src*$^-$ mice (Ignelzi et al., 1994). Cerebellar neurons from *src*$^-$ mice exhibit a reduced neurite extension rate on L1 substrate, but respond normally on top of laminin. Knockout mutants lacking other *src*-related non-receptor tyrosine kinases, such as *fyn* and *yes*, do not have this effect on L1 promoted neurite outgrowth. This specific impairment in *src*$^-$ neurons points to the importance of *src* in L1 signaling.

(c) Kinase Activities Associated with L1 Cytoplasmic Domain

The cytoplasmic domain of L1 is highly conserved among species and it is phosphorylated *in vivo* (Cervello et al., 1991; Hlavin et al., 1991). Several groups have demonstrated that protein kinases co-immunoprecipitate with L1 (Sadou et al., 1989; Wong et al., 1996a; Wong et al., 1996b). Two of them have been identified to be casein kinase II and p90^{rsk} (Wong et al., 1996a,b). Casein kinase II is an ubiquitous serine/threonine kinase enriched in the brain (Girault et al., 1990), and it phosphorylates Ser^{1181} in the L1 cytoplasmic domain (Wong et al., 1996a). p90^{rsk} belongs to the S6 kinase family and is another serine/threonine kinase. It has been found to phosphorylate Ser^{1152} (Wong et al., 1996b). Furthermore, tyrosine phosphorylation on both NgCAM and human L1 cytoplasmic domain has been reported recently and the receptor tyrosine

kinase Cek5 is believed to be involved (Zisch et al., 1997). Therefore, phosphorylation/dephosphorylation of the cytoplasmic domain of L1 may play a key role in the regulation of L1 functions, such as neurite extension and axon fasciculation (Cervello et al., 1991; Garver et al., 1997; Saffell et al., 1997).

(d) Association of L1 with Cytoskeleton Components

L1-mediated cell migration and neurite outgrowth are accompanied by cytoskeleton rearrangements. The link of L1 with the cytoskeleton component was reported by Davis and Bennett (1994) in their search of ankyrin-binding proteins. Ankyrins are a family of proteins that provide a linkage between membrane-spanning proteins, including ion channels and cell adhesion molecules, and the spectrin-based membrane skeleton located on the cytoplasmic surface of the plasma membrane of many cell types (Bennett and Gilligan, 1993). Ankyrin has been localized to the cell-cell contact region (Dubreuil et al., 1996). The specific recruitment of ankyrin to the plasma membrane in response to L1 adhesion might play a role in L1 signal-transduction.

F. L1-RELATED NEUROLOGICAL DISEASES

The human L1 gene has been mapped to the Xq28 locus (Djabali et al., 1990), and mutations in the L1 gene have been associated with several neurological disorders. These include X-linked hydrocephalus HSAS (hydrocephalus as a result of stenosis of the aqueduct of sylvius), MASA (mental retardation, aphasia, shuffling gait, and adducted

thumbs) syndrome, and spastic paraplegia type 1 (SPG1) (Wong et al., 1995; Jouet et al., 1995; Hortsch, 1996). HSAS is the most common form of congenital hereditary hydrocephalus, with a range of clinical features, including mental retardation and absence of the corticospinal tract and corpus callosum (Rosenthal et al., 1992; Camp et al., 1993). MASA syndrome and SPG1 are also X-linked disorders with an overlapping profile of clinical signs but milder presentation and longer life expectancy than HSAS (Bianchine et al., 1974; Kenwrick et al., 1986; Schrander-Stumpel et al., 1990). To date, more than fifty mutations of L1 have been associated with these diseases (Figure I.8), and they include missense mutations, deletions, premature truncations, and alternative splicing of L1 (Wong et al., 995; Jouet et al., 1995). These mutations are evenly distributed over the entire L1 molecule. However, direct link of these mutations to L1 functions is yet to be made. It is, therefore, of interest to elucidate the molecular basis of these L1 related clinical phenotypes.

IV. DESCRIPTION OF THE PROJECT

The specific aims of this project are: (1) to map the homophilic binding site of L1, (2) to identify the structural domain with neuritogenic activity, and (3) to investigate the relationship between these two intrinsic functions of L1.

In Chapter II, the question of which domain of L1 is involved in homophilic binding and neuritogenic activity is addressed. Recombinant L1 fragments were expressed in bacteria and then subjected to the Covasphere binding assay to determine which L1 fragment still retained the homophilic binding activity. The second Ig-like domain was found to mediate homophilic binding. When L1 fragments were tested for neuritogenic activity, the Ig2 domain again was found to promote neurite outgrowth from neural retinal cells.

Two HSAS/MASA mutations localized in Ig2 were tested for their effects on L1 homophilic binding and neuritogenic activities in Chapter III. The HSAS mutation (R184Q) completely abolished these two intrinsic activities associated with the second Ig-like domain of L1, while the MASA mutation (H210Q) had only partial effects. The differential effects of these two mutations on L1 functions correlate very well with their clinical phenotypes, and my results also point to the importance of R184 in L1 homophilic binding.

In Chapter IV, the requirement of the second Ig-like domain of L1 in homophilic binding was confirmed by expressing wildtype and mutant L1 in LR73 cells. Also, the homophilic binding site of L1 was mapped to a 14 amino acid sequence flanking the R184 residue. Peptide L1-A containing the sequence HIKQDERVTMGQNG was able to block L1 mediated cell-cell aggregation, as well as L1-Ig2 homophilic interaction,

whereas peptide pL1-B, which contained sequences flanking the H210 residue, did not display significant inhibitory effects. Studies using peptide analogues show that both charged residues and hydrophobic residues within pL1-A are important to its function. Inhibition studies using these peptides also demonstrate that the homophilic binding and neurite outgrowth promotion activities are closely related.

I have used the yeast two-hybrid system in an attempt to identify new components that associate with the L1 cytoplasmic domain. The L1 cytoplasmic domain, as well as the FNIII repeats, were used as bait to screen for protein interactors in a human fetal brain cDNA library. A cDNA encoding the human laminin binding protein (LBP) 32/67 was one of the positive clones obtained in the L1-cytoplasmic domain screen. Both LBP and L1 are expressed in the human melanoma cell line WM239. L1 was found to associate with LBP in the co-capping experiment. LBP was also found to be able to bind specifically to L1 cytoplasmic domain using affinity columns. These results suggest that LBP binds to the cytoplasmic domain of L1. These preliminary results and their implications are summarized in Appendix I of this thesis.

Table I.1. Cadherin family members

Type of cadherins	Representative members	HAV motif	EC domains	CP domains	TK domain
Classical cadherins*	E-cadherin P-cadherin N-cadherin B-cadherin R-cadherin EP-cadherin	yes	5	2	no
Atypical cadherins	OB-cadherin M-cadherin cadherin-5 cadherin-8 cadherin-12 DE-cadherin K-cadherin	no	5	2	no
Peptide transporters	intestinal proton-dependent peptide transporter (LI-cadherin)	no	7	1 short	no
Truncated cadherins*	T-cadherin	no	5	0	no
Protocadherins	protocadherin 43	no	6	1	no
Desmocollins*	desmocollin 1 desmocollin 2 desmocollin 3	no	5	2	no
Desmogleins*	desmoglein 1 desmoglein 2 desmoglein 3	no	5	6	no
Receptor tyrosine kinases	c-ret	no	7	1	yes
Others	fat protein	no	34	1 large	no

* cell adhesion molecules. EC, extracellular cadherin repeat. CP, cytoplasmic domain. TK, tyrosine kinase.

Table I.2: Ig-superfamily Molecules Involved in Cell Adhesion

Name	Ig domains		FNIII	Membrane anchor		Glycosylation		Ligands
	V	C2	repeats	GPI	TM	O-link	N-link	
NCAM	0	5	2	yes	yes	no	yes	NCAM, L1
L1	0	6	5	no	yes	no	yes	L1, NCAM, F11, TAG-1,neurocan, αvß3,
NrCAM	0	6	5	no	yes	no	yes	NrCAM, F11
neurofascin	0	6	5	no	yes	yes	yes	
F11	0	6	4	yes	no	no	yes	L1, NrCAM, tenascin-R
TAG-1	0	6	4	yes	no	no	yes	TAG-1, L1
DCC	0	4	6	no	yes	no	yes	netrins
MAG	1	4	0	no	yes	no	yes	collagens
P_0	1	0	0	no	yes	no	yes	P_0
DM-GRASP	2	3	0	no	yes	no	yes	DM-GRASP
ICAM-1	0	5	0	no	yes	no	yes	CD43, Mac-1, LFA-1
ICAM-2	0	2	0	no	yes	no	yes	LFA-1
ICAM-3	0	5	0	no	yes	no	yes	LFA-1
VCAM-1	0	3 or 7	0	no	yes	no	yes	VLA-4, α4ß7
MAd-CAM-1	1	2	0	no	yes	yes	yes	α4ß7, L-selectin
PECAM	0	6	0	no	yes	no	yes	PECAM
CEA	1	6	0	yes	no	no	yes	CEA, BGP, NCA
CD2	1	1	0	no	yes	no	yes	LFA-3, CD48, CD45, CD3
CD48	1	1	0	yes	no	no	yes	CD2
LFA-3	1	1	0	yes	yes	no	yes	CD2

Table I.3 L1 homologs and gene family members

Specie	Name	Reference
I. L1 homologs:		
Human	L1-CAM	Hlavin and Lemmon, 1991 Kobayashi et al., 1991
Mouse	L1	Moos et al., 1988
Rat	NILE	Miura et al., 1991
Chick	Ng-CAM/G4/8D9	Burgoon et al., 1991
Zebrafish	L1	Tongiorgi et al., 1995
Goldfish	L1	Giordano et al., 1996
Fugu rupripes	L1	Coutelle et al., 1997
C. elegan	L1-like sequence	Wilson et al., 1994
Tobacco hornworm	neuroglian	Chen et al., 1997
Drosophila	neuroglian	Bieber et al., 1989
II. Members of the L1 gene family:		
Human	Nr-CAM	Lane et al., 1996
Mouse	Nr-CAM/Bravo	Moscoso and Sanes, 1995
	neurofascin/ABGP	Moscoso and Sanes, 1995
	CHL1 (close homolog of L1)	Holm et al., 1996
Rat	Nr-CAM	Bennett et al., unpublished
	neurofascin	Davis et al., 1996
Chick	Nr-CAM/Bravo	Grumet et al., 1991
	neurofascin	Volkmer et al., 1992

Figure I.1 Integrin Subunit Associations and Ligand Specificities. Known integrin heterodimer pairs are indicated by connecting lines between α and β subunits. Described extracellular ligands for the specific heterodimer are found in parentheses. ICAM-1, ICAM-2: Intercellular adhesion molecule 1 and 2; iC3b: the cleavage fragment of complement protein C3; FB: Fibrinogen; LAM: laminin; COL: collagen (all types); FN: fibronectin; VCAM-1: vascular cell adhesion molecule 1; VN: vitronectin; VWF: von Willebrand factor; TSP: thrombospondin; ?: unknown ligand.

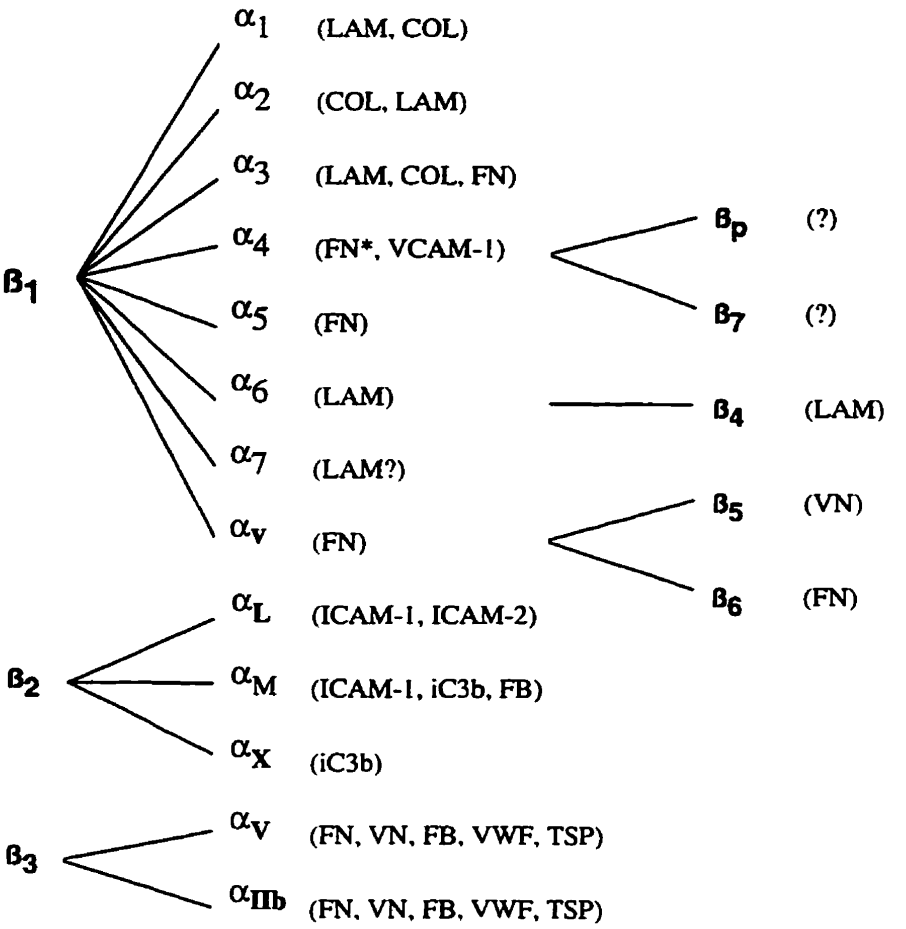

β1
α1 (LAM, COL)
α2 (COL, LAM)
α3 (LAM, COL, FN)
α4 (FN*, VCAM-1)
α5 (FN)
α6 (LAM)
α7 (LAM?)
αv (FN)
βp (?)
β7 (?)
β4 (LAM)
β5 (VN)
β6 (FN)
β2
αL (ICAM-1, ICAM-2)
αM (ICAM-1, iC3b, FB)
αX (iC3b)
β3
αv (FN, VN, FB, VWF, TSP)
αIIb (FN, VN, FB, VWF, TSP)

Figure I.2: Schematic Integrin Structure. An integrin is comprised of a noncovalently associated α subunit and ß subunit. Each subunit is anchored in the plasma membrane by a single short transmembrane region, with its C-terminus in the cytoplasm of the cell. All α subunits contain consensus sequences for divalent cation interaction in the extracellular portion (Ca^{++}). Some α subunits contain an "I domain". All ß subunits contain areas rich in cysteine (C) residues. All ß subunits are folded and stabilized by disulfide linkages. Both subunits contribute to an extracellular ligand binding region, and their cytoplasmic domains are involved in cytoskeletal interactions and signal transduction. ILK: integrin linked kinase; FAK: focal adhesion kinase. (This figure was modified from Dedhar and Hannigan, 1996).

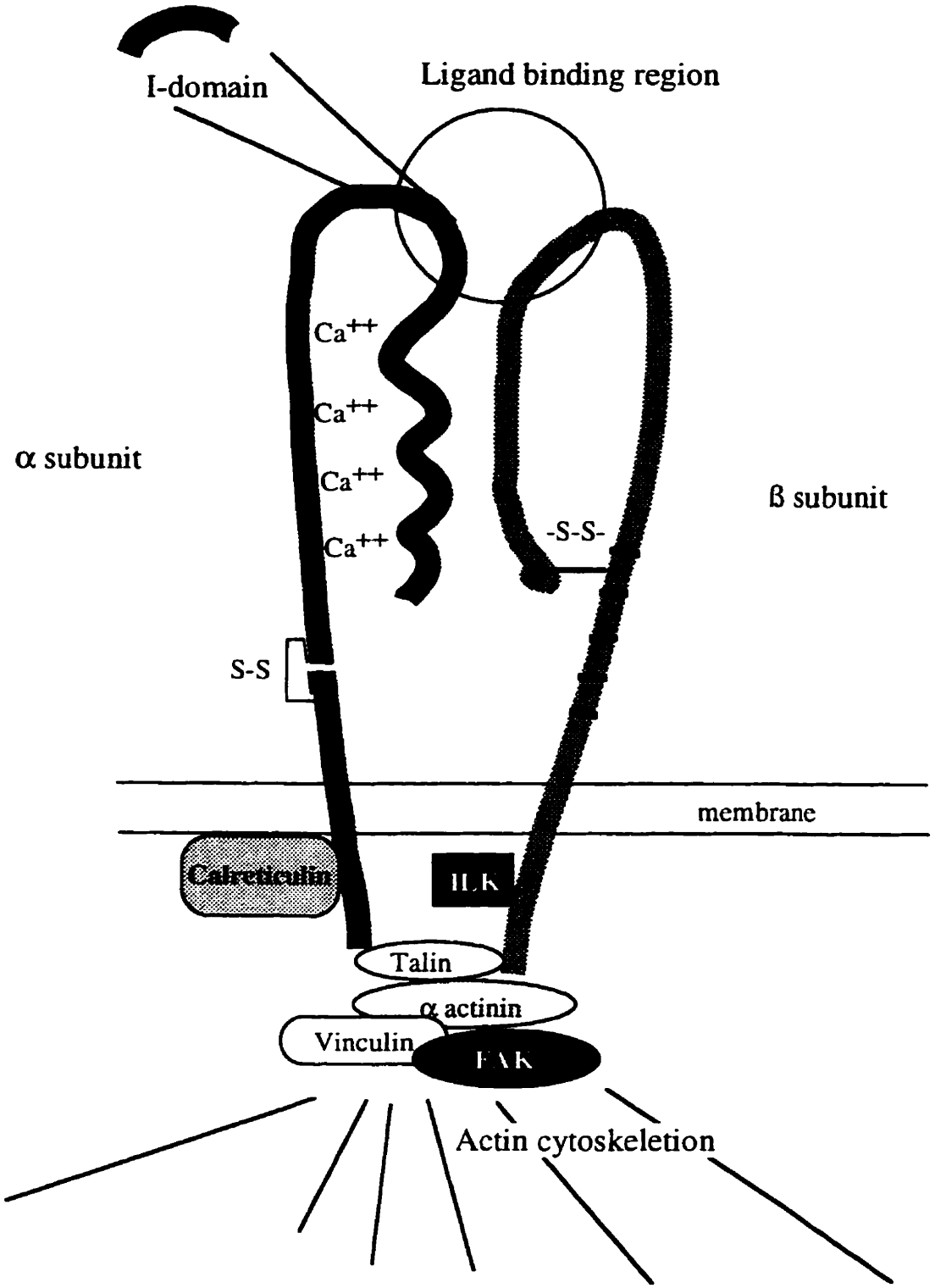
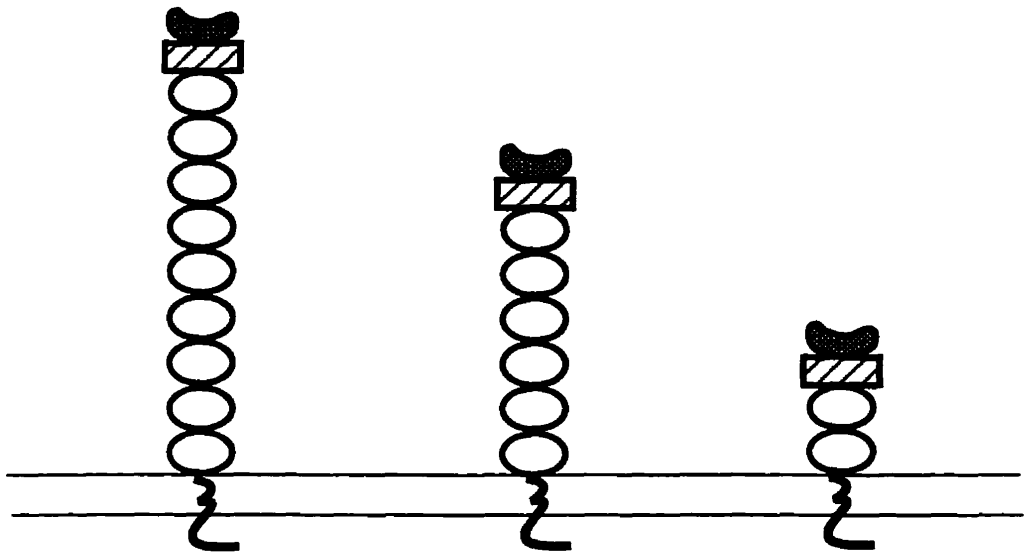

I-domain
Ligand binding region
α subunit
ß subunit
Ca++
Ca++
Ca++
Ca++
-S-S-
S-S
membrane
Calreticulin
ILK
Talin
α actinin
Vinculin
FAK
Actin cytoskeletion

Figure I.3: The Schematic Selectin Structure. The domain organizations of the three selectins are shown.  : C-type lectin domain;  : E G F domain;  :SCR (short consensus repeats).



Name	P-selectin	E-selectin	L-selectin
CD nomenclature	CD62P	CD62E	CD62L
Previous names	GMP-140, PADGEM	ELAM-1	LECAM-1, LCAM-1, $gp90^{MEL}$
ligands	PSGL-1 (P selectin glysoprotein ligand 1)	ESL-1 (E-selectin ligand 1)	GLYCAM-1, CD34, MADCAM-1
Distribution	α-Granules of platelets, megakaryocytes, endothelial cells, Weibel-Palade granules	On activated endothelial cells	On all circulating leukocytes, including subpopulations of lymphocytes
Regulation	Rapidly elicited to cell surface by thrombin, histamine, etc.; transcriptionally induced by cytokines	Transcriptionally induced by IL-1, TNF-α, lipopolysaccharide	Subject to complex regulation upon activation of leukocytes

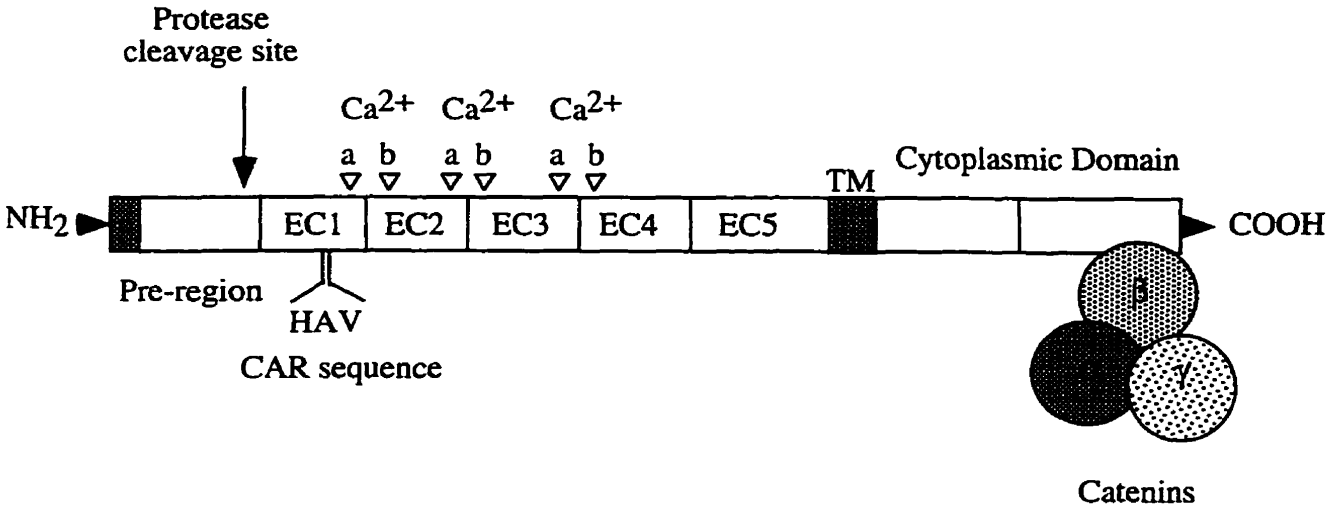
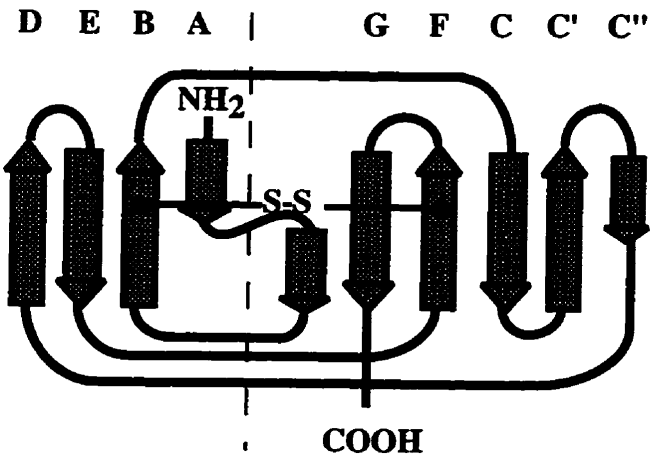


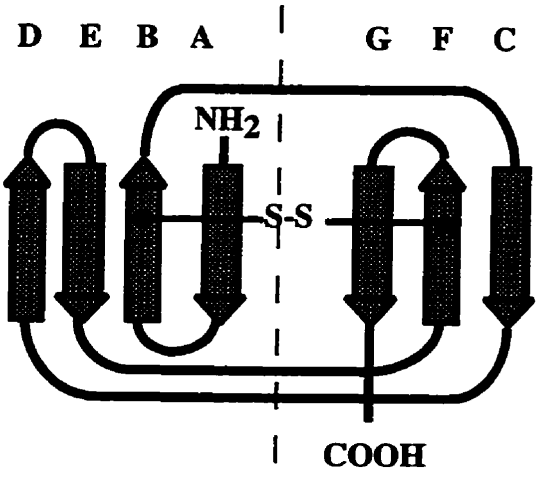
Figure I.4: Structure Organization of the Classical Cadherins. Classic cadherins are transmembrane proteins derived from a precursor by cleavage of the pre-region. The classical cadherins are composed of five extracellular cadherin repeats, one transmembrane (TM) domain and two cytoplasmic domains. The extracellular domain contains calcium-binding motifs (a: DXNDN and b: DXD sequence) and CAR: Cadherin Adhesion Recognition sequence (HAV). The cytoplasmic domain 2 contains the ß-catenin binding site.

Figure I.5: Topology diagrams of Ig-like domains. ß strands are represented by broad arrows pointing in the amino-to-carboxy direction, and their connecting loops by thin lines. Only the topological connectivity of the ß strands is shown, with no attempt to indicate the lengths of the strands or their connections. (A): Ig-like variable domain. (V domain); (B): Ig-like constant C1-set domain; and (C): Ig-like constant C2-set domain and FN-like type III domain. For C2-set Ig-like domain, the ß strand D is associated with the GFC sheet and is also referred as C' strand. In case of FNIII domain, no disulphide bond exists between two ß sheets. (D): Ig-like I-set domain. (The schematic drawings were modified from Williams et al., 1988; Harpaz and Chothia, 1994).

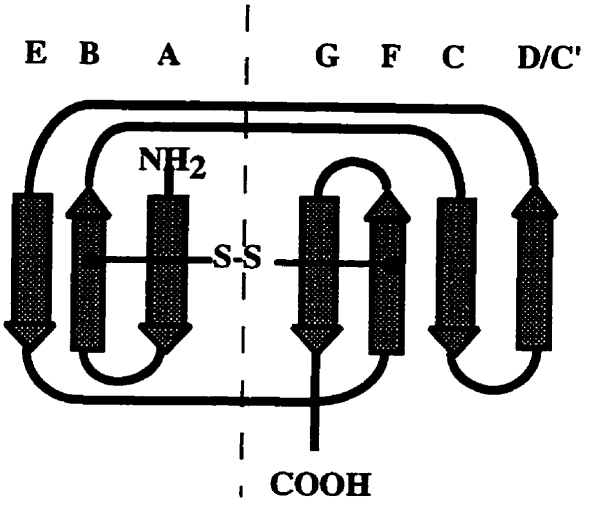
A


D E B A
G F C C' C"
NH2
S-S
COOH

B


D E B A
G F C
NH2
S-S
COOH

C


E B A
G F C D/C'
NH2
S-S
COOH

D

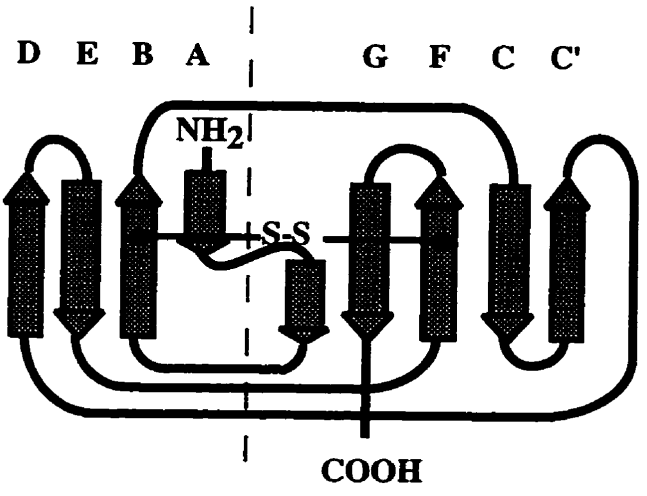

D E B A
G F C C'
NH2
S-S
COOH

Figure I.6: Peptide and domain structure of human cell adhesion molecule L1. A: The domain structure of cell adhesion molecule L1. L1 contains six Ig-like domains (represented by loops near the amino terminus (NH_2), followed by five fibronectin type III repeats (rectangle boxes), a transmembrane domain and a cytoplasmic tail near the carboxy terminus (COOH). The RGD sequence localized in the Ig-like domain 6 is shown, and the putative N-glycosylation sites are indicated. B: The peptide structure of cell adhesion molecule L1. The 135 kD and 80 kD components are derived from the 200 kD by proteolysis. The 135 kD contains all the six Ig-like domains and the first 2 and a half fibronectin type III domains, while the 80 kD component contains the remaining part of L1 molecule.

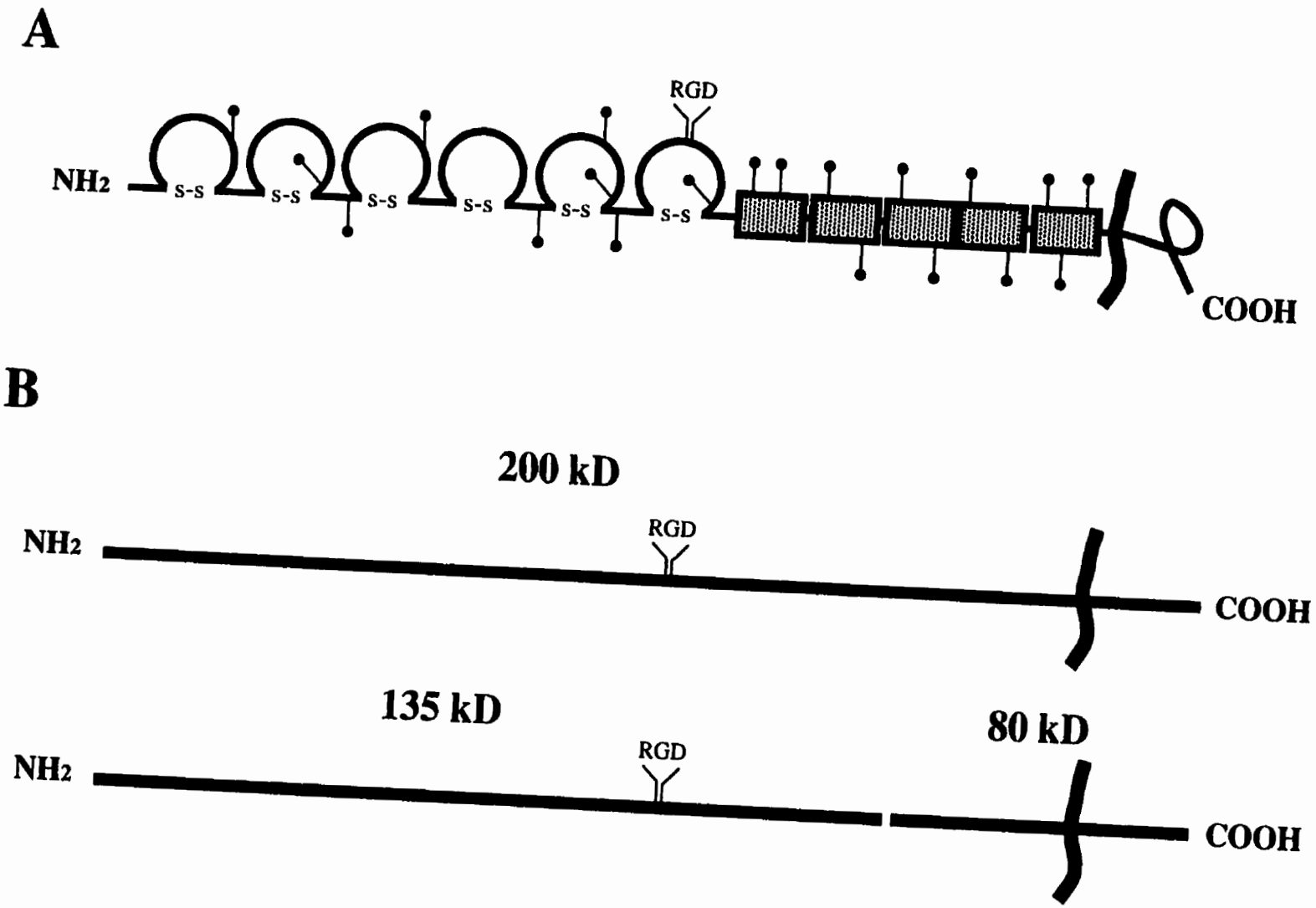

A
RGD
NH2
S-S
S-S
S-S
S-S
S-S
S-S
COOH
B
200 kD
NH2
RGD
COOH
135 kD
80 kD
NH2
RGD
COOH

Figure I.7: Alignment of the Ig-like domains of human cell adhesion molecule L1. All six Ig-like domains of L1 are aligned. Amino acids conserved in more than four domains are underlined, and amino acids conserved in all six domains were bolded.

```
Ig-1                                VITEQSPRRLVVFPTDDISLK
Ig-2                             AEGAPKWPKETVKPVEVEEGESVVLP
Ig-3                      KATNSMIDRKPRLLFPTNSSSHLVALQGQPLVLE
Ig-4                              YWLHKPQSHLYGPGETARLD
Ig-5                             AKILTADNQTYMAVQGSTAYLL
Ig-6                               QITQGPRSTIEKKGSRVTFT
```

```
          •     •     •                                                 • •
Ig-1      CEASGKPEVQF-R--WTR-DGVHFKPKEELGVTVYQSPHSGSFTITGNNSNFAQRFQGIYRC
Ig-2      CNPP--PSAEPLRIYWMNSKILHIKQDE--RVTMGQNGN--LYFANVLT----SDNHSDYIC
Ig-3      CIAEGFPT--PT-IKWR-PSGPMPA-D---RVT-YQNHNKTLQL---LKVG-EED-DGEYRC
Ig-4      CQVQGRPQ--PE-VTWRIN-GIPVEELAKD--QKYRIQRGAL--ILSN-VQ-PSD-TMVTQC
Ig-5      CKAFGAP-V-PS-VQWLDEDGTTVLQDERFFP--YANGT--LG-IRDLQ---AND-TGRYFC
Ig-6      CQASFDPSLQPS-ITWRG-DGRDLQELGDSD--KYFIEDGRLV-IHSLD---YSD-QGNYSC
          •     •     •                                                 • •
```

```
          •
Ig-1      FASNKLGTAMSHEIRLM
Ig-2      HAHFP-GTRTIIQKEPIDLRV
Ig-3      LAENSLGSARH-AYY-VTVEAAP
Ig-4      EARNRHGLLLANAYIYVVQLP
Ig-5      LAANDQNNVTIMANLKVKDAT
Ig-6      VASTELDVVESRAQLLVVGSPGP
          •
```

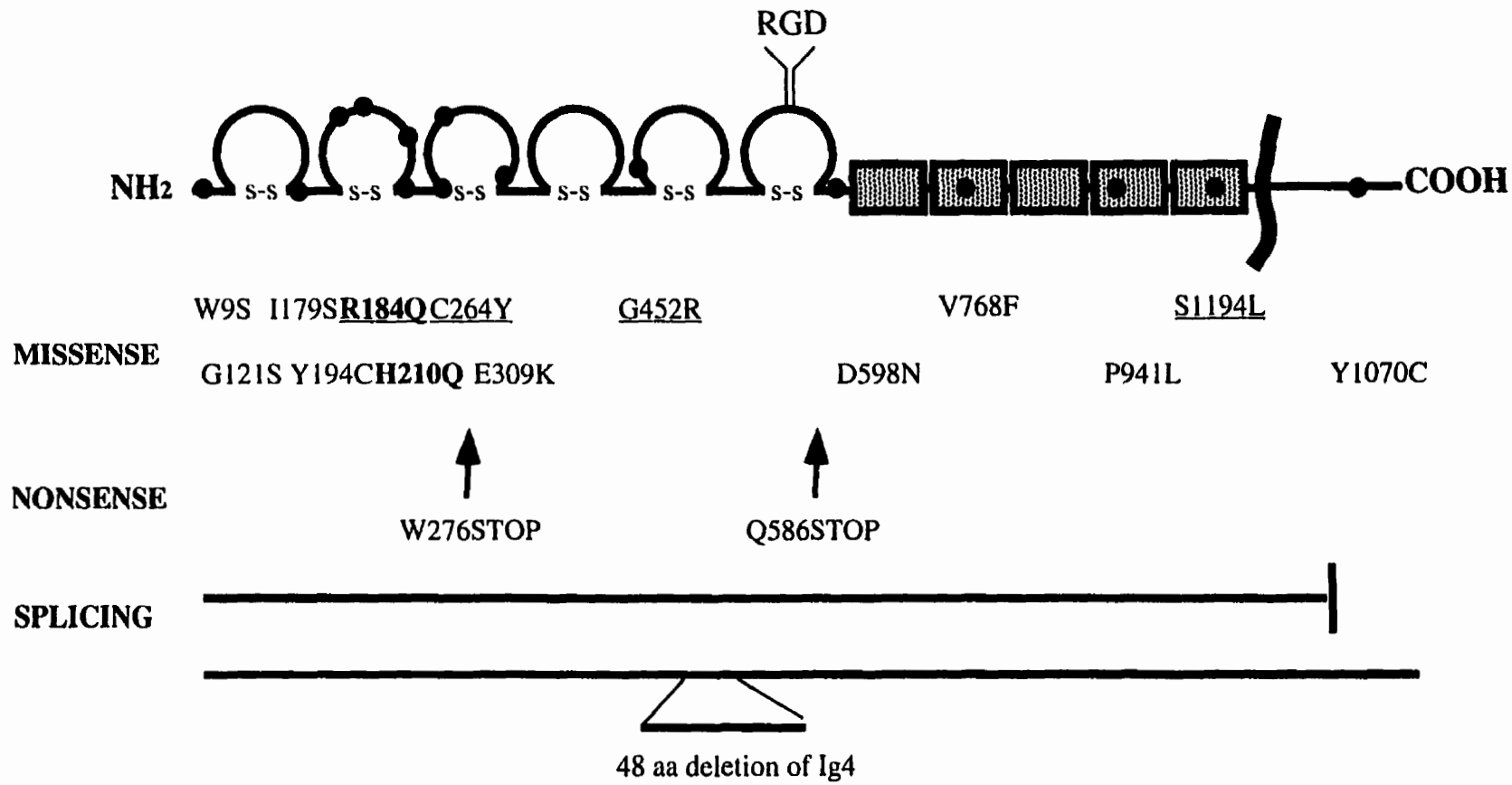


Figure I.8 List of L1 Mutations Implicated in Hydrocephalus and MASA Syndrome Some of the HSAS/MASA mutations are indicated in the map. Underlined mutations are found in Hydrocephalus patients.

CHAPTER TWO

COLOCALIZATION OF THE HOMOPHILIC BINDING SITE AND THE NEURITOGENIC ACTIVITY OF THE CELL ADHESION MOLECULE L1 TO ITS SECOND IG-LIKE DOMAIN

The contents of this chapter have been published in the

Journal of Biological Chemistry **270**:29413-29421 (1995).

I. INTRODUCTION

Intercellular adhesion is of prime importance in morphogenesis and maintenance of tissue integrity. This is especially important during neural development, when specific synaptic connections are established primarily by extension of axons along restricted pathways (Carpenter and Hollday, 1992; Martini, 1994). The molecular basis of these processes involves cell adhesion molecules and diffusible factors. In recent years, an increasing number of cell adhesion molecules have been found associated with the neuronal cell surface. These cell adhesion molecules have been categorized according to their structure into three major groups: the cadherins (Takeichi, 1991), the integrins (Reichardt and Tomaselli, 1991), and proteins of the immunoglobulin (Ig) superfamily (Rathjen and Jessel, 1991; Grumet et al., 1991).

The cell adhesion molecule L1 was first described as a 200 kDa transmembrane glycoprotein in the central nervous system, and it belongs to the Ig-superfamily (Lindner et al., 1983; Moos et al., 1988). L1 consists of six C2-type Ig-like domains in the amino-terminal region, followed by five fibronectin type III-like repeats, a transmembrane domain and a cytoplasmic domain (Moos et al., 1988). NILE in rat, NgCAM, G4 and 8D9 in chicken are the species homologues of mouse L1 (Grumet et al., 1984b; Rathjen et al., 1987; Lemmon and McLoon, 1986). L1 cDNAs have been cloned from mouse (Moos et al., 1988), rat (Miura et al., 1991; Prince et al., 1991), and human (Hlavin and Lemmon, 1991; Kobayashi et al., 1991). The L1 gene in human has been mapped to chromosome Xq28 (Chapman et al., 1990; Djabali et al., 1990). It has been reported that the X-linked hydrocephalus phenotype is associated with mutations in the L1 gene (Rosenthal et al., 1992; Camp et al., 1993; Jouet et al., 1994).

L1 can undergo homophilic binding as well as heterophilic interactions with several other cell adhesion molecules, such as NCAM(Kadmon et al., 1990a; Horstkorte et al., 1993), TAG-1/axonin-1 (Kuhn et al., 1991; Felsenfeld et al., 1994), F3/F11 (Brümmendorf et al., 1993), Glia (Grumet and Edelman, 1988; Lemmon et al., 1989), and the extracellular matrix protein laminin (Grumet et al., 1993). Some of these heterophilic interactions are known to modulate L1 functions. For instance, NCAM has been shown to undergo *cis*-interactions with L1, which in turn facilitates L1-L1 homophilic binding (Kadmon et al., 1990a). Neurocan, on the contrary, is able to bind to L1 and inhibit neuronal adhesion and neurite extension promoted by the L1 substrate (Friedlander et al., 1994).

L1 has been implicated in a wide range of neuronal cell differentiations. Substrate-coated L1 is a potent inducer of neurite outgrowth from a number of primary neurons (Hlavin and Lemmon, 1991; Williams et al., 1992; Seilheimer and Schachner, 1988). Axonal growth involves both adhesion and the transmission of extracellular signals into the interior of a growth cone to activate intracellular events (Brümmendorf and Rathjen, 1993; Doherty and Walsh, 1992). L1 appears to play an important role in this signal transduction process (Schuch et al., 1989; Williams et al., 1994b). A L1-Fc chimeric protein has been reported to induce protein tyrosine phosphorylation in neuronal cells (Williams et al., 1994a) as well as promote neurite outgrowth (Doherty et al., 1995), suggesting that the clustering of L1 molecules may trigger the signaling pathway leading to neurite extension.

It is therefore evident that the formation of adhesion complexes via L1 homophilic interactions may serve as an initiation point for many important signaling events. However, very little is known about the homophilic binding site of L1 and the

mechanism of its interaction. In this report, experiments were carried out to investigate the relationship between L1 homophilic binding and its neuritogenic activity. To identify these functional domains, we expressed fusion proteins containing various segments of the extracellular portion of L1, which were examined in several *in vitro* assays. We found that the second Ig-like domain of L1 was capable of binding to cell membrane-associated L1 as well as undergoing homophilic binding by itself. In addition, the Ig-like domain 2 of L1 was capable of promoting neurite outgrowth from retinal ganglion cells, suggesting an intimate relationship between L1 homophilic binding and L1-mediated neurite outgrowth.

II. EXPERIMENTAL PROCEDURES

A. Materials

The pGEX-3X plasmid and glutathione-Sepharose 4B were purchased from Pharmacia (Toronto, ON). The pQE-8 plasmid and nickel-nitrilotriacetic acid resin were purchased from QIAGEN (Chatsworth, CA). Covaspheres were obtained from Duke Scientific Corporation (Palo Alto, CA). Freund's adjuvant, trypsin, α-MEM, and N2-supplement were purchased from GIBCO (Toronto, ON). Poly-L-lysine and BSA were purchased from Sigma (St. Louis, MO). DiI was purchased from Molecular Probes (Eugene, OR). The BCA (bincinchoninic acid) protein assay kit was purchased from Pierce (Rockford, IL). Human L1 cDNA is a kind gift from Dr. Vance Lemmon (Case Western Unniversity, Cleveland, OH).

B. Construction of Expression Vectors

Standard recombinant DNA methods were followed in the construction of expression vectors (Sambrook et al., 1989). DNA fragments encoding different portions of L1 were obtained using appropriate restriction enzymes or PCR amplification. The cDNA fragment coding for the Ig-like domains 1, 2 and 3 (Ig1-2-3) between amino acid positions 24 to 351 (amino acid and nucleotide numbering according to Hlavin et al. (1991)) was released by *Nar* I at nucleotide position 113 and *Bam* HI at nucleotide position 1114, followed by treatment with Klenow enzyme and addition of *Eco* RI linkers at both ends. This fragment was subcloned into the unique *Eco* RI site of the pGEX-3X

vector for expression of the fusion protein GST-Ig1-2-3. The cDNA fragment coding for the Ig-like domains 4, 5 and 6 (Ig-4-5-6) between amino acid positions 352 and 595 was obtained by *Bam* HI digestion of a PCR product, which was generated using the forward primer 5'-GCCCCGGGACCATGGTCATCACGGAACAGTCT-3' and the reverse primer 5'-GCGAATTCTGGGATCCCGGCCCAGGGCTCCCCAC-3'. This fragment was subcloned into the unique *Bam* HI site of the pGEX-3X vector for expression of the fusion protein GST-Ig4-5-6. The fragment coding for all the five fibronectin type III-like repeats (Fn) between amino acid positions 596 and 1094 was amplified by PCR using the forward primer 5'-CCGGATCCCCATGGTGCCACGGCTGGTGCTG-3' and the reverse primer 5'-GGGAATTCTGAGATCTAGGGAGCCTCACGCGGCC-3'. The PCR product was digested by *Eco* RI and *Bam* HI and then subcloned into these two sites of pGEX-3X to create GST fusion proteins, such that GST was linked to the amino-terminus of all three fusion proteins. DNA fragments coding for Ig-like domains 1 and 2 (Ig1-2) between amino acid positions 17 and 209), Ig-like domain 1 (Ig1) between amino acid positions 17 and 113, and Ig-like domain 2 (Ig2) between amino acid positions 114 and 209 were also obtained by PCR amplification. Oligonucleotide primers 5'-AGGGATCCGTCATCACGGAACAGT-3' and 5'-GTGGATCCGACCCGGAGGTCAA TG-3' were used for generating the Ig1-2 cDNA, primers 5'-AGGGATCCGTCATCACG GAACAGT-3' and 5'-CGGAATTCCATGAGCCGGATCTCA-3' for Ig1 cDNA, and primers 5'-TGGGCGCCTCATGGCCGAGGGTGC-3' and 5'-GTGGATCCGACCCGGA GGTCAATG-3' for Ig2 cDNA. These DNA fragments were either digested with *Bam* HI or ligated with *Bam* HI linkers. They were then subcloned into the *Bam* HI site of the pQE-8 plasmid which was designed to fuse a His_6-tag to the amino-terminus of the recombinant protein. The nucleotide sequences of these inserts were confirmed by

double-stranded DNA sequencing using a T7 Sequencing™ kit (Phamacia, Ont. Canada). The *E. coli* strain JM101 was used for the transformation of pGEX-derived plasmids and the *E. coli* strain M15 for the transformation of pQE-derived plasmids.

C. Expression and Refolding of Recombinant L1 Fusion Proteins

To produce GST-L1 fusion proteins, transformed *E. coli* cells were grown at 37° C in 250 ml LB medium with 100 µg/ml ampicilin. Protein synthesis was induced by adding 1 mM isopropyl ß-D-thiogalactopyranoside when A_{600} reached 0.6 - 0.8. Cells were collected 3 h after induction by centrifugation at 4,000 x *g* for 10 min. The pellet was resuspended in 10 ml lysis buffer (50 mM Hepes buffer, pH 7.9, 5% glycerol, 2 mM EDTA, 0.1 M DTT). The fusion protein was isolated as inclusion bodies by sonication of the cell suspension on ice, followed by centrifugation for 10 min at 10,000 x *g* at 4° C. Inclusion bodies were washed two times using washing buffer (50 mM Hepes, pH 7.9, 5% glycerol, 2 mM EDTA, 0.1 M DTT, 0.05% deoxycholic acid and 1% Triton X-100) followed by washing once with lysis buffer. Then they were solubilized by overnight incubation in 10 ml of 6 M guanidine buffer (10 mM Hepes, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, and 10 mM DTT) at 4° C. After centrifugation at 10,000 x *g* for 15 min to remove the insoluble material, the supernatant was slowly diluted by adding 40 ml dilution buffer (10 mM Hepes, pH 7.9, 0. 2mM EDTA, 2 mM DTT) and was allowed to sit at 4° C overnight. Then the denaturant and reducing reagents were slowly removed by dialyzing against the storage buffer (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM EGTA). The refolded GST-fusion proteins were passed through a glutathione-Sepharose 4B column according to the manufacturer's protocol. Eluted

proteins were dialyzed against PBS at 4° C. His-Ig1-2, His-Ig1, and His-Ig2 were purified and refolded as discribed previously (Rao et al., 1994). In brief, His-tagged proteins were purified under denaturing conditions following the manufacturer's description. Recombinant proteins were then pooled and adjusted to ~2mg/ml. They were completely reduced by first adding ß-mercaptoethanol to a final concentration of 0.1M and then incubated at 37° C for 90 min. To reoxidise, His-tagged proteins were dialysed against 8 M urea, 150 mM NaCl, 50 mM Tris/HCl, pH 8.0 at 4° C overnight. Samples were diluted 1:20 in PBS and concentrated by ultrafiltration followed by dialysis against 20 mM Tris-HCl, pH 7.5, overnight to reduce the denaturant.

D. Preparation of Domain-specific Antibodies

Polyclonal antibodies were raised in rabbits against the purified and refolded GST-fusion proteins: GST-Ig1-2-3, GST-Ig4-5-6, and GST-Fn. 100 µg protein was used in the first immunization, 50 µg protein was used in three subsequent boosts at two-week, two-week and four-week intervals. Serum samples were taken one week after each boost. To prepare acetone powder of GST-expressing bacteria for absorption of antisera, *E. coli* cells transformed with pGEX-3X were induced to synthesize GST for 3 h, and then collected and resuspended in 2 ml of saline (0.9% NaCl). The cell/saline suspension was kept on ice for 5 min. Then 8 ml of acetone (-20° C) was added and mixed vigorously. The acetone suspension was kept on ice for 30 min and the precipitate was collected by centrifugation at 10,000 x *g* for 10 min. The supernatant was discarded and the pellet was resuspended with fresh acetone and mixed vigorously. The mixture was kept on ice for another 10 min. The pellet was collected and air-dried at room temperature. The dried

acetone powder was added to the antiserum at a final concentration of 1% (w/v) and the mixture was incubated at 4° C overnight with gentle rotation. After spinning at 10,000 x *g* for 10 min, the supernatant was dialyzed and passed through an Econo-Pac™ Serum IgG Purification Column (Bio-Rad) according to the manufacturer's instruction.

E. Covasphere-to-Substratum Attachment Assay

Green or red MX Covaspheres (50 µl) were briefly sonicated before the addition of 10 µg of fusion protein and the final volume was adjusted to 100 µl with PBS. After rotation at room temperature for 75 min, the Covaspheres were centrifuged at 10,000 x *g* for 10 min at 4° C. The pellet was resuspended in 1 ml of PBS containing 1% BSA for 15 min to block the remaining active sites on the Covaspheres. The Covaspheres were pelleted and resuspended in 50 µl PBS.

The Covaspheres-to-substratum attachment assay was carried out as described previously (Rao et al., 1994). Recombinant proteins (5 µl of a 1 µM solution) or anti-domain IgG (5 µl at 10 µg/ml) in PBS was used to coat spots (~2.5 mm in diameter) on 35-mm plastic Petri dishes at room temperature for 1 h, followed by blocking with 1% BSA in PBS for 30 min. Then 2 µl of fusion protein-conjugated Covaspheres were added to 100 µl of PBS and dispersed with sonication for 5 min in a chilled water bath sonicator. Samples of the diluted Covaspheres (30 µl each) were added to the coated spots and incubated at room temperature for 30 min. After 5 gentle washes with PBS, the binding of Covaspheres to the substratum was observed by epifluorescence microscopy and images were recorded on video tapes. The relative amounts of bound Covaspheres were calculated by counting the number of Covaspheres per unit area. In competition

experiments, Covasperes were incubated with competitors at room temperature for 10 min before the binding assay.

F. Covasphere-to-Cell Binding Assay

Neural retinal cells from day 6 chick embryos were isolated as described previously (Rao et al., 1993) and seeded sparsely on coverslips in N2 medium. Cells were kept in culture for 5 to 6 h at 37° C. Coverslips were then blocked with 1% BSA in HBSS for 5 min at room temperature. In inhibition studies, inhibitors at different final concentrations were incubated with cells for 25 min at room temperature after blocking with BSA. After the removal of excess inhibitor and one gentle wash with HBSS, 100 µl of fusion protein-conjugated Covaspheres were added on to the coverslip. All coverslips were incubated for 45 min at room temperature on a platform shaker. After washing several times with PBS, cells were observed using epifluorescence microscopy. Cells with more than 5 Covaspheres attached on the surface were scored positive and generally 100-200 cells were scored for each coverslip.

G. Neurite Outgrowth Assay

Neural retinal cells from day 5 chick embryos were isolated as described previously (Sandig et al., 1994). Round glass coverslips (12-mm diameter) were coated with 0.01% (w/v) poly-L-lysine at room temperature for 3 h. After washing three times with distilled water, 80 µl samples of recombinant proteins at 1 µM were used to coat the coverslips overnight at 4° C. To determine the efficiency of protein coating, protein

adsorbed to coverslips was solublized by incubation in 1% SDS (10 µl) at 37° C for 10 min. The relative amounts of bound and unbound protein were determined using the BCA protein assay. Protein samples were adjusted to a final volume of 50 µl and transferred to a 96-well plate. Then 200 µl working reagent was added to each sample. After incubation at 37° C for 2 h, colour development was stopped by rapid cooling and absorbance at 570 nm was measured using a microtiter plate reader. Approximately 65% of the input protein was found adsorbed to the substratum, and similar results were obtained for both GST-fusion proteins and His-tagged proteins.

The unbound protein was removed by washing with distilled water and the coverslips were blocked with 1% BSA in α-MEM at room temperature for 30 min. These coverslips were then transfered in 24-well Linbro plates and retinal cells suspended in N2 medium were seeded on top of them. Rat L1 protein was either adsorbed on to a nitrocellulose substrate according the method of Lagenaur and Lemmon (Lagenaur and Lemmon, 1987) or on to a poly-L-lysine substrate. In inhibition studies, Fab or recombinant proteins were used to precoat either the substratum or the retinal cells at final concentrations of 250 µg/ml and 40 µg/ml, respectively. Neurite extension was allowed to proceed for 16-18 h. Retinal cells were fixed for 20 min in 3.7% formaldehyde in PBS by gradually replacing the culture medium with the fixative. After three washes with PBS, coverslips were mounted in vinol, containing 1,4-diazabicyclo(2,2,2,) octane and *p*-phenylenediamine to retard photobleaching. Samples were examined by epifluorescence microscopy. Only cells with neurites longer than the cell body width were scored. Consistently, 15-20% of retinal cells sent out neurites. Retinal ganglion cells bearing neurites were recorded on video tapes. The length of ~100 neurites were measured in each experiment.

III. RESULTS

A. Expression and Refolding of GST-Fusion Protein

To investigate the structure/function relationships of L1, cDNA fragments encoding three different extracellular segments of L1 were fused to glutathione-S-transferase (GST) for expression in *E. coli* (Figure II.1A). These three GST-fusion proteins contained Ig-like domains 1, 2, 3 (GST-Ig1-2-3), Ig-like domains 4, 5, 6 (GST-Ig4-5-6) and fibronectin type III-like domains 1 to 5 (GST-Fn), respectively. All three GST-fusion proteins were isolated from inclusion bodies. The inclusion bodies were solubilized in 6 M guanidine-HCl in the presence of 10 mM DTT and 2 mM EDTA. The denatured proteins were refolded by dialysis and by gradually decreasing the concentration of denaturant and reducing agents. The refolded proteins were further purified by passing through a glutathione-4B Sepharose column. Fusion proteins were eluted by glutathione and dialysed against PBS. To estimate the purity of the refolded fusion proteins, samples were subjected to SDS/polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining (Figure II.1B). Under reducing conditions, fusion proteins migrated with apparent *M*r of 65,000, 55,000, and 85,000 for GST-Ig1-2-3, GST-Ig4-5-6 and GST-Fn, respectively. These values corresponded closely to the expected molecular size of these fusion proteins. Several minor bands of lower molecular size were observed in these protein preparations. Since these bands were recognized by anti-GST antibodies in Western blots, they were probably due to partial degradation of the recombinant proteins. The yield of refolded fusion protein was ~5 mg/L of bacteria culture in all three cases.

To obtain L1 domain-specific antibodies, rabbits were immunized with the purified fusion proteins. The antisera were absorbed against acetone powder to remove antibodies that recognized bacterial protein and the GST moiety of these fusion proteins. The IgG fraction was isolated from each antiserum to obtain L1 domain-specific antibodies. Western blots were carried out using these purified IgG to ensure that they did not cross-react with the other two fusion proteins (Figure II.2).

B. Binding of Fusion Protein-conjugated Covaspheres to Substrate-Coated Proteins

To determine which fusion protein contained the L1 homophilic binding activity, a Covasphere-to-substratum attachment assay was developed. Fusion proteins were conjugated to Covaspheres, which were tested for their ability to adhere to different substrate-coated proteins. As a positive control, IgG that recognized specific L1 segments was adsorbed on to Petri dishes for Covasphere binding (Figure II.3a). Covaspheres were also incubated on substrate-coated GST to monitor background resulting from GST-to-GST interactions (Figure II.3b). Covaspheres conjugated with GST-Ig1-2-3 attached very well to the GST-Ig1-2-3 substrate, whereas the level of binding on GST-Ig4-5-6 was close to background level (Figure II.3c, d). These results indicate that the fusion protein GST-Ig1-2-3 is capable of undergoing homophilic binding.

The relative percentages of Covaspheres bound per unit area were estimated by normalizing the results to the level of Covasphere binding in the IgG-coated substrate (Figure II.4). The amount of GST-Ig1-2-3-conjugated Covaspheres bound to the GST-Ig1-2-3 substrate was ~25-fold higher than that attached on the GST substrate. In

contrast, binding of GST-Ig4-5-6-conjugated Covaspheres to the GST-Ig4-5-6 substrate was at the background level (Figure II.4). In the case of GST-Fn-conjugated Covaspheres, a higher background level of binding to the GST substrate was observed, but there was no significant difference between the level of binding to the GST-Fn substrate and the level of binding to GST (Figure II.4). Furthermore, GST-Ig1-2-3-conjugated Covaspheres did not attach to substrate-coated GST-Ig4-5-6 or GST-Fn (data not shown). It was evident that the Ig-like domains 1, 2, 3 did not interact with other extracellular segments of L1. These results thus demonstrate that the L1 homophilic binding site resides within the first three Ig-like domains, and that the last three Ig-like domains and the fibronectin domains may not be directly involved in L1 homophilic interactions.

The dose effect of substratum-associated GST-Ig1-2-3 on Covasphere binding was also examined. When equal amounts of Covaspheres were loaded onto substrates coated with different concentrations of GST-Ig1-2-3, Covasphere attachment was found to be dose-dependent and maximal binding was achieved when the substratum was coated with 1 μM GST-Ig1-2-3 (Figure II.5A). When binding was carried out on a GST-coated substratum, no significant binding was observed up to a concentration of 5 μM.

To demonstrate the specificity of Covasphere binding, competition experiments were carried out using either anti-Ig1-2-3 Fab or soluble GST-Ig1-2-3. Anti-Ig1-2-3 Fab blocked the binding of GST-Ig1-2-3-conjugated Covaspheres to substrate-coated GST-Ig1-2-3 in a dose-dependent manner (Figure II.5B). Fifty percent inhibition was achieved at ~35 nM of anti-Ig1-2-3 Fab. However, neither anti-Ig4-5-6 Fab nor goat-anti-mouse Fab had significant inhibitory effects up to a concentration of 5 μM. The attachment of GST-Ig1-2-3-conjugated Covaspheres to substrate-coated GST-Ig1-2-3 was also inhibited

by soluble GST-Ig1-2-3 (Figure II.5C). The inhibition was dose-dependent and 50% inhibition was achieved at ~80 nM soluble GST-Ig1-2-3. In contrast, the attachment of GST-Ig1-2-3 Covaspheres to GST-Ig1-2-3 substrate was not affected by GST or GST-Ig4-5-6 even at a concentration of 3 μM.

C. Localization of the Homophilic Binding Site to Ig-like Domain 2 of L1

To identify the domain which contains the homophilic binding site, smaller fragments of the extracellular portion of L1 were expressed as fusion proteins using the pQE expression vectors. All these recombinant proteins contained 6 histidine residues at the amino-terminus (Figure II.6A). His-Ig1-2 contained the first two Ig-like domains of L1, His-Ig1 and His-Ig2 contained the first and the second Ig-like domain, respectively. The recombinant proteins were solubilized using 8 M urea and purified by passing through a Ni-resin column, which specifically bound the His-tag incorporated in the recombinant proteins. Recombinant proteins were eluted in low pH buffer, followed by the renaturing steps described in Materials and Methods. Refolded proteins were analyzed by SDS/polyacrylamide gel electrophoresis and they were at least 90% pure (Figure II.6B).

The fusion proteins were used to coat Petri dishes and then assayed for their ability to bind GST-Ig1-2-3-conjugated Covaspheres. A large number of GST-Ig1-2-3-conjugated Covaspheres attached to the His-Ig1-2 substrate, suggesting that the third Ig-like domain of L1 is not needed for homophilic interactions. To determine which of the first two Ig-like domains was involved in homophilic binding, Covaspheres were deposited on substratum coated with either His-Ig1 or His-Ig2. When recombinant

proteins containing a single Ig-like domain were tested, Covaspheres attached to the His-Ig2 substrate, but not to the His-Ig1 substrate (Figure II.7A), suggesting that it is Ig2, and not Ig1, that is directly involved in L1-L1 binding. Consistent with this observation, His-Ig2 was able to function as a competitor to displace GST-Ig1-2-3-conjugated Covaspheres in the attachment assay. In the presence of His-Ig2, GST-Ig1-2-3 homophilic interaction was inhibited. Only residual binding (~5%) was observed when binding was carried out in the presence of 10 μM soluble His-Ig2 (Figure II.7A). In contrast, a relative level of 70% binding was retained when the same concentration of His-Ig1 was included in the assay.

Whether His-Ig2 was able to interact with L1 molecules expressed by neural retinal cells was also examined. Retinal cells were isolated from day 6 chick embryos and cultured on coverslips. His-Ig2-conjugated Covaspheres were deposited on top of these cells and the number of cells showing positive Covasphere binding was estimated. About 40% of retinal cells were decorated with His-Ig2-conjugated Covaspheres, whereas binding of His-Ig1-conjugated Covaspheres to these cells was at the background level (Table II.1). To demonstrate that His-Ig2 was interacting with L1 molecules on the surface of retinal cells, cells were first incubated with either soluble His-Ig2 or anti-Ig1-2-3 Fab. After removal of the excess protein, His-Ig2 conjuaged Covaspheres were placed on top of the precoated cells. Both soluble His-Ig2 and anti-Ig1-2-3 Fab were able to block the binding of Covaspheres to retinal cells and the inhibition was dose-dependent. The data thus indicate that His-Ig2 was binding to L1 molecules on the surface of retinal cells.

To determine whether Ig2 can bind to Ig2, His-Ig2-conjugated Covaspheres were assayed for their ability to attach to substrate-coated His-Ig2. Binding of Covaspheres

was observed on the His-Ig2 substrate, but not on GST (Figure II.7B). Positive results were also obtained when these Covaspheres were deposited on substratum coated with either GST-Ig1-2-3 or His-Ig1-2. However, His-Ig2-conjugated Covaspheres did not bind to substrate-coated His-Ig1. These results are consistent with the notion that L1-L1 binding is mediated by homophilic interactions between the second Ig-like domains of two apposing L1 molecules.

D. Promotion of Neurite Outgrowth by L1 Fusion Proteins

L1 as a substrate has been found to be a potent neurite outgrowth promoter (Hlavin and Lemmon; 1991; Williams et al., 1992; Seilheimer and Schachner, 1988). To determine which fusion protein contained the stimulatory activity of L1-dependent neurite outgrowth, neural retinal cells were isolated from day 5 chick embryos and cultured on top of substrate-coated coverslips. The protein coating efficiency on coverslips has been determined to be similar (Figure II.8). The length of neurites extending from retinal ganglion cells was measured after 18 h. Relatively long neurites were observed in cells cultured on the GST-Ig1-2-3 substrate (Figure II.9a). However, only short neurites were observed on substratum coated with either GST, GST-Ig4-5-6, or GST-Fn (Figure II.9b, c).

Quantitative analysis showed that the majority of neurites (>80%) extending from retinal cells cultured on GST-Ig4-5-6, GST-Fn or GST were <25 μm, with mean neurite lengths ranging between 15 and 20 μm. (Figure II.10). In contrast, retinal cells cultured on top of GST-Ig1-2-3 sent out much longer neurites, with a wider range of size distribution (Figure II.10A). Approximately 90% of them were >25 μm. As a positive

control, retinal cells were cultured on rat L1-coated substratum. The patterns of neurite length distribution for GST-Ig1-2-3 and intact L1 were almost identical (Figure II.10A), and their mean neurite lengths were 42.3 μm and 47.4 μm, respectively (Figure II.10B). The data indicated that GST-Ig1-2-3 retained most of the neuritogenic activity of native L1. In comparison to substratum coated with GST where cells yielded a mean neurite length of 13 μm, retinal cells cultured on the GST-Ig1-2-3 substrate extended neurites with a 3-fold increase in their average length, whereas GST-Ig4-5-6 and GST-Fn did not lead to a significant increase in neurite outgrowth over the GST control.

Competition experiments were carried out using either soluble fusion protein or anti-Ig1-2-3 Fab. When retinal cells were cultured in the presence of soluble GST-Ig1-2-3, neurite outgrowth was reduced to the background level. The pattern of neurite length distribution was similar to that of cells cultured on GST (Figure II.11). Similar inhibitory effects were observed when cells were cultured in the presence of anti-Ig1-2-3 Fab. In both cases, the active L1 sites on retinal cells and substratum were blocked by the competitor. Nevertheless, the number of cells attached to the coverslip did not decrease, suggesting that the anchorage of cells to the substratum per se was not sufficient to promote neurite outgrowth and that neurite outgrowth was dependent on L1-L1 interactions.

E. Localization of the Neuritogenic Activity of L1 to Ig-like Domain 2

To further narrow down the segment of L1 polypeptide that harbored the neuritogenic activity, the effects of soluble His-Ig1-2, His-Ig1 and His-Ig2 on neurite outgrowth were examined using neural retinal cells cultured on substrate-coated GST-

Ig1-2-3. His-Ig1 had relatively little effect on neurite outgrowth. However, the neurite outgrowth promotion activity of the substrate was abolished in the presence of His-Ig1-2 and His-Ig2 (Figure II.12). These results suggest that the second Ig-like domain also contains the neuritogenic activity of L1.

To directly demonstrate that the second Ig-like domain of L1 was capable of promoting neurite outgrowth, retinal cells were cultured on top of substrate-coated His-Ig2 (Figure II.13). Relatively long neurites were extended by retinal ganglion cells cultured on top of the His-Ig2 substrate, with a mean neurite length of 52 μm (Figure II.13B). Similar results were obtained when cells were cultured on substrate-coated His-Ig1-2. Their length distribution patterns were similar to that of cells cultured on GST-Ig1-2-3 (Figure II.13A). In contrast, only short neurites were found on the His-Ig1 substrate, which yielded a mean neurite length of 18.5 μm. These results thus indicate that the second Ig-like domain alone is sufficient to promote neurite outgrowth from retinal cells.

IV. DISCUSSION

Homophilic interactions between L1 molecules not only result in cell-cell adhesion, but also elicit neurite outgrowth (Horstkort et al., 1993; Lemmon et al., 1989). In this report, we first focused on mapping the L1 homophilic binding site. Fusion proteins containing different segments derived from the extracellular region of L1 were assayed for homophilic binding activity. Only fusion proteins that contained Ig-like domain 2 were capable of undergoing homophilic interactions, suggesting that the homophilic binding of L1 resides within its Ig-like domain 2. Since these fusion proteins do not interact with each other, it becomes evident that Ig-like domain 2 interacts directly with Ig-like domain 2 on an apposing L1 molecule. The initial interactions centered at Ig-like domain 2 may lead to subsequent interactions at other secondary sites on L1, further stabilizing the homophilic binding reaction. It is of interest to note that Ig-like domain 2 has the greatest interspecies sequence similarity among the extracellular domains of L1 (Hlavin and Lemmon, 1991). This probably accounts for the ability of L1 to mediate homophilic interactions among several vertebrate species (Hlavin and Lemmon, 1991).

A similar binding mechanism has been reported for NCAM, which also mediates cell-cell adhesion by homophilic interactions (Hoffman and Edelman, 1983; Grumet et al., 1982; Edelman, 1988; Peck and Walsh, 1993). The homophilic binding site of NCAM has been mapped to a decapeptide sequence (KYSFNYDGSE) within its third Ig-like domain (Rao et al., 1992), corresponding to the C' ß-strand and the C'-E loop of the Ig fold (Rao et al., 1993; Rao et al., 1992). The charged residues as well as the aromatic side-chains appear to play a crucial role in NCAM homophilic binding (Rao et al., 1993).

The NCAM homophilic binding sequence is unique to Ig-like domain 3 and it probably interacts isologously with the same sequence on NCAM present on apposing cells (Rao et al., 1994). A similar strategy of binding is used by the cell adhesion molecule gp80 in *Dictyostelium discoideum* (Siu et al., 1985; Siu et al., 1987; Kamboj et al., 1988; Siu and Kamboj, 1990). gp80 is a primitive member of the Ig superfamily of recognition molecules (Siu and Kamboj, 1990; Matsunaga and Mori, 1987), and it mediates cell-cell adhesion in a Ca^{2+}-independent manner. The homophilic binding site has been mapped to an octapeptide sequence (YKLNVNDSI) which is also predicted to adopt a ß-strand conformation followed by the beginning of a ß-turn structure (Kamboj et al., 1989). As in NCAM, both the amino-terminal Tyr residue and the two internal charged residues are vital to the homophilic binding activity of gp80. Furthermore, the homophilic binding site of gp80 is capable of undergoing isologous interaction with the same sequence in an anti-parallel manner (Kamboj et al., 1989).

The exact location of the homophilic binding site within Ig-like domain 2 of L1 is not yet known. However, two point mutations within this domain have been implicated in X-linked hydrocephalus and mental retardation. One of the mutations resulted in the substitution of Arg-184 with Gln, while the other mutation substitutes Gln for His-210 (Jouet et al., 1994). Both mutations may affect the folding of the Ig-like domain 2, resulting in the abolition or reduction of the affinity of L1 homophilic interactions. It is of particular interest to note that Arg-184 lies within a region corresponding to the predicted C' ß-strand of the Ig fold (Hlavin and Lemmon, 1989), suggesting that Arg-184 and its flanking sequences may participate in L1 homophilic binding in a manner similar to the C' strand in Ig-like domain 3 of NCAM.

Whereas L1 and NCAM undergo homophilic binding via interactions between two identical domains, the carcinoembryonic antigen (CEA), which is also member of the Ig superfamily, adopts a heterologous binding mechanism. This involves the reciprocal interactions between the amino-terminal Ig-like domain of one molecule and an internal Ig-like domain of the apposing molecule (Zhou et al., 1993). Since the Ig superfamily consists of a great variety of recognition molecules, it is conceivable that different mechanisms may be utilized in the adhesive processes mediated by different molecules. It remains to be determined whether the two mechanisms utilized by L1/NCAM and CEA are widely adopted by other members of the Ig superfamily.

In addition to being able to undergo homophilic binding, the Ig-like domain 2 of L1 is a potent inducer of neurite outgrowth. Our results showed that the Ig-like domains 3 to 6 and all five fibronectin type III repeats did not promote neurite outgrowth. In contrast, Appel et al. (1993) reported that L1 fusion proteins containing Ig-like domains 1-2, 3-4, 5-6, or fibronectin type III repeats 1-2 were all capable of promoting neurite outgrowth from small cerebellar neurons. Interestingly, a more recent study on Ng-CAM, a chicken homolog of L1, showed that only the fourth and fifth fibronectin-like domains of NgCAM were required for stimulating neurite outgrowth from dorsal root ganglia cells (Burgoon et al., 1995). It is possible that, depending on the relative levels of endogenous L1 and L1 receptors, different types of primary neurons may respond differently to these external peptide substrates. These apparently conflicting results may also reflect the complexities involved in neurite outgrowth.

It should be pointed out that the second Ig-like domain alone is sufficient to stimulate neurite outgrowth. Since the potency of His-Ig-2 in our neurite outgrowth assay was comparable to that of intact L1, the other structural domains of L1 do not seem to be

required in the initial step of activating the neurite outgrowth pathway. Our results suggest that an intimate relationship exist between L1 homophilic binding and L1-induced neurite outgrowth. Similar observations have been made when retinal cells were cultured on top of a monolayer of NCAM-expressing L cell transfectants. Here retinal neurons extend much longer neurites than those cultured on control cells (Sandig et al., 1994). However, mutations in the NCAM homophilic binding site abrogates the ability of NCAM to stimulate neurite outgrowth (Sandig et al., 1994).

Lemmon et al. (1989) have shown that L1 stimulated neurite outgrowth via a homophilic binding mechanism. It is therefore conceivable that the substrate-coated His-Ig2 may interact with the Ig-like domain 2 of L1 on cells, which in turn generates neurite outgrowth signals either by inducing conformational changes in the L1 molecules or by altering L1 interactions with neighboring membrane and cytoplasmic components. Direct association between the cytoplasmic domain of L1 and ankyrin has been reported (Davis and Bennett, 1994). The cytoplasmic domain of L1 has also been found to associate with both protein kinase C and non-protein kinase C activities and it can be phosphorylated (Sadoul et al., 1989; Bixby and Jhabvala, 1990). Both tyrosine phosphorylation and Ca2+ influx have been found to be key steps in the signaling pathways initiated by cell adhesion molecules (Williams et al., 1992; Williams et al., 1994b; Doherty et al., 1995).

A recent study (Doherty et al., 1995) showing that L1 clustering by a soluble bivalent L1-Fc chimeric protein leads to an increase in neurite outgrowth is consistent with the notion that L1-L1 homophilic interaction serves as the first step in the signaling cascade that leads to neurite extension. It is of interest to note that NCAM behaves somewhat differently in this respect. We have previously found that a synthetic peptide which contains the NCAM homophilic binding site within a 21-amino acid sequence is a

potent inducer of neurite outgrowth from retinal cells (Sandig et al., 1994). This suggests that while NCAM homophilic binding is required, clustering of NCAM molecules may not be essential.

The signaling cascade involved in L1-dependent neurite outgrowth is a subject of considerable debate. Several recent reports implicate an essential role for the FGF receptor in neurite outgrowth induced by several cell adhesion molecules, including L1, NCAM and N-cadherin (Williams et al., 1994a; Doherty et al., 1995). A similar neurite outgrowth response can be elicited by treatment with basic FGF (Williams et al., 1994c), suggesting the L1-L1 binding may lead to the activation of FGF receptor. Since both the L1-Fc chimera and basic FGF induce increases tyrosine phosphorylation on a common set of neuronal proteins (Williams et al., 1994a), a common signaling pathway has been postulated for all cell adhesion molecule-dependent neurite outgrowth (Williams et al., 1994a; Williams et al., 1994c). On the other hand, studies using *src* or *fyn* knock-out mice have indicated that $pp60^{c\text{-}src}$ is an essential component of the intracellular signaling pathway in L1-mediated neurite outgrowth (Ignelzi et al., 1994). Whereas the L1 response is dependent on the nonreceptor tyrosine kinase $pp60^{c\text{-}src}$, NCAM-stimulated neurite outgrowth is dependent on $p59^{fyn}$, since neuronal cells derived from *fyn*-minus mice fail to respond to NCAM (Beggs et al., 1994). These results argue for the involvement of distinctly different components in the early steps of signaling pathways induced by L1 and NCAM. However, the nature of the association between either L1 and *src* or NCAM and *fyn* is still not known. Similarly, there is no direct evidence to demonstrate physical interactions between L1 and FGF receptor. Future experiments to address this and related issues will be required to resolve the discrepancy between these models.

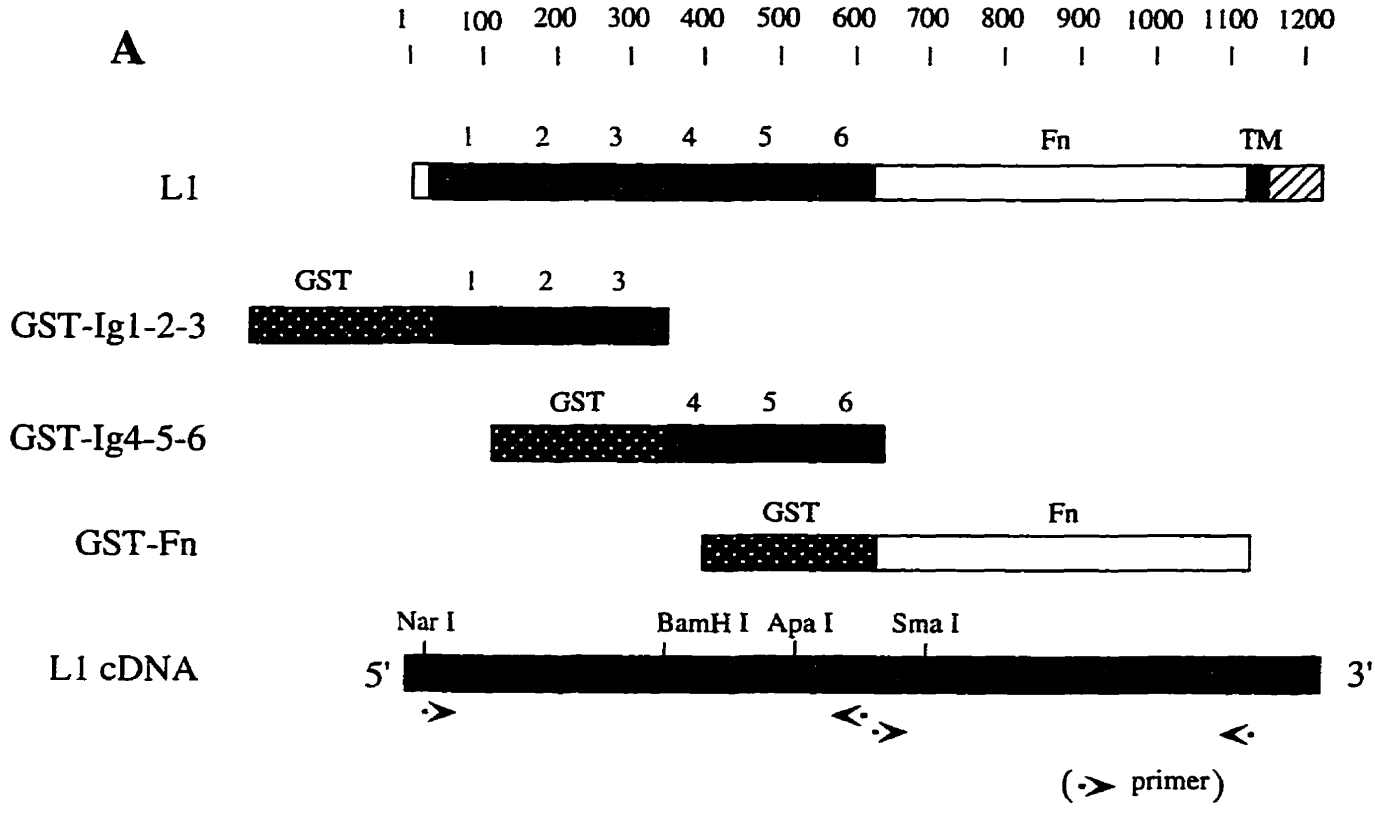
L1 is a multidomain molecule and is known to undergo heterophilic interactions with other molecules such as NCAM (Kadmon et al., 1990a; Horstkorte et al., 1993; Kadmon et al., 1990b), TAG-1/axonin-1 (Kuhn et al., 1991; Felsenfeld et al., 1994), F3/F11 (Brümmendorf et al., 1993) and brain proteoglycans (Friedlander et al., 1994; Milev et al., 1994). Interactions with these membrane and extracellular components may have important roles at specific stages of brain development. In addition to those detected in Ig-like domain 2, several other mutations in L1 have been reported to co-segregate with X-linked hydrocephalus (Rosenthal et al., 1992; Camp et al., 1993; Wong et al., 1995). Some mutations have been found to affect the expression of L1, others may have a deleterious effect on L1 interactions with its ligands. Further investigation of the role of L1 during embryonic development will depend on the identification of its homophilic and heterophilic binding sites as well as the elucidation of their mechanism of interaction. These studies should help us better understand how the hydrocephalus-related mutations affect L1 functions and provide new insights in the cause of X-linked hydrocephalus and mental retardation.

Table II.1: Binding of fusion protein-conjugated Covaspheres to neural retinal cells

In competition experiments, cells were first incubated with the soluble His-Ig2 protein or Fab fragments directed against Ig1-2-3 for 25 min and the excess competitor was removed by washing. Then fusion protein-conjugated Covaspheres were added, and incubation was carried out at room temperature for 45 min.

Protein conjugated to Covaspheres	Competitor added	Cells with bound Covaspheres	Inhibition
		%	%
BSA		8.3 ± 0.3	
His-Ig1		14.0 ± 1.3	
His-Ig2		40.7 ± 1.5	
His-Ig2	His-Ig2 (10 μg/ml)	27.0 ± 3.6	42.3
His-Ig2	His-Ig2 (40 μg/ml)	14.7 ± 3.1	80.2
His-Ig2	Fab (10 μg/ml)	16.7 ± 1.5	74.1
His-Ig2	Fab (75 μg/ml)	12.0 ± 2.0	88.6

Figure II.1. Construction and expression of GST fusion proteins. (A) Schematic drawings of GST fusion proteins: GST-Ig1-2-3, GST-Ig4-5-6 and GST-Fn. The restriction enzyme sites Nar I, and BamH I were used in the construction of GST-Ig1-2-3. The PCR product from the BamH I site at nucleotide position 1114 to the end of Ig-like domain 6, at L1 nucleotide position 1848, was used to construct GST-Ig4-5-6. The PCR fragment from nucleotide position 1849 to 3344 was used to construct GST-Fn. All the fragments were subcloned into pGEX-3T vector, and the GST protein was incorporated to the amino-terminus of the recombinant proteins. (B) Gel profiles of the GST fusion proteins after purification. Protein samples were separated on 10% SDS/polyacrylamide gels and stained with Coomassie Brilliant Blue. Lane a: purified GST-Ig1-2-3. Lane b: purified GST-Ig-4-5-6. Lane c: purified GST-Fn.


A
1 100 200 300 400 500 600 700 800 900 1000 1100 1200
1 2 3 4 5 6 Fn TM
L1
GST 1 2 3
GST-Ig1-2-3
GST 4 5 6
GST-Ig4-5-6
GST Fn
GST-Fn
Nar I BamH I Apa I Sma I
L1 cDNA
5'
3'
(➔ primer)

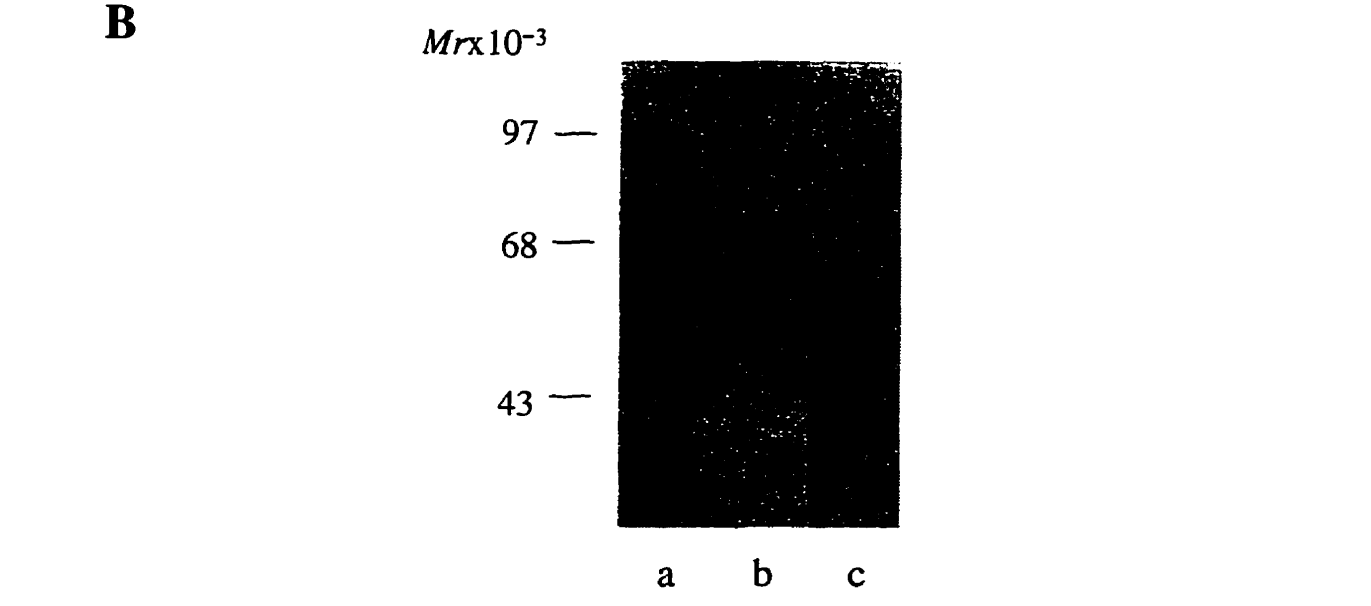

B
Mrx10-3
97
68
43
a b c

Figure II.2: Specificity of anti-domain antibodies. Purified L1-GST fusion proteins were loaded on the gel and western blots were done using pre-adsorbed anti-domain serum at 1:2000 dilution. lane a: GST-Ig1-2-3; lane b: GST-Ig4-5-6; and lane c: GST-Fn. Panels from left to right: anti-Ig1-2-3 antibody; anti-Ig-4-5-6 antibody; and anti-Fn antibody.

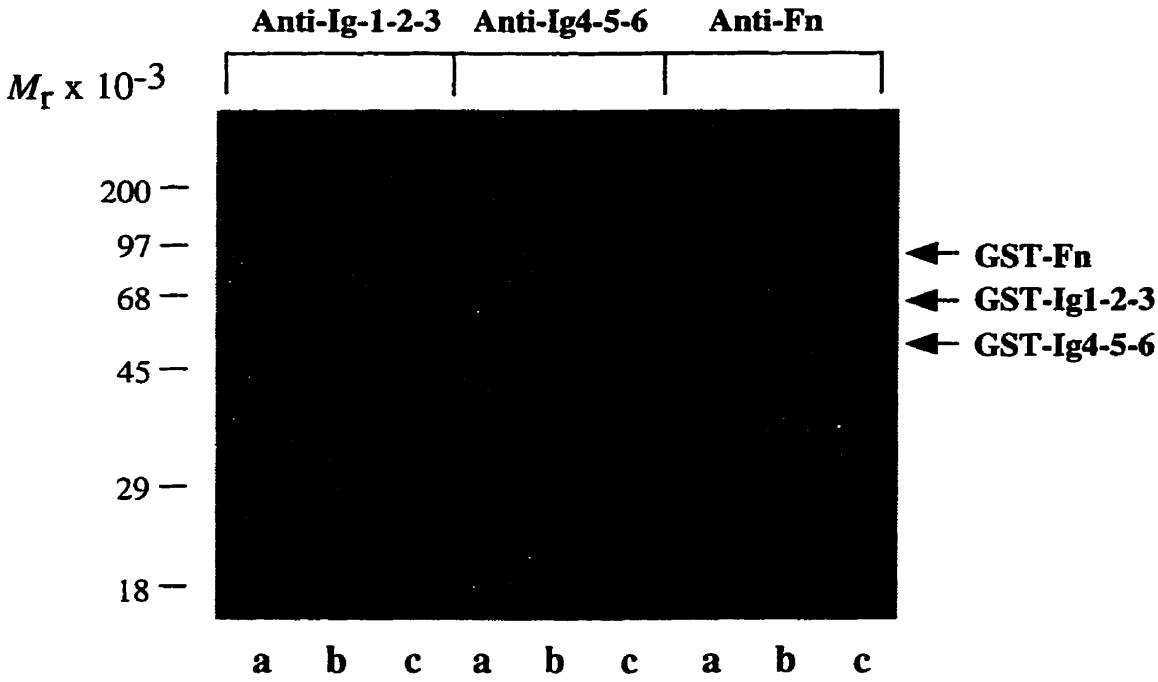
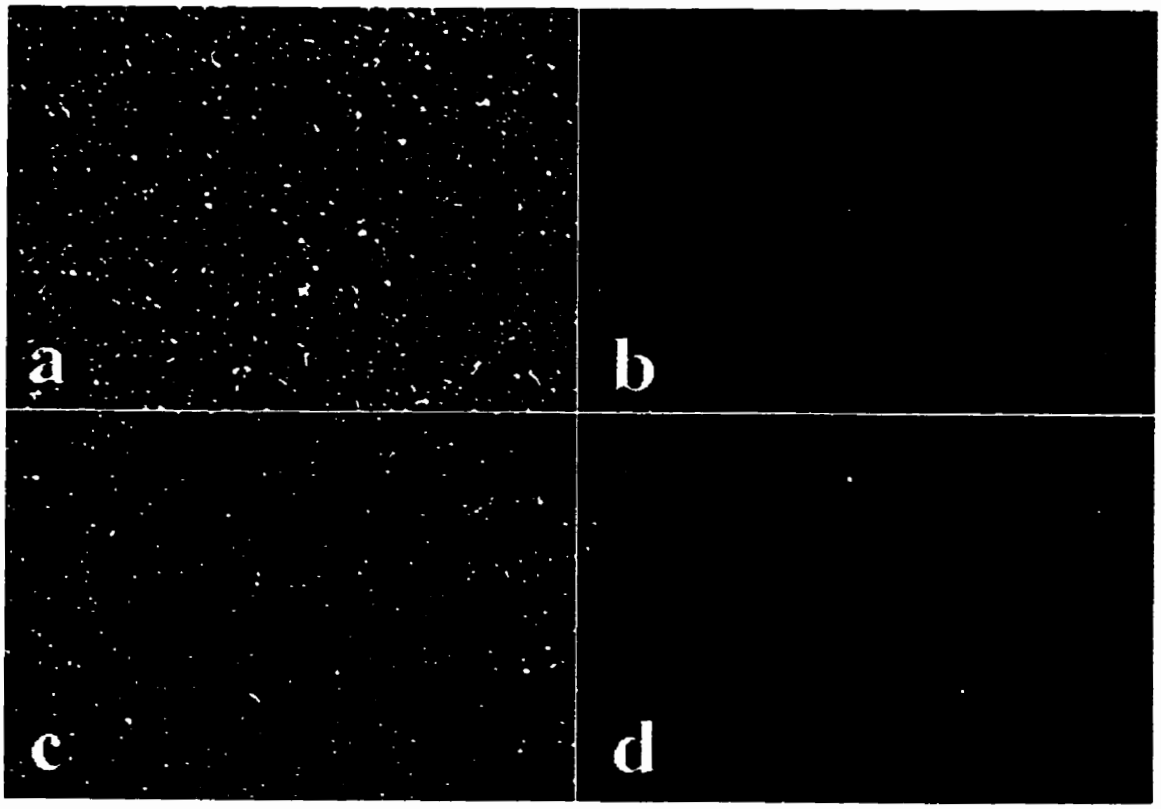

Anti-Ig-1-2-3
Anti-Ig4-5-6
Anti-Fn
M_r x 10^{-3}
200
97
68
45
29
18
GST-Fn
GST-Ig1-2-3
GST-Ig4-5-6
a b c a b c a b c

Figure II.3. Epifluorescence micrographs showing the binding of the GST-Ig1-2-3-conjugated Covaspheres to substrate-coated fusion proteins. Round spots on Petri dishes were coated with (a) anti-Ig1-2-3 IgG, (b) GST, (c) GST-Ig1-2-3 or (d) GST-Ig4-5-6. GST-Ig1-2-3-conjugated Covaspheres were allowed to adhere to the substratum for 30 min. After washing five times with PBS, the binding of Covaspheres to the substratum was observed by epifluorescence microscopy.


a
b
c
d

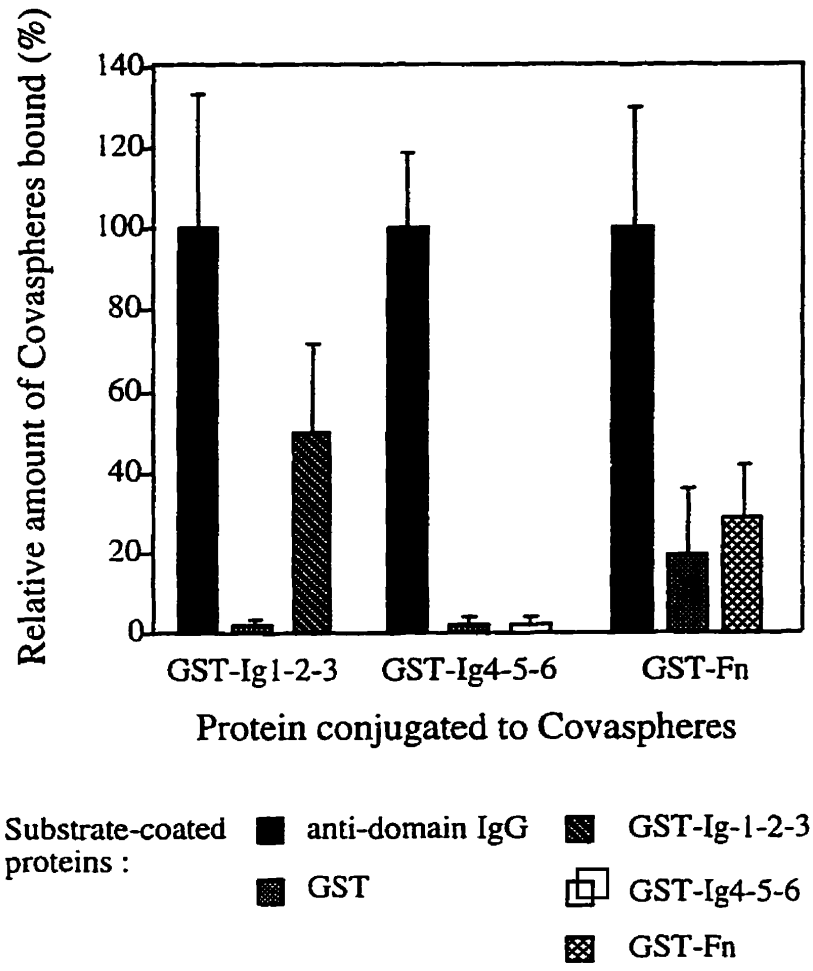


Figure II.4. Binding of fusion protein-conjugated Covaspheres to different substrates. Round spots on Petri dishes were coated with different proteins. GST-Ig1-2-3-conjugated Covaspheres were allowed to adhere to the substrate for 30 min. The binding of Covaspheres to substrate was recorded on video tapes, and the number of bound Covaspheres per unit area was counted. The relative amounts of Covaspheres attached were calculated and the results were normalized to the amount of Covaspheres bound to the substratum coated with domain-specific IgG. Data represent the mean ± S.D. (n = 6 to 9).

Figure II.5. Binding specificity of GST-Ig1-2-3-conjugated Covaspheres to GST-Ig1-2-3 substrate. (A) Dose effect of GST-Ig1-2-3 fusion protein used to coat the substratum on Covasphere binding. Spots on Petri dish were coated with different concentrations of either GST-Ig-1-2-3 (●) or GST (○). GST-Ig1-2-3-conjugated Covaspheres were allowed to adhere to coated spots. The relative amounts of Covaspheres attached were estimated relative to the amount bound to anti-Ig1-2-3 IgG. (B) Inhibition of the attachment of GST-Ig1-2-3-conjugated Covaspheres to substrate-coated GST-Ig1-2-3 by domain-specific antibodies. GST-Ig-1-2-3-conjugated Covaspheres were mixed with different concentrations of anti-Ig1-2-3 Fab (○), anti-Ig4-5-6 Fab (▲), or goat-anti-mouse-IgG Fab (●) before being placed on GST-Ig-1-2-3-coated spots. The relative amounts of Covaspheres bound per unit area were estimated. (C) Inhibition of the binding of GST-Ig1-2-3-conjugated Covaspheres by soluble GST-Ig1-2-3. Binding of Covaspheres to substrate-coated GST-Ig-1-2-3 was carried out in the presence different concentrations of GST-Ig-1-2-3 (○), GST-Ig-4-5-6 (▲), or GST (●). Data represent the mean ± S.D. (n = 6 to 9).

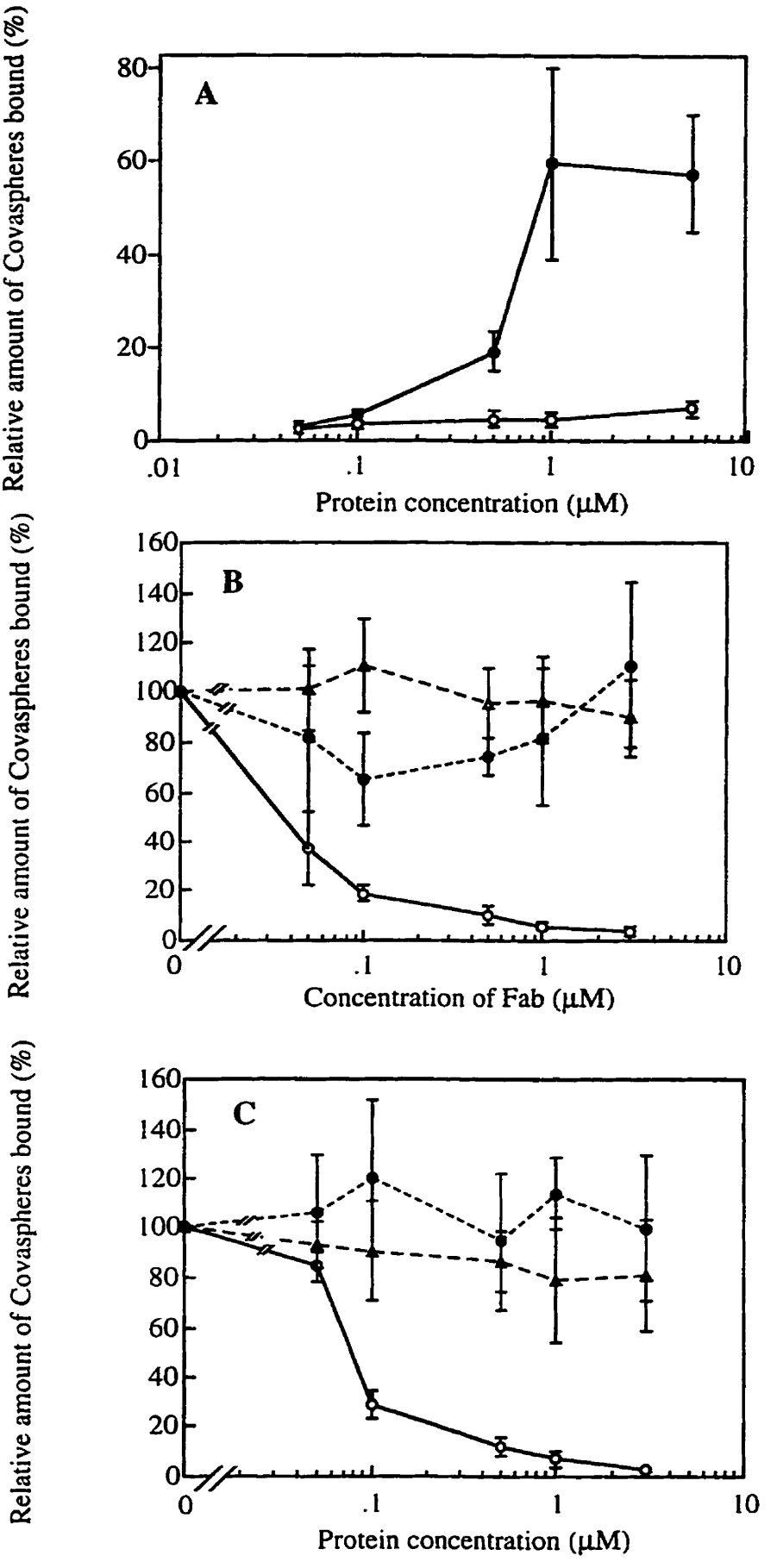
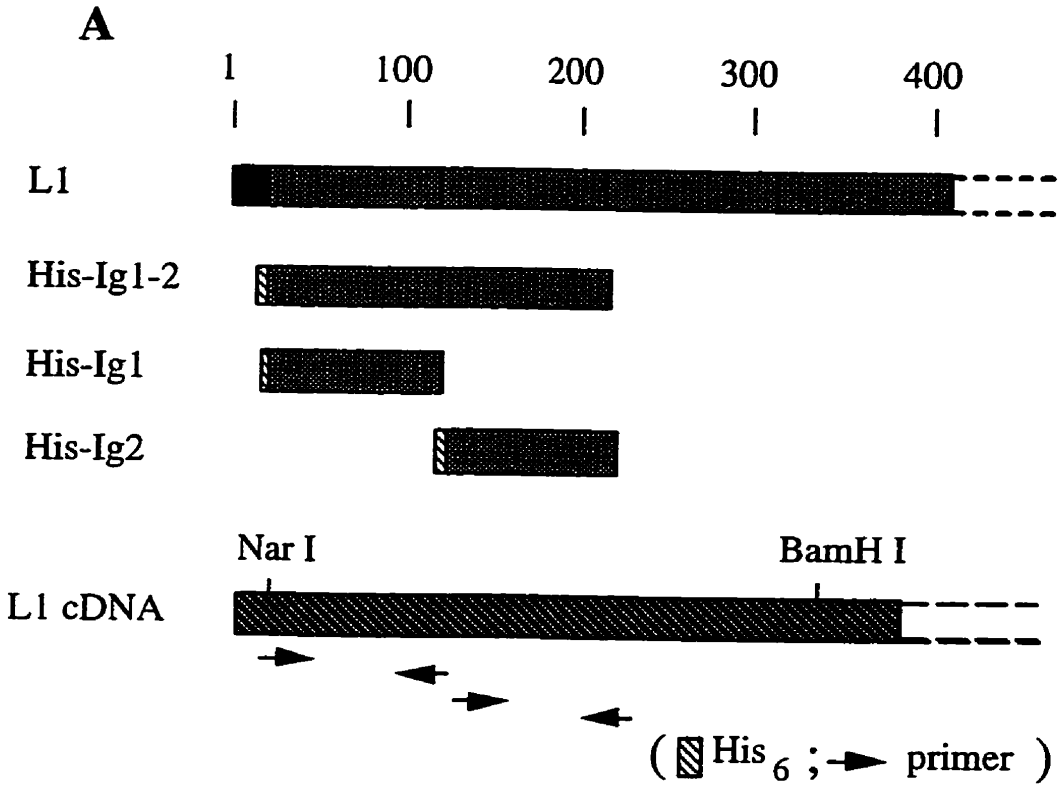

A
Relative amount of Covaspheres bound (%)
80
60
40
20
0
.01
.1
1
10
Protein concentration (μM)
B
Relative amount of Covaspheres bound (%)
160
140
120
100
80
60
40
20
0
0
.1
1
10
Concentration of Fab (μM)
C
Relative amount of Covaspheres bound (%)
160
140
120
100
80
60
40
20
0
0
.1
1
10
Protein concentration (μM)

Figure II.6. Construction and expression of His-tagged domain proteins. (A) Schematic diagrams of the three recombinant domain proteins. PCR fragments containing the coding sequences for Ig1-2, Ig1 and Ig2 were generated and subcloned into the pQE-8 expression vector. (B) Gel profiles of the recombinant proteins under reducing conditions. Proteins samples were separated on 12% SDS/polyacrylamide gels and then stained with Coomassie Brilliant Blue. Lane a, His-Ig1-2; lane b, His-Ig1; lane c, His-Ig2.


A
1
100
200
300
400
L1
His-Ig1-2
His-Ig1
His-Ig2
Nar I
BamH I
L1 cDNA
(His6 ; primer)

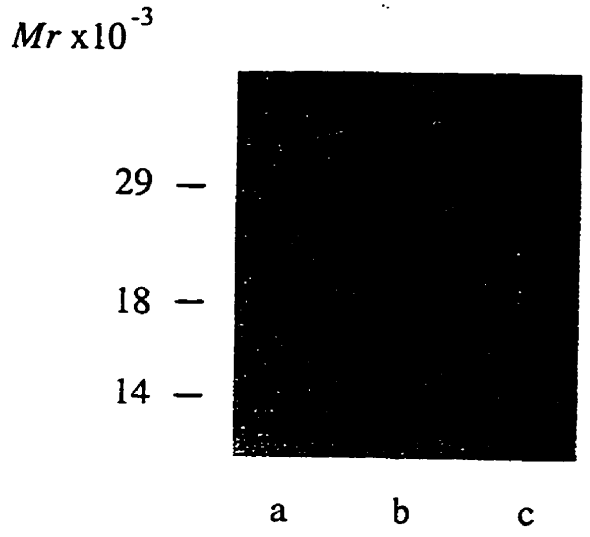
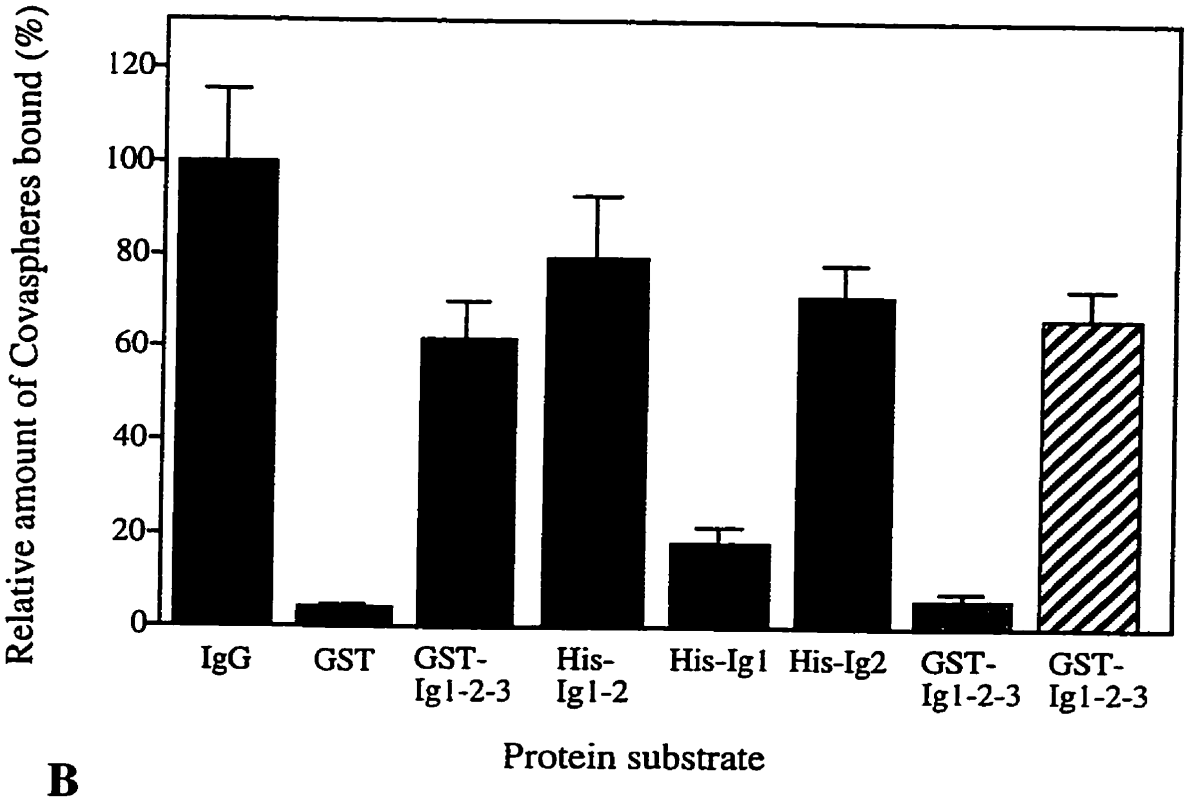
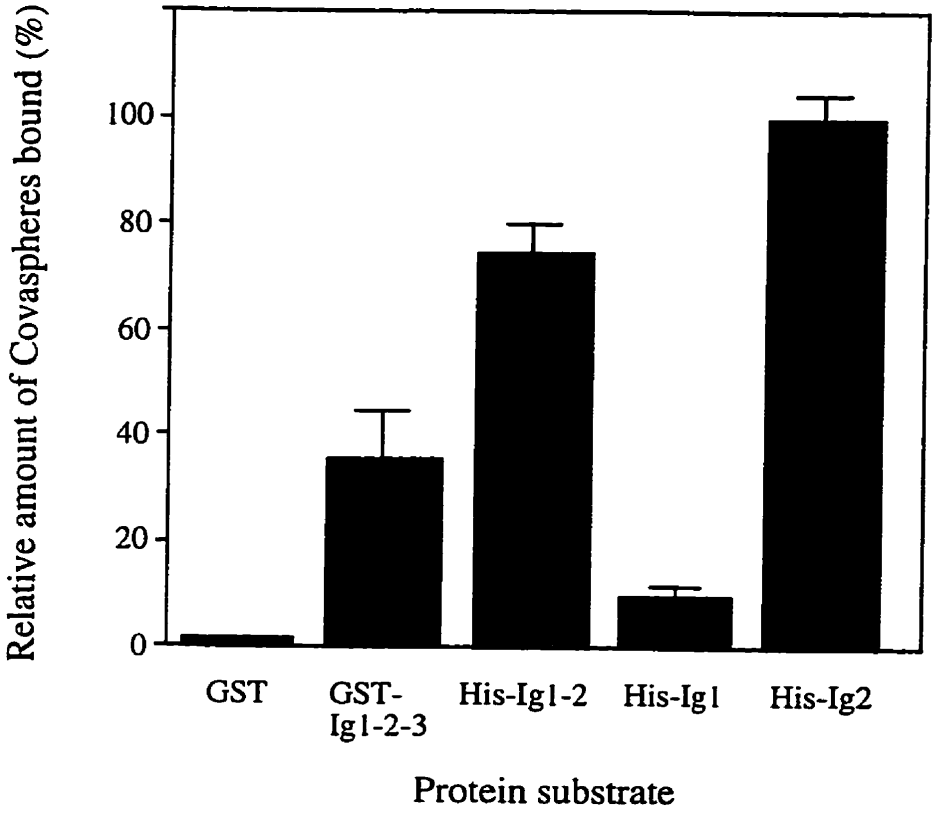

B
Mr x10-3
29
18
14
a
b
c

Figure II.7. Binding of GST-Ig1-2-3-conjugated Covaspheres to substrate-coated domain proteins. In A, Petri dishes were coated with different recombinant proteins: GST-Ig1-2-3, His-Ig1-2, His-Ig1, His-Ig2 or GST at 3 μM concentration. GST-Ig1-2-3-conjugated Covaspheres were allowed to adhere to the substrate for 30 min. In case of inhibition, Covaspheres were mixed with 10 μM His-Ig2 (*stippled bar*) or His-Ig1 (*hatched bar*) recombinant proteins before placing on substrate coated GST-Ig1-2-3. The relative amounts of Covaspheres bound were estimated relative to the amount of Covaspheres bound to substrate-coated anti-Ig1-2-3 antibodies. In B, His-Ig2-conjugated covaspheres were assayed for their ability to attach to substrate-coated His-tagged recombinant proteins. Values were normalized to the amount of Covaspheres bound to the His-Ig2 substrate. Data represent to the mean ± S.D. (n = 9)

A


Relative amount of Covaspheres bound (%)
120
100
80
60
40
20
0
IgG
GST
GST-Ig1-2-3
His-Ig1-2
His-Ig1
His-Ig2
GST-Ig1-2-3
GST-Ig1-2-3
Protein substrate

B


Relative amount of Covaspheres bound (%)
100
80
60
40
20
0
GST
GST-Ig1-2-3
His-Ig1-2
His-Ig1
His-Ig2
Protein substrate

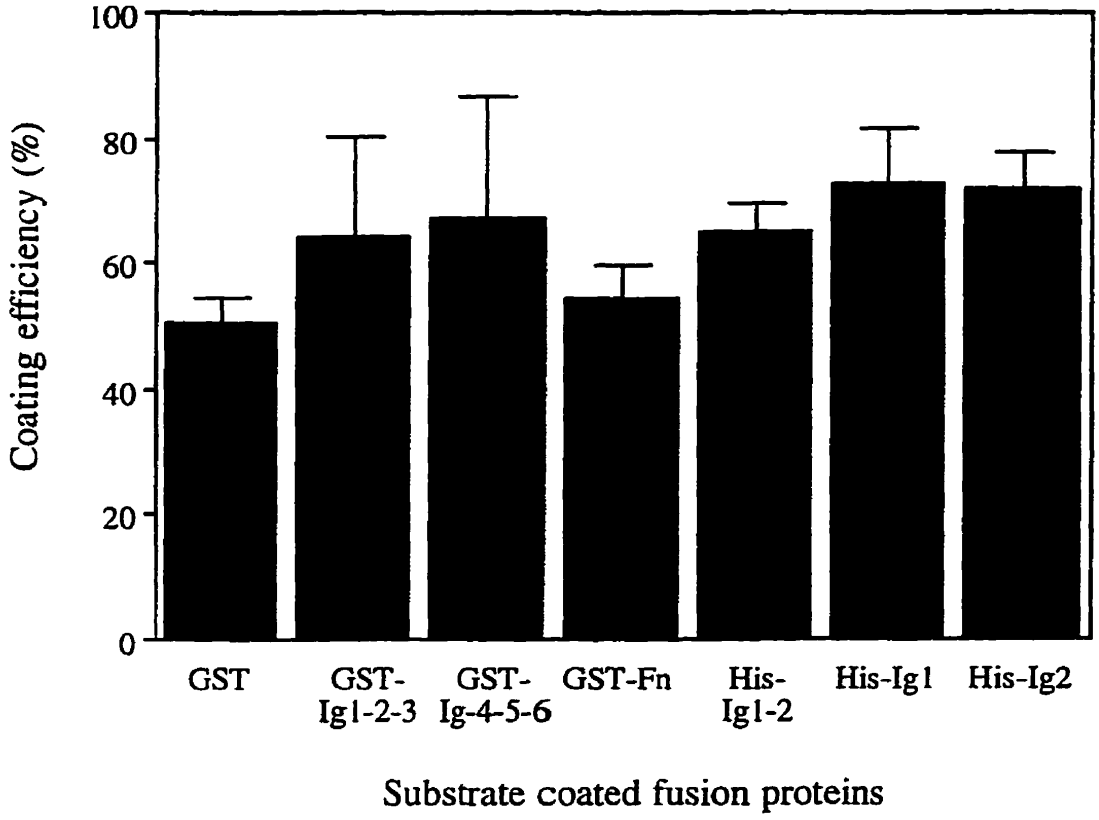


Figure II.8 Coating efficiency of fusion proteins on coverslips. Round glass coverslips (12-mm diameter) were coated with 0.01% (w/v) poly-L-lysine at room temperature for 3 h. After washing three times with distilled water, 80 µl samples of recombinant proteins at 1 µM were used to coat the coverslips overnight at 4°C. To determine the efficiency of protein coating, protein adsorbed to coverslips was solublized by incubation in 1% SDS (10 µl) at 37°C for 10 min. The relative amounts of bound and unbound protein were determined using the BCA protein assay. Data represented the Mean ± S.D. of three experiments.

Figure II.9: Epifluorescence micrographs of neurites extended by retinal cells. Neural retinal cells were isolated from E5 chick embryos and labeled with DiI. Retinal cells were cultured for 18 h on different fusion protein-coated substrates: GST-Ig1-2-3 (a), GST-Ig4-5-6 (b), and GST (c). *Bar*, 10 μm.

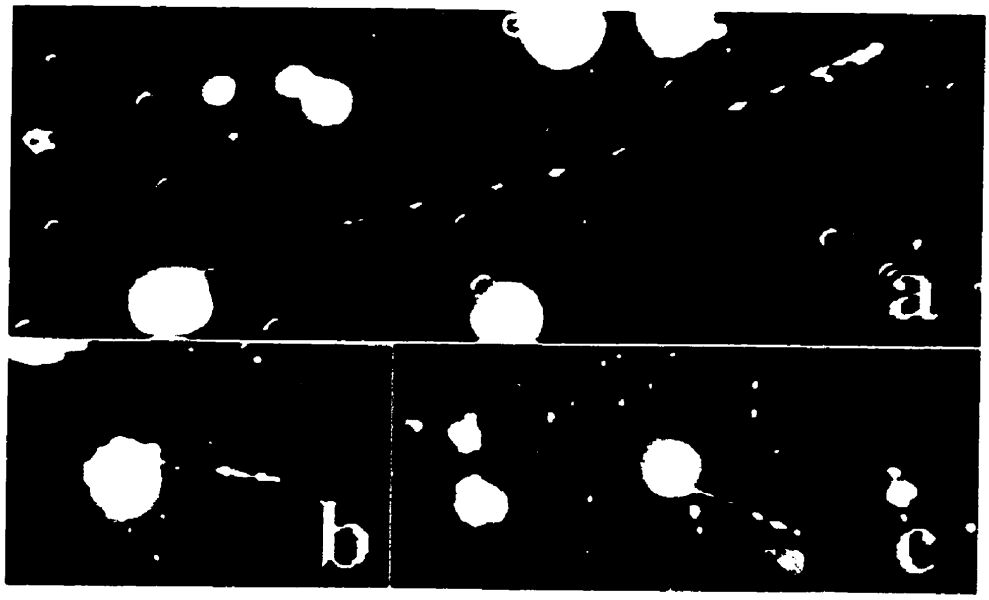
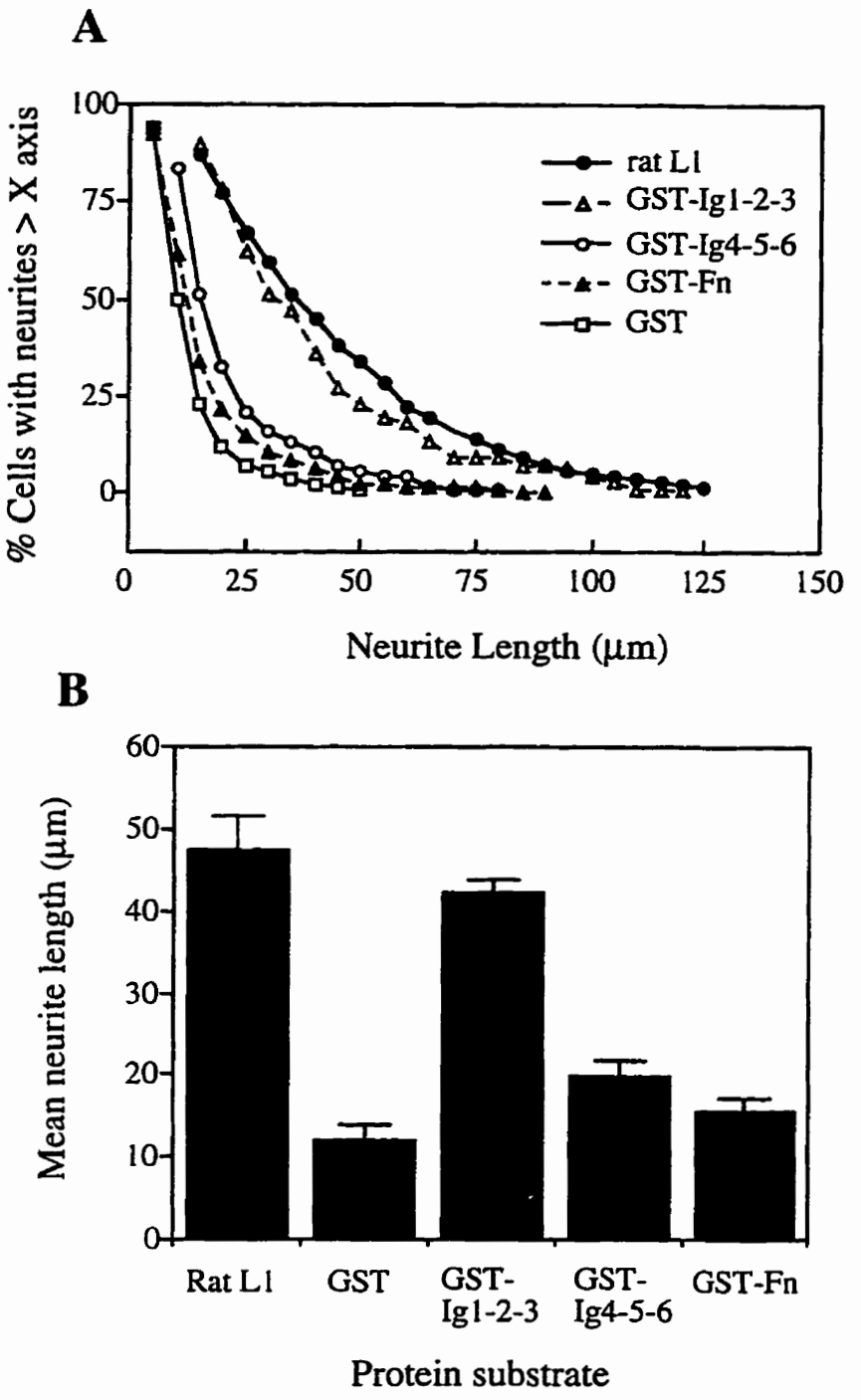

a
b
c

Figure II.10: Neurite outgrowth from retinal cells on different substratum. Coverslips were first coated with 0.01% poly-L-lysin, followed by one of the GST fusion proteins at 1 μM concentration. Purified rat L1 was used as the positive control. Retinal cells were seeded onto the coverslips in N2 medium. Cultures were incubated at 37°C for 18 h. Cells were fixed with formaldehyde and observed under a epifluorescence microscope. Images were recorded on VCR for neurite length measurements. (A) Size distribution of neurites extending from retinal cells cultured on different fusion protein substrates (●, rat L1; Δ, GST-Ig-1-2-3; O, GST-Ig-4-5-6; ▲, GST-Fn; ❑,GST). (B) Mean neurite lengths for cells cultured on different protein substrates. Data represent the mean ± SD of three experiments


A
% Cells with neurites > X axis
100
75
50
25
0
0
25
50
75
100
125
150
Neurite Length (μm)
rat L1
GST-Ig1-2-3
GST-Ig4-5-6
GST-Fn
GST
B
Mean neurite length (μm)
60
50
40
30
20
10
0
Rat L1
GST
GST-Ig1-2-3
GST-Ig4-5-6
GST-Fn
Protein substrate

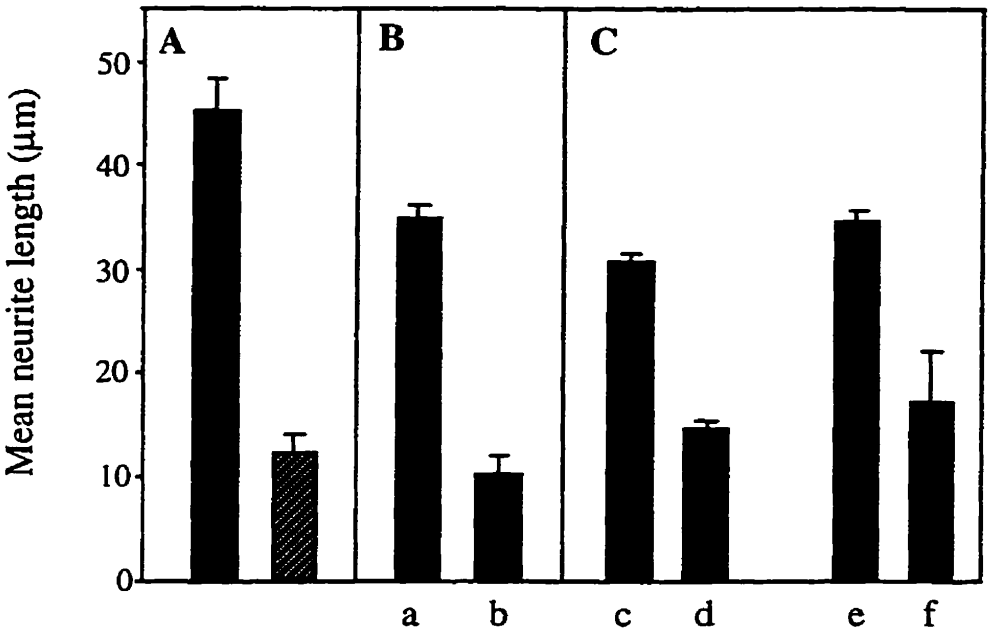


Figure II.11: Inhibitory effect of GST fusion proteins and anti-domain Fab on neurite outgrowth. (A) Retinal cells were cultured on GST-Ig-1-2-3-coated substratum (Solid bar) or on GST (hatched bar) in the absence of competiters. (B) The GST-Ig-1-2-3 substrate was preincubated with the inhibitor for 15 min at room temperature and then washed before retinal cells were deposited on the coverslip. (C) inhibitor was added to retinal cells prior to seeding on protein substrate. (a,c): 5 µM Goat-anti-mouse Fab used as control, (b,d):5 µM Fab against GST-Ig-1-2-3 , (e) 40 µg/ml GST , (f) 40 µg/ml GST-Ig-1-2-3.

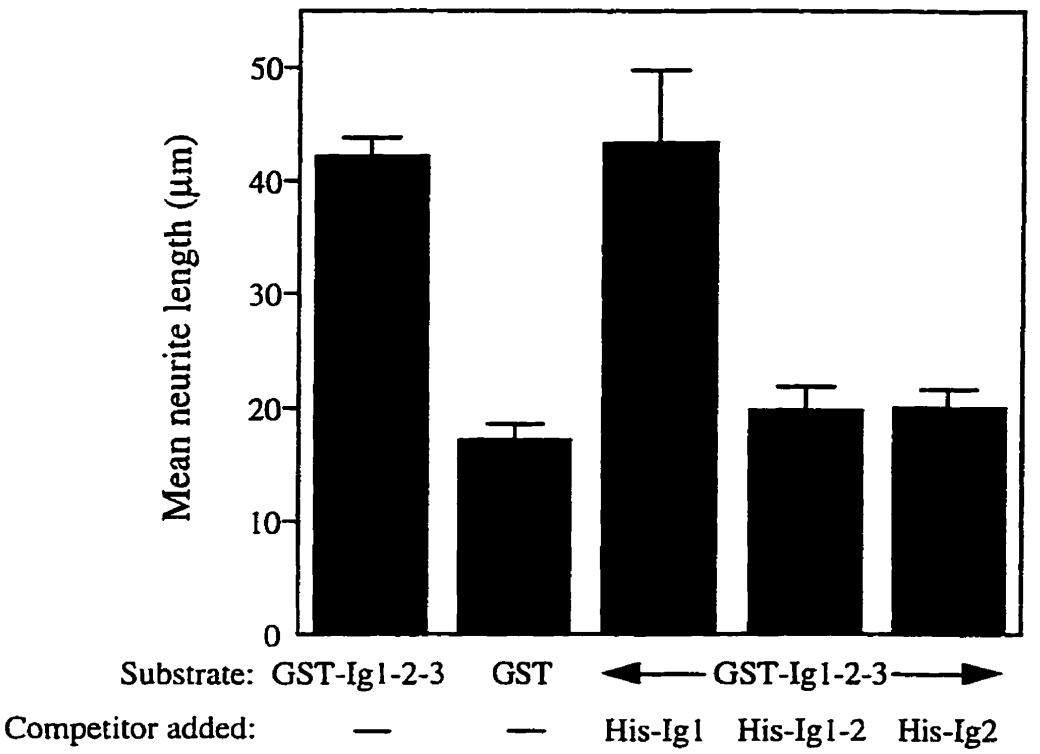
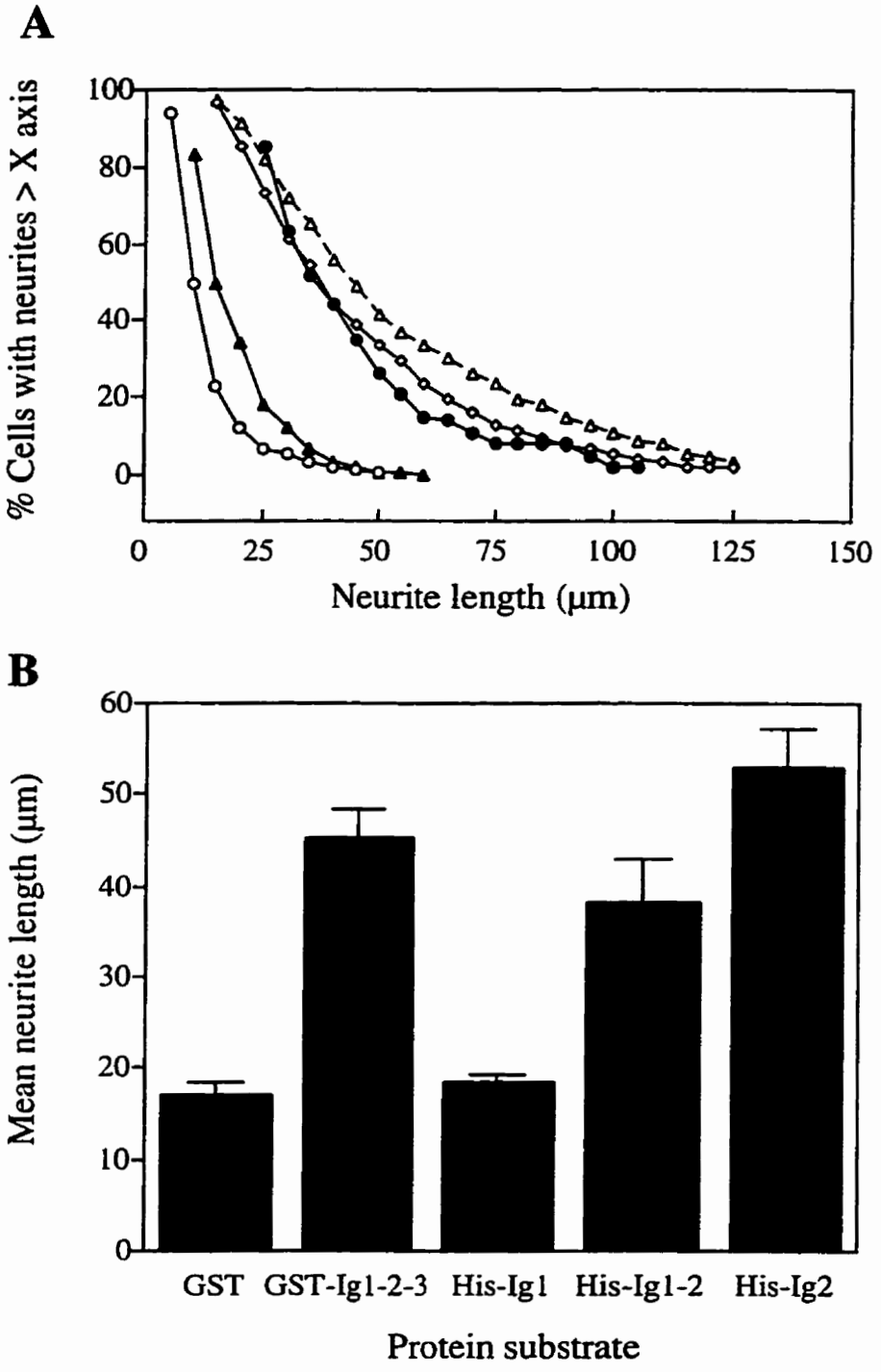


Figure II.12: Inhibition of neurite outgrowth by recombinant domain proteins. Retinal cells were cultured on top of Ig1-2-3-GST-coated coverslips in the presence of Ig1-2, Ig1, or Ig2 at 1μM. Mean neurite length represent the mean ± SD of three experiments.

Figure II.13: Neurite outgrowth on domain protein-coated substratum. Nitrocellulose coated coverslips were coated with 1μM proteins, and retinal cells were deposited on them and cultured for 18 h. Cells were fixed and the neurite lengths were measured. (A): Size distribution of neurites extending from retinal cells cultured on different protein substrates: GST (○), GST-Ig1-2-3 (✧), His-Ig1 (▲), His-Ig2 (Δ), His-Ig1-2 (●). (B): Mean neurite lengths of retinal cells. Data represent the mean ± SD of three experiments.


A
% Cells with neurites > X axis
100
80
60
40
20
0
0
25
50
75
100
125
150
Neurite length (μm)
B
Mean neurite length (μm)
60
50
40
30
20
10
0
GST
GST-Ig1-2-3
His-Ig1
His-Ig1-2
His-Ig2
Protein substrate

CHAPTER THREE

DIFFERENTIAL EFFECTS OF TWO HYDROCEPHALUS/MASA SYNDROME-RELATED MUTATIONS ON THE FUNCTION OF THE CELL ADHESION MOLECULE L1

The contents of this chapter have been published in the

Journal of Biological Chemistry **271**:6563-6566 (1996).

I. INTRODUCTION

The cell adhesion molecule L1 is expressed primarily in postmitotic neurons and has been implicated in neural migration, neurite outgrowth and fasciculation during brain development (for a review, see Wong, et al., 1995). L1 is a 200-kDa transmembrane glycoprotein and a member of the immunoglobulin (Ig)[1] superfamily of cell adhesion molecules. It contains six Ig-like domains in the amino-terminal region, followed by five fibronectin type III repeats, one transmembrane domain and a cytoplasmic domain (Moos, et al., 1988; Prince, et al., 1991). L1 can undergo homophilic interactions with L1 (Lemmon, et al., 1989; Miura, et al., 1992), as well as heterophilic interactions with other adhesion molecules, such as NCAM (Kadmon et al., 1990), TAG-1/axonin-1 (Kuhn et al., 1991; Felsenfeld et al., 1994), F3/F11 (Brümmendorf et al., 1993), glia (Grumet and Edelman, 1988) and components of the extracellular matrix (Grumet et al., 1993; Friedlander et al., 1994). In addition to cell adhesion, substrate-coated L1 is a potent inducer of neurite outgrowth from primary neurons (Lemmon et al., 1989; Miura et al., 1992).

The human L1 cDNA has been cloned (Hlavin and Lemmon, 1991) and the gene has been mapped to chromosome Xq28 (Djabali et al., 1990). Several recent reports show that a group of heterogeneous mutations in L1 are responsible for X-linked hydrocephalus and two related neurological disorders, MASA (mental retardation, aphasia, shuffling gait and adducted thumbs) syndrome and spastic paraplegia type 1 (SPG1) (Vits et al., 1994; Jouet et al., 1994; Jouet et al., 1995). Most of them are missense mutations, resulting in amino acid changes in extracellular and cytoplasmic domains, while others are nonsense, deletion, or splicing mutations resulting in the

truncation or secretion of L1. However, little is known about how these mutations give rise to these related neurological diseases. An investigation of the role of the mutated residues in L1 function is, therefore, crucial to our understanding of these defects.

We recently demonstrated that the Ig2 domain of L1 harbors both homophilic binding and neuritogenic activities (Zhao and Siu, 1995). Interestingly, two missense mutations have been localized to Ig2. One results in the replacement of Arg184 with Gln. This mutation is found in patients with severe hydrocephalus, which is characterized by the absence of the corticospinal tract and stenosis of the aqueduct of sylvius (Jouet et al., 1994). The other mutation results in the substitution of His210 with Gln and is detected in MASA patients with a milder phenotype (Jouet et al., 1994). Those who survived suffered from mental retardation. We have investigated the effects of these two mutations on the homophilic binding and neuritogenic activities associated with L1 Ig2. These activities are completely lost in the Arg184Gln mutation, but are only partially affected in the His210Gln mutation.

II. EXPERIMENTAL PROCEDURES

A. Construction and Expression of Mutated Forms of L1 Ig-like Domain 2

The human L1 cDNA fragment coding for Ig2 was obtained by PCR amplification using the forward primer 5'-CGGGATCCTGGCCGAGGGTGCCCC-3' and the reverse primer 5'-GTGGATCCGACCCGGAGGTCAATG-3'. The PCR product was digested with *Bam*HI and then subcloned into pBluescript SKII(+) for mutation and DNA sequencing. The Transformer™ site-directed mutagenesis kit (Clontech) was used according to the manufacturer's protocol. The oligonucleotides 5'-GACGAGCAGGTGA CGA-3' and 5'-TCTGCCAGGCCCACTT-3' were used as mutagenic primers for Ig2mtl (Arg184Gln) and Ig2mt2 (His210Gln), respectively. The oligonucleotide 5'-ATAAGCTTGATCGCGAATTCCTGC-3' was used as the selection primer. Mutations were confirmed by double-stranded DNA sequencing. The inserts were then released from pBluescript and subcloned into the pGEX-3T vector. Expression and refolding of the two mutant GST-Ig2 fusion proteins were carried out as described previously (Zhao and Siu, 1995).

B. Cell Transfection

The full-length L1 cDNA (16) was subcloned into the unique *Hin*dIII site of the expression vector pRc/CMV (Invitrogen). An antisense-L1 construct was made by inserting the L1 cDNA in the reverse orientation. The CHO cell line LR73 (Zhou et al., 1993) was cultured in α-MEM medium containing 10% fetal calf serum until

subconfluence. Cells were collected and plated on 10-cm dishes (at 1:10 dilution) 1 day before transfection. LR73 cells were transfected with 20 μg of plasmid DNA using the calcium phosphate precipitation method. Transfected clones were selected using 400 μg/ml of G418, followed by limiting dilution and clonal analysis for L1 expression.

C. Binding of L1-conjugated Covaspheres to Cells

Rat L1 or recombinant GST-Ig2 was conjugated to Covaspheres as previously described (Zhao and Siu, 1995). To assay for the binding of L1-Covaspheres to cells, L1-transfected LR73 cells were seeded sparsely on coverslips 20 h before the assay. Coverslips were blocked with α-MEM containing 1% BSA for 5 min at 22° C. To inhibit binding, 100 μl of α-MEM containing the competitor (0.1 mg/ml) was placed on coverslips and incubated with cells at 22° C for 25 min. After one wash with α-MEM to remove the unbound protein, 100 μl of L1-conjugated Covaspheres (diluted at 1:30) were added and incubated for 30 min at 22° C on a platform shaker. After several washes with PBS, cells were observed using epifluorescence microscopy. Cells with >5 Covaspheres attached on the surface were scored as positive cells. Generally, 100-250 cells were scored for stable transfectants and >1000 cells were scored for transiently transfected cells.

D. Neurite Outgrowth Assay

L1 transfected LR73 cells were grown on round coverslips until confluent. Neural retinal cells were isolated from day 5 chick embryos and suspended in N2 medium

before seeding on top of the LR73 cell monolayer. Co-cultures were carried out in N2 medium at 37° C for 16 h. Cells were fixed with 3.7% formaldehyde in PBS and then examined by epifluorescence microscopy. Images of neurites were recorded and ~100 neurites were measured for each experiment. In inhibitory studies, competitors were included in the coculture medium at 0.1 mg/ml. When protein-coated substratum was used to induce neurite outgrowth, coverslips were first coated with 0.01% poly-L-lysine at room temperature for 3 h, and then with GST-fusion proteins at 4° C overnight. The coverslips were washed once and then plated with retinal cells.

III. RESULTS

A. Expression of mutant GST-Ig2 fusion proteins

To investigate the effects of Arg184Gln and His210Gln mutations on the homophilic binding and neuritogenic activities of L1, Ig2 containing these mutations were expressed in bacteria as GST fusion proteins. GST-Ig2mt1 contained the Arg184Gln mutation and GST-Ig2mt2 contained the His210Gln mutation. Purified proteins were analyzed by gel electrophoresis (Figure III.1). Under reducing conditions, both wildtype and mutant GST-Ig2 fusion proteins migrated with an M_r of 41,000. The purified and refolded proteins were used in subsequent assays.

B. Expression of full-length L1 on LR73 cell surface

To facilitate the analysis of these fusion proteins, LR73 cells were transfected with the L1 cDNA. LR73 cells do not express L1 and are especially suitable for the analysis of cell adhesion molecules (Zhou et al., 1993). Sense or antisense L1 cDNAs were inserted into the pRc/CMV (Figure III.2) for expression in LR73 cells. Immunoblot analysis revealed a major L1 band at ~200 kDa and two cleavage products of smaller size in cells transfected with the sense L1 cDNA, but not in those with the antisense construct (Figure III.2). Cell surface expression of L1 on transfectants was confirmed by immunofluorescence staining (see Figure IV.I in chapter IV).

C. Homophilic binding activity of mutant GST-Ig2 to full length L1 expressed on LR73 cells

The Covasphere-to-cell binding assay demonstrated that the L1 molecules expressed in these cells were functional. About 50% of L1-expressing cells bound L1-conjugated Covaspheres, while only 3% of antisense-L1 transfectants showed positive binding (Figure III.3). Binding of L1-Covaspheres to L1-transfectants was inhibited by anti-Ig1-2-3 Fab, but not by anti-Ig4-5-6 Fab (Figure III.4). This is consistent with our previous finding that L1-L1 binding is dependent on the Ig2 domain (Zhao and Siu, 1995).

To assess the effects of the Arg184Gln and His210Gln mutations on the ability of Ig2 to compete for L1 binding, the Covasphere-to-cell binding assay was performed in the presence of these fusion proteins (Figure III.4). Fifty percent inhibition was achieved at 50 μg/ml of GST-Ig2 and binding was abolished at 100 μg/ml. In contrast, GST-Ig2mt1 failed to inhibit the binding reaction at the same concentration. Similar results were obtained with GST as the control competitor. Interestingly, only partial competition was observed with 100 μg/ml of GST-Ig2mt2, which inhibited the binding of Covaspheres to cells by ~70%.

D. Homophilic binding activity of mutant GST-Ig2

To directly test the homophilic binding activity of the mutant fusion proteins, binding of fusion protein-conjugated Covaspheres to substrate-coated protein was carried out. All fusion proteins adsorbed to Petri dishes with similar efficiency (Figure III.5). As

a positive control, IgG directed against Ig1-2-3 of L1 (Zhao and Siu, 1995) was adsorbed on to Petri dish for Covasphere binding, while substrate-coated GST was used as a negative control. GST-Ig2-conjugated Covaspheres attached very well to substrate-coated GST-Ig2. However, binding of GST-Ig2-Covaspheres to GST-Ig2mt2 was reduced by ~50% , but no significant binding to the GST-Ig2mt1 substrate was observed (Figure III.6A). In addition, GST-Ig2mt1-conjugated Covaspheres attached to substrate-coated GST-Ig2mt1 only at background level (Figure III.6B), indicating that the Arg to Gln substitution led to the loss of its homophilic binding activity. In contrast, GST-Ig2mt2 still retained significant homophilic binding activity (Figure III.6C). Thus, the His210Gln mutation had a milder effect on the homophilic binding activity of Ig2.

E. Inhibitory activity of mutant GST-Ig2 in neurite outgrowth assay

Next, the effects of these fusion proteins on neurite outgrowth were examined. The mean neurite length of neural retinal cells cultured on L1-expressing LR73 cells were ~3 times longer than those cultured on the antisense transfectants (Figure III.7A). When individual fusion proteins were included in this assay, GST-Ig2, being a strong competitor for L1 binding, reduced the mean neurite length to near background level, while GST and GST-Ig2mt1 exhibited no significant inhibitory effects. However, GST-Ig2mt2 was ~80% as effective as GST-Ig2 in the inhibition of neurite outgrowth from retinal cells (Figure III.7B).

F. Neurite outgrowth promotion activity of mutant GST-Ig2 proteins.

We have previously demonstrated that the L1 Ig2 fragment can serve as a potent substrate for neurite outgrowth from retinal cells (Zhao and Siu, 1995). The effects of the two hydrocephalus/MASA syndrome-related mutations on the neuritogenic activity of Ig2 were examined. In comparison to cells cultured on the GST substrate, GST-Ig2 stimulated a 2.8-fold increase in the mean neurite length of retinal cells (Figure III.8). In contrast, GST-Ig2mt1 failed to promote neurite outgrowth from these cells. GST-Ig2mt2, on the other hand, retained substantial neuritogenic activity and only a 20% reduction in the mean neurite length was observed.

IV. DISCUSSION

The above results, taken together, demonstrate that the Arg184Gln mutation abolishes both the homophilic binding activity and the neuritogenic activity associated with the Ig2 domain of L1, whereas the His210Gln mutation results only in a partial loss of these two functions. It is likely that Arg184 plays a crucial role in the structure and function of L1. Arg184 is highly conserved in L1 among different species (Figure III.9), including mouse (Moos et al., 1988), rat (Prince et al., 1991), chicken (Burgoon et al., 1991) and the *Drosophila* homolog (Bieber et al., 1989). In addition, this residue lies within a region corresponding to the predicted C' β-strand of the Ig fold, suggesting that the C' region of L1 Ig2 may participate directly in L1-L1 homophilic binding. It is conceivable that the forward binding reaction may depend on electrostatic interactions involving Arg184. Interactions centered at this region may then lead to other interactions at secondary sites along the length of the extracellular segments of two apposing molecules, further stabililizing the binding interactions. This possibility has been observed in the neural cell adhesion molecule NCAM, which is also a member of the Ig superfamily. NCAM homophilic interaction is centered around the C' β-strand and the C'-E loop in the third Ig-like domain (Rao et al., 1992). This region of the NCAM molecule is capable of undergoing isologous interactions with the same region of an apposing molecule (Rao et al., 1994). The charged residues in this region also appear to play a crucial role (Rao et al., 1992).

In both L1 and NCAM, homophilic binding is closely coupled to their ability to induce neurite outgrowth from neuronal cells (Zhao and Siu, 1995; Sandig et al., 1994). Although several other structural domains in L1 have been implicated in neurite

outgrowth promotion (Appel et al., 1993; Burgoon et al., 1995), the severity of the neuropathological phenotype of patients with the Arg184Gln mutation attests to the importance of the homophilic binding and neuritogenic activities centered around Arg184. It is conceivable that homophilic binding may generate neurite outgrowth signals by inducing conformational changes in the molecule and alter its interactions with other membrane or cytoplasmic components. Potential candidates involved in down-stream events of L1-dependent neurite outgrowth include FGF receptor (Doherty et al., 1995), $pp60^{c\text{-}src}$(Ignelzi et al., 1994), and ankyrin (Davis and Bennett, 1994).

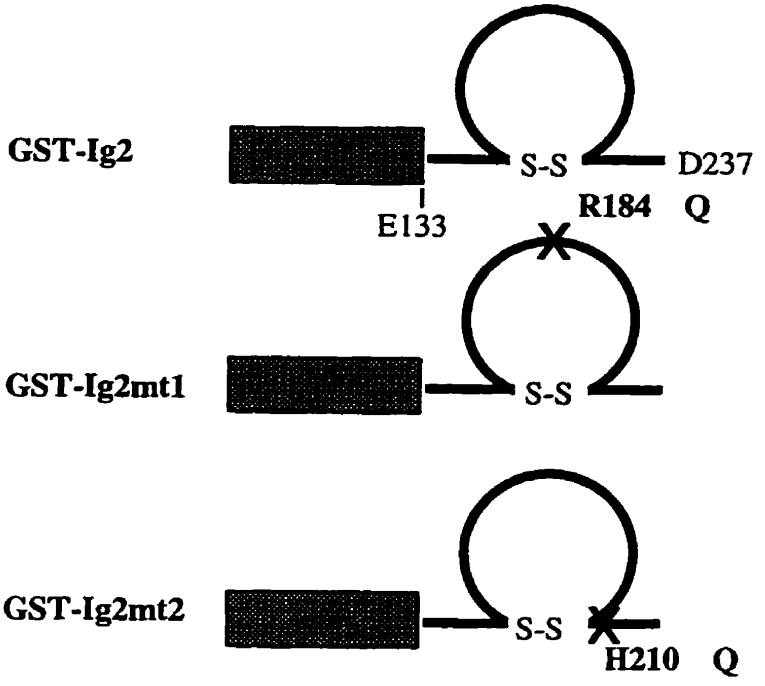
His210 is predicted to lie within the F β-strand of the Ig fold, with its charged side-chain pointing outward on the surface of the molecule. Substitutions with another charged or polar residue are likely to be tolerated. Indeed, this residue is less conserved in L1 homologs (Figure III.9) and His210 is replaced by Asn in mouse and rat (Moos et al., 1988; Prince et al., 1991) and by Ser in the Drosophila homologue (Bieber et al., 1989). This is supported by the less deleterious effects of the His210Gln mutation. Therefore, a close correspondence exists between the *in vitro* functions of the mutant proteins and the pathological phenotype caused by these mutations. Exactly how these two mutation affect neural development is not known. Further studies will depend on the availability of transgenic animals that express these mutant forms of L1.

To date, 23 mutations in L1 have been reported in hydrocephalus, MASA and SPG1 patients. These mutations are evenly distributed along the L1 molecule (Wong et al., 1995; Jouet et al., 1994), indicating that these structural domains may have important biological functions. Alternatively, some of the amino acid substitutions may induce conformational changes, causing the inactivation of functional domains at a distance. Mutations outside the Ig2 domain may affect heterophilic interactions of L1 with other

matrix and membrane components. Recently, the RGD sequence located in the sixth Ig-like domain of L1 has been found to interact with the $\alpha_v\beta_3$ integrin (Montgomery et al., 1996). α_v is present predominantly in the glia of the central nervous system (Hirsch et al., 1994), suggesting a role for the L1 RGD sequence in neuron-glia interaction in the brain cortex and in the cerebellum. L1 is also known to interact heterophilically with axonin-1/TAG-1, F3/F11, and brain proteoglycans. It is evident that L1 has a very complex biology and mutations affecting its interactions with different binding partners may have far-reaching effects on neuron migration and neurite outgrowth. Our future understanding of the diverse biological roles of L1 in brain development will depend on the identification of its binding sequences and the elucidation of their mechanisms of interaction.

Figure III.1: Construction and expression of mutant GST-L1 fusion proteins. (A) Schematic drawings of GST-mutant Ig2 fusion proteins. (B) Gel profiles of purified GST fusion proteins. Protein samples were separated on 10% SDS/polyacrylamide gel and stained with Coomassie Blue. Lane a, GST-Ig2; lane b, GST-Ig2mt1; lane c, GST-Ig2mt2; lane d, GST.

A


GST-Ig2
S-S
D237
E133
R184
Q
GST-Ig2mt1
S-S
GST-Ig2mt2
S-S
H210
Q

B

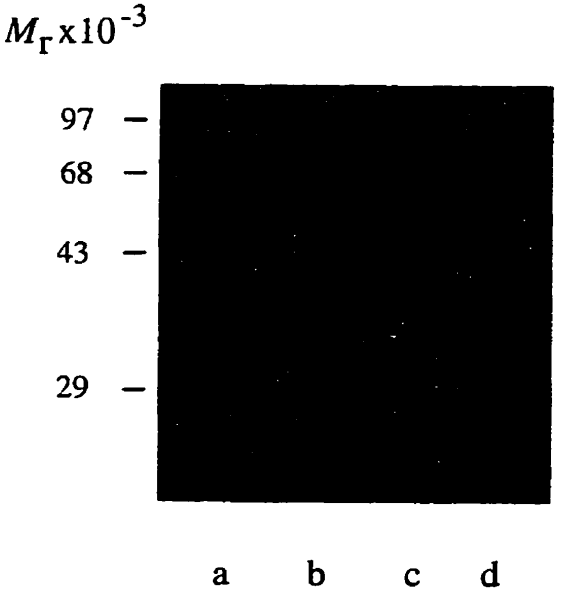
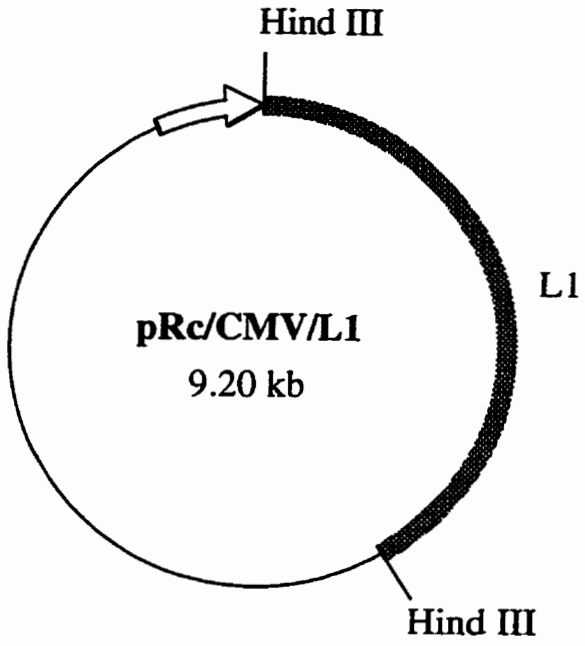

M_r x10^{-3}
97
68
43
29
a
b
c
d

Figure III.2: Construction of the pRc/CMV/L1 expression plasmid and L1 expression in transfected LR73 cells. Immunoblots of transfectants were stained with 10 μg/ml of rabbit anti-Ig4-5-6 IgG: lane a, control LR 73 cells; lane b, antisense transfectants; lane c, L1 transfectants.

A



B

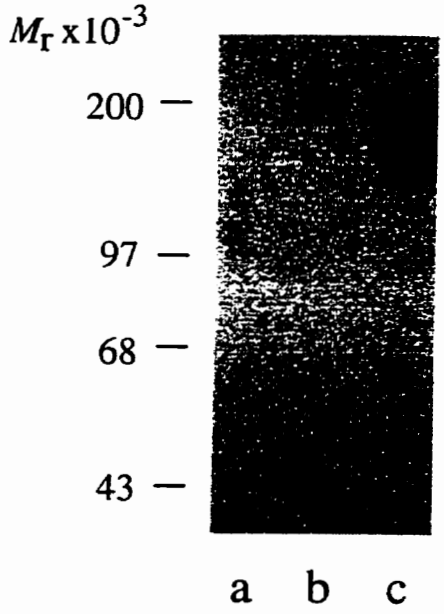


Figure III.3: Binding of L1-conjugated Covaspheres to L1-transfected LR73 cells

Epifluorescence micrographs showing the binding of L1-conjugated Covaspheres to L1-expressing LR73 cells (a) or antisense transfectants (b).

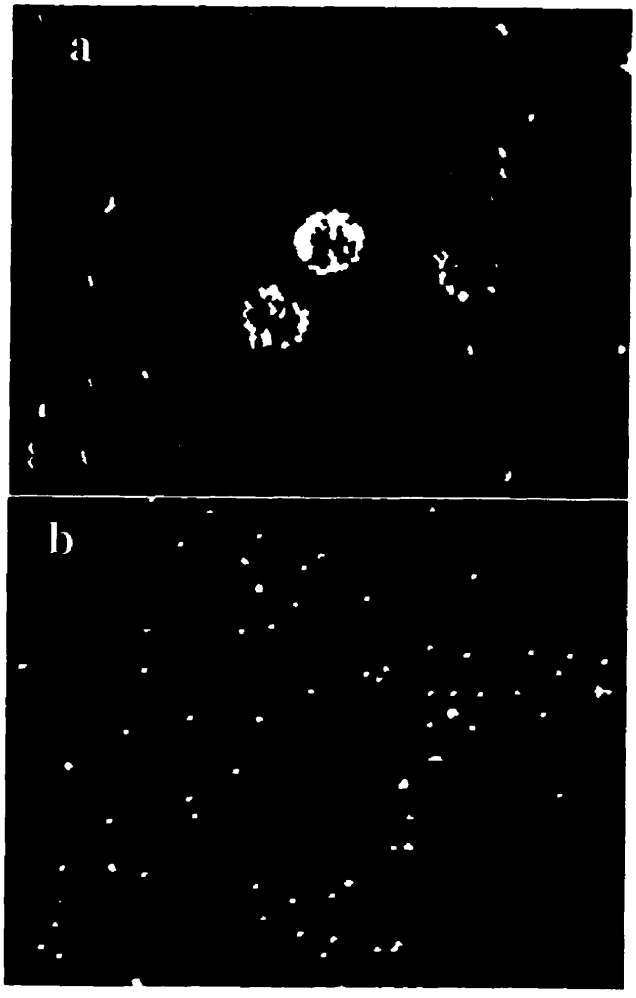
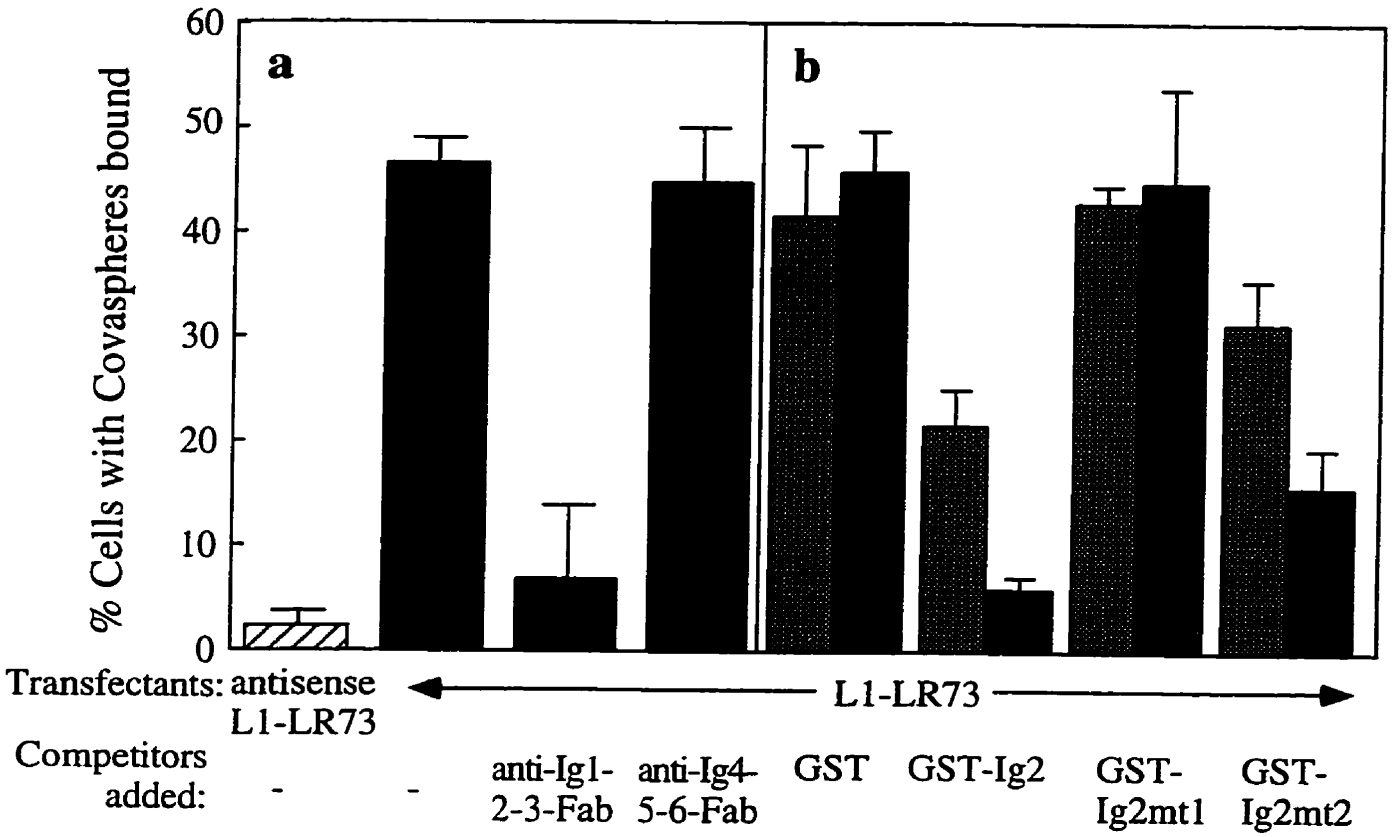

a
b

Figure III.4: Effects of mutant GST-Ig2 fusion proteins on the binding of L1-conjugated Covaspheres to L1 transfectants. In (a), binding of L1-Covaspheres to antisense-L1-LR73 cells and L1-LR73 cells. L1-LR73 cells were also precoated with either anti-Ig1-2-3 Fab or anti-Ig4-5-6 Fab (100 μg/ml) and their effects were determined. In (b), binding of L1-Covaspheres were carried out after the preincubation of L1-LR73 cells with various protein competitors at 50 μg/ml (stippled bars) or 100 μg/ml (solid bars). Identical cell samples were stained with anti-L1 antibody. The percentage of cells with bound Covaspheres were normalized to the percentage of L1-expressing cells.


60
50
40
30
20
10
0
% Cells with Covaspheres bound
a
b
Transfectants: antisense L1-LR73
L1-LR73
Competitors added:
-
-
anti-Ig1-2-3-Fab
anti-Ig4-5-6-Fab
GST
GST-Ig2
GST-Ig2mt1
GST-Ig2mt2

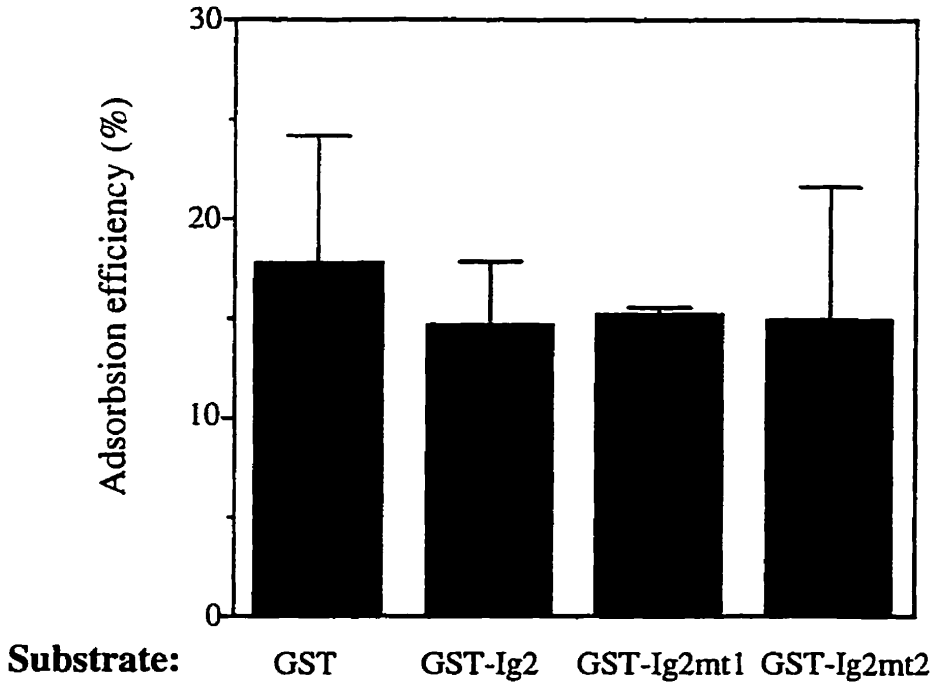


Figure III.5 Coating efficiency of L1-Ig2 GST-fusion proteins. Recombinant proteins (10 μl) in PBS was used to coat spots (~ 3 mm in diameter) on plastic Petri dishes at room temperature for 1 h. To determine the eficiency of protein coating, protein adsorbed to coverslips was solublized by incubation in 1% SDS (10 μl) at 37°C for 10 min. The relative amounts of bound and unbound protein were determined using the BCA assay. Data represent the mean ± S.D of three experiments.

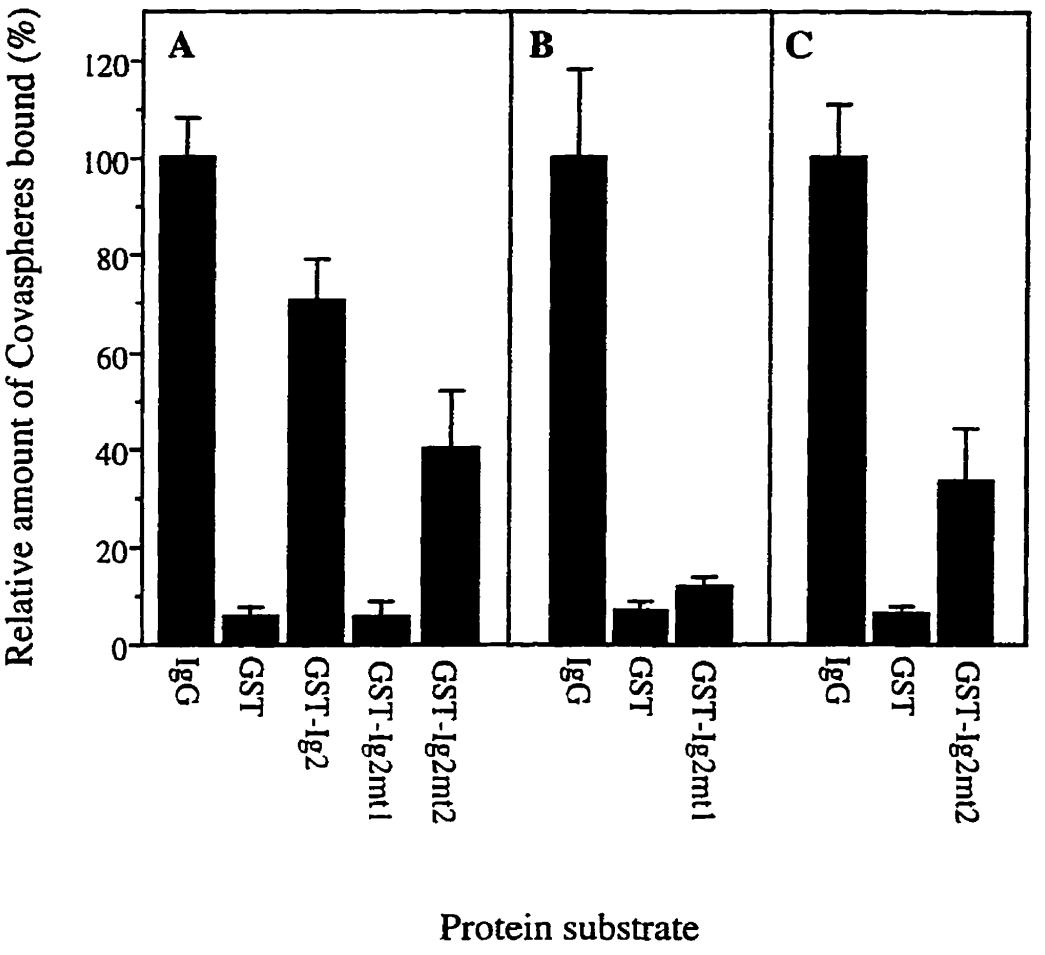


Figure III.6: Binding of Covaspheres conjugated with mutant GST-Ig2 fusion proteins to different substrates. Round spots on Petri dishes were coated with 5 µl of different proteins (1 µM). Covaspheres conjugated with GST-Ig2 (A), GST-Ig2mt1 (B), or GST-Igmt2 (C) were allowed to adhere to the protein substrate for 30 min. The number of Covaspheres attached per unit area was estimated, and the results were normalized to the amount of Covaspheres bound to the substratum coated with anti-Ig1-2-3 IgG. Data represent the mean ± S.D. (n=9).

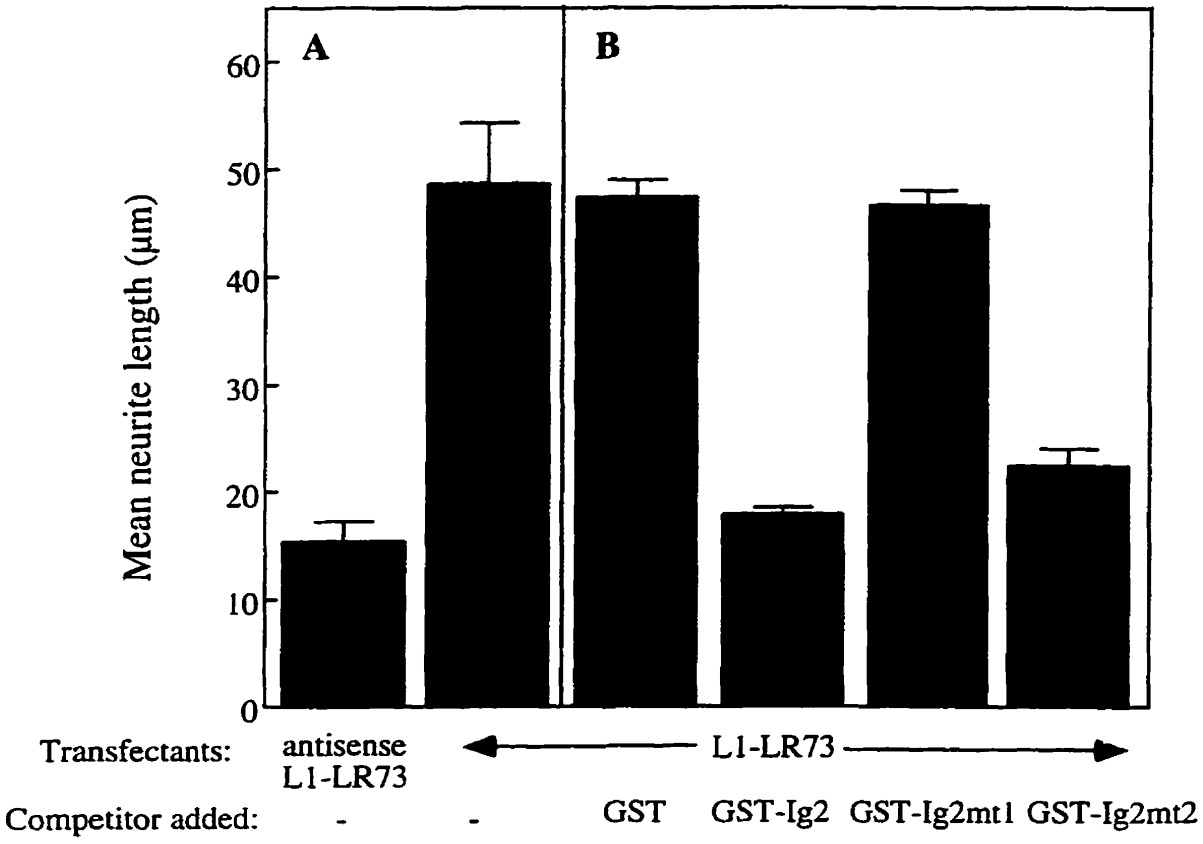
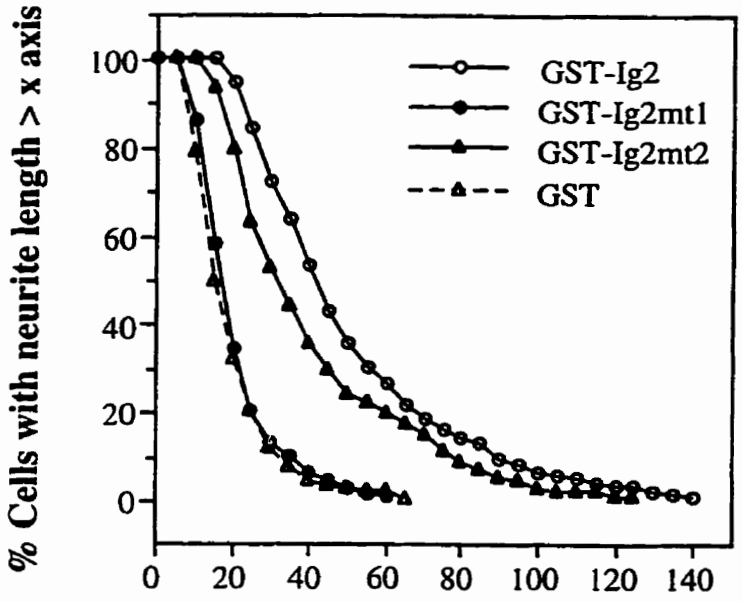
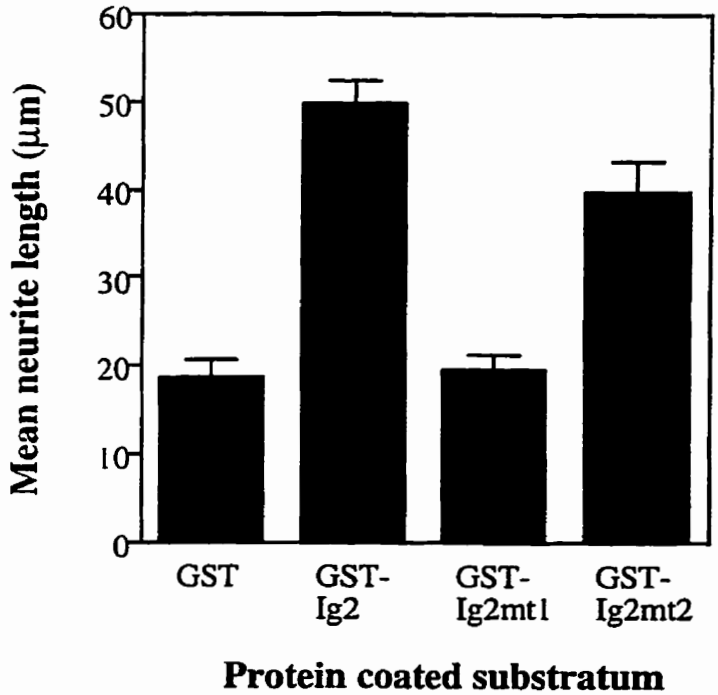


Figure III.7: Differential effects of mutant GST-Ig2 fusion proteins on neurite outgrowth. (A) Retinal cells were labeled with DiI and then deposited on a monolayer of LR73 transfectants. After 18 h of coculture, cells were fixed and neurites extended from retinal neurons were measured. (B) In competition experiments, cocultures were carried out in the presence of different competitors at 1 μM concentration. Data represent the mean ± S.D. of three experiments.

Figure III.8: Neurite outgrowth promotion activity of mutant GST-Ig2 fusion proteins. Coverslips were coated with 80 µl of GST-Ig2 fusion protein at 1 µM concentration. Retinal cells were seeded on different protein substrates and cultured for 18 h. (A) Size distribution of neurite length on mutant GST-Ig2 fusion protein substratum. (○) GST-Ig2, (●) GST-Ig2mt1, (▲) GST-Ig2mt2, and (Δ) GST control. (B) Mean neurite lengths for cells cultured on these protein substrates. Data represent the mean ± S.D. of three experiments.


% Cells with neurite length > x axis
100
80
60
40
20
0
0
20
40
60
80
100
120
140
GST-Ig2
GST-Ig2mt1
GST-Ig2mt2
GST
Neurite length (μm)


Mean neurite length (μm)
60
50
40
30
20
10
0
GST
GST-Ig2
GST-Ig2mt1
GST-Ig2mt2
Protein coated substratum

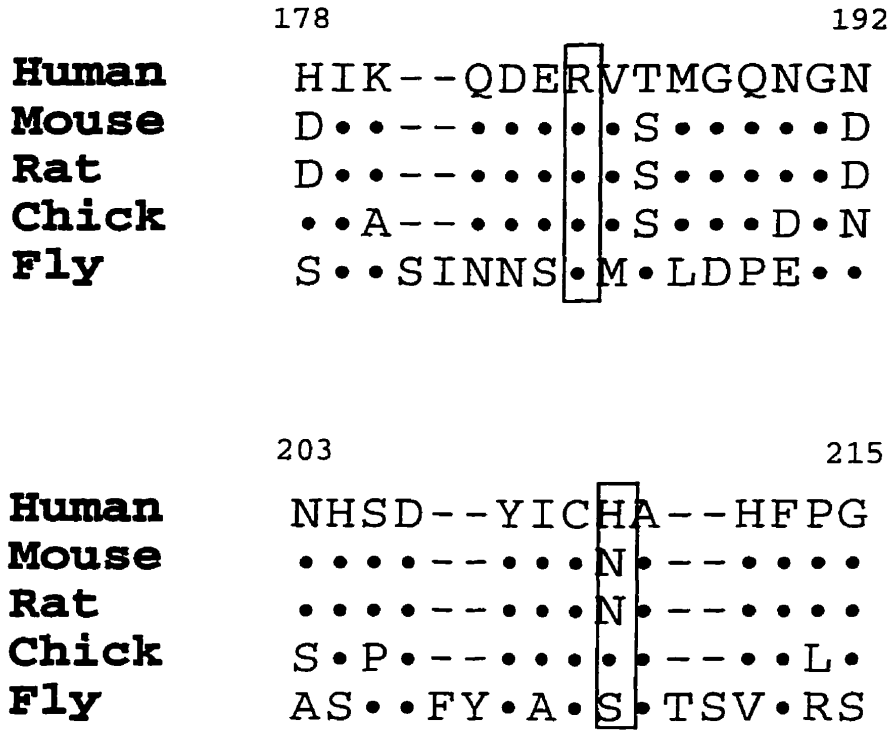


Figure III.9 Alignment of sequence flanking R184 and H210 region. Sequences flanking R184 and H210 regions from human, mouse, and rat L1, chick NgCAM, and Drosophila L1 related molecule neuroglian are aligned. Identical amino acids were represented by dots.

CHAPTER FOUR

IDENTIFICATION OF THE HOMOPHILIC BINDING SITE IN IMMUNOGLOBULIN-LIKE DOMAIN 2 OF THE CELL ADHESION MOLECULE L1

A version of this chapter has been submitted for publication.

I. INTRODUCTION

During neural development, axons are extended from numerous neurons along restricted pathways and eventually specific synaptic connections are established with their targets. These complex processes require the participation of a variety of diffusible factors and cell surface molecules (Tissier-Lagvin and Goodman, 1996). Cell adhesion molecules often constitute a favorable substrate for cell attachment and provide the permissive cues for growth cone migration and axonal extension of neuronal cells. L1 is a cell adhesion molecule expressed predominantly in the developing peripheral and central nervous system (Rathjen and Schachner, 1984). It has been implicated in a variety of developmental processes, such as neuronal migration (Lindner et al., 1983), neurite outgrowth (Lagenaur et al., 1987), axon fasciculation and path finding (Landmesser et al., 1988; Honig et al., 1996), axon regeneration (Kobayashi et al., 1995; Aubert et al., 1995), and memory (Lüthi et al., 1994; Rose et al., 1995).

L1 was first cloned and identified to be a 200 kDa transmembrane glycoprotein from mouse (Moos et al., 1988). L1 belongs to the Ig-superfamily of cell adhesion molecules, consisting of six Ig-like domains in the N-terminal region, followed by five fibronectin type III-like repeats, a transmembrane domain and a short cytoplasmic domain (Moos et al., 1988). The cell adhesion molecule L1 is known to mediate cell-cell adhesion using a Ca^{2+}-independent homophilic binding mechanism (Grumet and Edelman, 1988; Miura et al., 1992). L1-L1 interactions have been implicted in L1-promoted neurite outgrowth (Lemmon et al., 1989; Zhao and Siu, 1995). On the other hand, L1 is kown to undergo heterophilic interactions with other adhesion molecules, such as NCAM (Kadmon et al., 1990a; Horstkorte et al., 1993), TAG-1/axonin-1 (Kuhn

et al., 1991; Felsenfeld et al., 1994), F3/F11 (Brümmendorf et al., 1993), DM-GRASP (DeBermardo et al., 1996), integrins (Ruppert et al., 1995; Montgomery et al., 1996; Ebeling et al., 1996), and the extracellular matrix protein laminin (Grumet et al., 1993; Hall et al., 1997). Many of these heterophilic interactions may also have a role in L1-mediated cell adhesion and/or the promotion of neurite outgrowth.

The human L1 gene has been mapped to chromosome Xq28 (Chapman et al., 1990; Djabali et al., 1990). Recently, mutations in the coding sequence have been implicated in several related neurological syndromes: X-linked hydrocephalus (HSAS), MASA syndrome, and X-linked spastic paraplegia (SPG1) (for reviews, see Wong et al., 1995; Fransen et al., 1995; Hortsch, 1996). Since L1 mutations are distributed evenly along the entire L1 protein, it is likely that defects in either homophilic interactions or heterophilic interactions with other membrane or substrate components may lead to pathological development in the brain. The identification and characterization of specific functional domains in L1 are, therefore, important to our understanding of these defects.

We have previously demonstrated that the Ig-like domain 2 (Ig2) of human L1 harbors both homophilic binding and neuritogenic activities (Zhao and Siu, 1995). However, fusion proteins containing several extracellular domains of mouse L1 have been found to undergo self-binding (Holm et al., 1995). In this report, we have reassessed the role of the extracellular domains of human L1 in homophilic binding. Results obtained with transfectants that express either wildtype or mutant human L1 confirm the crucial role of Ig2. Interestingly, substitution of Arg-184 with Gln, a known HSAS mutation (Jouet et al., 1994) in Ig2 leads to a drastic reduction in its ability to compete for the homophilic binding site. We therefore predict that Arg-184 plays a key role in L1 homophlic interaction. Using synthetic peptides in competition experiments,

we have localized the homophilic binding site to a short peptide sequence within L1 Ig2. Peptides containing sequences flanking Arg-184 are potent inhibitors of L1-mediated cell-cell adhesion and L1-promoted neurite outgrowth from retinal cells. Taken together, these results identify a L1 homophilic binding site between His-178 and Gly-191 and demonstrate the importance of L1 homophilic binding in the promotion of neurite outgrowth.

II. EXPERIMENTAL PROCEDURES

A. Materials

The pRc/CMV vector was purchased from Invitrogen (San Diego, CA). The T7 Sequencing™ kit was purchased from Pharmacia Biotech (Baie d'Urfe, Quebec). The Geneticen disulfate (G-418 Sulfate) was purchased from ICN Biochemicals (Aurora, OH). Trypsin, α-minimal essential medium, and N2 supplement were purchased from Life Technologies, Inc. (Toronto, ON). Poly-L-lysine was purchased from Sigma (Oakville, ON), and DiI was purchased from Molecular Probes (Eugene, OR). Covaspheres were obtained from Duke Scientific Corp. (Palo Alto, CA). Synthetic oligopeptides were purchased from Vetrogen (London, ON). GST-L1 fusion proteins and domain-specific antibodies were prepared as previously described (Zhao and Siu, 1995; 1996).

B. Construction of Deletion Mutations in L1 cDNA

Standard recombinant DNA methods were followed in the construction of expression vectors (Sambrook et al., 1989). The human L1 cDNA (phL1/Blue) was obtained from Dr. Vance Lemmon (Case Western Reserve University, Cleverland, OH). A 1.1 kb *Hind* III-*Bam* HI fragment was isolated from phL1/Blue and inserted in pBluescript SKII(+) HB/SK for domain 2 deletion (L1Δ2) and DNA sequencing. The first PCR product was obtained from the HB/SK template using M13 universal primer and a reverse primer (5'-CGGAATTCCATGAGCCGGATCTCA-3') corresponding to

the coding region of L1 Ig1, and the second PCR product was obtained using the M13 reverse primer and a Ig3 forward primer (5'CGGAATTCATGATTGACAGGAGC3'). They were cut with *Hind* III/*Eco* RI and *Eco* RI/*Bam* HI, respectively, and then subcloned into the *Hind* III/*Bam* HI sites of the Bluescript vector (Δ2HB/SK), the deletion was verified by double-stranded DNA sequencing using a T7 Sequencing™ kit. The 0.9 kb *Hind* III-to-*Bam* HI fragment was isolated from Δ2HB/SK and used to replace the 1.1 kb *Hind* III-to-*Bam* HI fragment in the phL1/Blue plasmid. The L1Δ2 construct encoded a protein with a deletion of 108 amino acids between Leu-131 and Arg-239. The full length L1 and L1 Δ2 cDNAs were subcloned into the expression vector pRc/CMV, which was used to transfect LR73 cells.

C. Cell Transformation

The LR73 cell line was derived from CHO cells and was obtained from Dr. Clifford Stanners of McGill University (Zhou et al., 1993). LR73 cells were cultured in α-MEM medium containing 10% fetal bovine serum until subconfluence. Cells were collected and plated on 10-cm dishes (at 1:10 dilution) 1 day before transfection. The cells were transfected with 20 μg of plasmid DNA using the calcium phosphate precipitation method. Transfected cells were selected with 400 μg/ml of G418. G418-resistant colonies were cloned. Stable clones were tested individually for L1 expression by immunobloting. Expression of L1 on the cell surface was ascertained by immunofluorescence staining.

D. Immunofluorescence Staining of L1

L1 transfectants were seeded sparsely on coverslips 24 h before staining. Cells were washed once with PBS and then fixed with 100% methanol at -20°C for 3 min, followed by blocking with 1% BSA in PBS at room temperature for 5 min. Coverslips were incubated with mouse anti-L1 monoclonal antibody 74-5H7 (Lemmon et al., 1989) at room temperature for 60 min. After five washes with PBS, the cells were incubated sequentially for 50 min with biotinylated goat anti-mouse IgG (1:1000 dilution with 1% BSA in PBS) and streptavidin-conjugated Texas Red (1:500 dilution in 1% BSA). Coverslips were mounted in 80% glycerol and observed using a Zeiss axiovert confocal microscope.

E. Cell Aggregation Assay

L1 transfectants were plated on dishes 20 h before assay to ensure that they were in their exponential growth phase. Cells were dissociated in PBS containing 2 mM EDTA and then collected. Cells were centrifuged at 500 rpm for 5 min, followed by resuspension in α-MEM medium containing 1% fetal bovine serum at 5 x 10^6 cells/ml. Cell reassociation was carried out in aliquots of 200 µl and rotated at 200 rpm on a platform shaker at 37°C. At regular time intervals, the number of nonaggregated cells was counted using a hemocytometer and the percentage of cell aggregation was calculated. In inhibition assays, different amounts of competitors were added to 100 µl aliquots of cells and the final volume was adjusted to 200 µl. The cell samples were incubated on ice for 10 min before the aggregation assay. Cells were allowed to

reassociate at 37° C for 45 min. The percentage of inhibition of aggregation was calculated relative to the percentage of aggregation of the control sample which did not contain any competitor.

F. Covasphere-to-Substratum Binding Assay

The Covasphere-to-substratum attachment assay was carried out as described previously (Zhao and Siu, 1995). Green MX Covaspheres (50 µl) were dispersed by brief sonication before the addition of 10 µg of GST-Ig2 and the final volume was adjusted to 100 µl with PBS. The sample was incubated for 75 min at room temperature on a rotator. Excessive binding sites on Covaspheres were blocked with 1% (w/v) BSA. After washing with PBS, the GST-Ig2-conjugated Covaspheres were resuspended in 50 µl PBS and stored at 4° C. GST-Ig2 (5 µl of a 3 µM stock solution) was used to coat 2-mm spots on plastic Petri dishes (microbiological grade) at room temperature for 1 h. The fusion protein-coated spots were blocked with 1% BSA in PBS. Then GST-Ig2-conjugated Covaspheres at 1:30 dilution was added to these spots. After 30 min of incubation at room temperature, the unbound beads were washed off gently using 5 changes of PBS. Samples were observed using a Wild Leitz orthoplan epifluorescence microscope. Random fields were recorded by VCR and the relative amounts of bound Covaspheres per unit area were estimated using the NIH Image program. In competition experiments, Covaspheres were pre-incubated with competitors at room temperature for 10 min.

G. Neurite Outgrowth Assay

The neurite outgrowth assay was carried out as previously described (Zhao and Siu, 1995). Neural retinal cells from day 5 chick embryos were isolated and labeled with DiI as described by Sandig et al. (1994). Round glass coverslips were first coated with 0.01% (w/v) poly-L-lysine at room temperature for 3 h and then with GST-Ig2 (1 μM) at 4° C overnight. The coverslips were washed once with sterilized H_2O and then seeded with retinal cells in N2 medium as described previously (Sandig et al., 1994). In inhibitory studies, peptides were included in the culture medium at 1 mg/ml.

Alternatively, DiI-labeled neural retinal cells were plated on top of confluent monolayers of LR73 transfectants. Co-cultures were carried out in N2 medium at 37° C for 16 h. Cells were fixed with 3.7% formaldehyde in PBS and then examined by epifluorescence microscopy. Images of neurites were recorded and ~100 neurites were measured for each experiment.

III. RESULTS

A. Differential Localization of Wildtype and Mutant L1 on Transfectants

Using fusion proteins in *in vitro* assays, we have shown that the homophilic binding of site of human L1 resides within Ig2 (Zhao and Siu, 1995). To extend these studies, LR73 cells were transfected with expression vectors containing cDNA encoding either intact L1 or mutant L1 with Ig2 deleted (L1 Δ2) (Figure IV.1). This approach allowed the presentation of L1 in its natural environment, the plasma membrane. LR73 cells were chosen because they do not express L1 and are unable to form large aggregates in cell reassociation assays (Zhou et al., 1993). As a control, LR73 cells were transfected with an antisense L1 cDNA. Stable transfectants expressing comparable amounts of L1 or L1 Δ2 were selected for further studies.

To examine the subcellular localization of L1, LR73 transfectants expressing either L1 or L1 Δ2 were subjected to immunofluorescence staining. L1 staining was predominantly associated with the plasma membrane of both L1-LR73 cells and L1 Δ2-LR73 cells. Cells transfected with the antisense-L1 cDNA construct did not show positive L1 staining. Laser scanning confocal microscopy revealed a biased surface distribution of L1 in cell aggregates. L1 was enriched in the cell-cell contact regions of L1-LR73 cells. X/Z optical sections showed that intense L1 staining was present uniformly along the long axis of the contact surface (Figure IV.2, panels a and b), consistent with the notion that L1 is a cell adhesion molecule. In contrast, L1 Δ2 distributed fairly evenly on the cell surface and failed to concentrate in the intercellular

contacts of L1 Δ2-LR73 transfectants (Figure IV.2, panels c and d). Quantitative analysis showed that >80% of the L1-LR73 cell pairs had an enrichment of L1 in contact regions, while only 8% of the L1 Δ2-LR73 cell pairs exhibited a similar staining pattern. These results, therefore, indicate that the enrichment of L1 in intercellular contact regions is dependent on the second Ig-like domain of L1.

B. Aggregation of LR73 Transfectants

When LR73 transfectants were subjected to the cell aggregation assay, cells expressing wildtype L1 showed an increase in intercellular cohesiveness and formed large aggregates (Figure IV.3). In contrast, transfectants expressing L1 Δ2 failed to form aggregates. A small number of L1 Δ2-LR73 cells, however, were able to form two- to five- cell aggregates after a prolonged period of shaking. Figure IV.4 shows the kinetics of cell reassociation by different transfected cell lines. Aggregates of L1-LR73 cells formed rapidly, and ~80% of the cells were recruited into large aggregates within the first 20 min. However, only 35% of L1 Δ2-LR73 cells were able to form small aggregates after 60 min of rotation (Figure IV.4). Similar results were obtained with cells transfected with the antisense construct, suggesting that the extracellular domains, other than Ig2, do not contribute significantly to the adhesive property of L1.

C. Inhibition of L1-LR73 Cell Aggregation by Antibodies and Fusion Proteins

To demonstrate that L1-mediated cell-cell adhesion among L1-LR73 cells was dependent primarily on the adhesive interactions centered at Ig2, antibody inhibition

experiments were carried out. Antibodies directed against Ig1-2-3 of L1 showed dose-dependent inhibitory effects on the reassociation of L1-LR73 cells (Figure IV.5). In contrast, antibodies directed against Ig4-5-6 of L1 did not exert significant effects on L1-mediated cell-cell adhesion. Furthermore, the fusion protein GST-Ig2 was able to inhibit L1-LR73 cell aggregation in a dose-dependent manner, whereas GST had no effect (Fig IV.5).

Previously, we found that the HASA mutation (Arg184Gln) could abolish the adhesive property of Ig2, while the MASA mutation (His210Gln) had a milder effect (Zhao and Siu, 1996). When L1-LR73 cells were reassociated in the presence of GST-Ig2 fusion proteins carrying these mutations, both GST-Ig2mt1 (R184Q) and GST-Ig2mt2 (H210Q) showed lower levels of inhibition than GST-Ig2, with the fusion protein carrying the HASA mutation being the least inhibitory (Figure IV.6).

D. Inhibitory Effects of Synthetic Peptides on Cell Aggregation

The above results point to the importance of the Arg-184 residue. It is likely that Arg-184 is either part of or in proximity to the homophilic binding site of L1. To test this hypothesis, synthetic peptides that spanned the flanking regions of Arg-184 and His-210 were prepared and their ability to compete for L1-L1 binding was determined. The peptide L1-A contained the sequence between His-178 and Gly-191, while peptide L1-B contained the sequence between Asp-202 and Gly-215 (Figure IV.7). When the reassociation of L1-LR73 cells was assayed in the presence of these peptides, peptide L1-A was found to be a potent inhibitor and the percentage of cell aggregation was reduced from 90% to 40% (Figure IV.8). Under these conditions, cells failed to form large

aggregates and most of the aggregates contained only a few cells. On the other hand, peptide L1-B did not exert significant effects on the reassociation of L1-LR73 cells, which were still capable of forming large aggregates (Figure IV.8). Similar observations were made with the control peptide P7.

E. Effects of Synthetic Peptides on Ig2-mediated Covasphere-to-substratum Attachment

To demonstrate that peptide L1-A could compete for the homophilic binding site in L1 Ig2, GST-Ig2-conjugated Covaspheres were mixed with different amounts of peptides before seeding onto a substratum coated with GST-Ig2. After 30 min, the unbound Covaspheres were removed and the number of Covaspheres attached on the substratum was counted. Peptide L1-A inhibited the binding of Covaspheres to substrate-coated GST-Ig2 in a dose-dependent manner, 50% inhibition was achieved at ~0.08 mg/ml (Figure IV.9). The control peptide P7 had no effect in this assay. Peptide L1-B, on the other hand, had only a small effect and the attachment to the GST-Ig2 to substrate was reduced by <20% (Figure IV.9). These results are consistent with the notion that the amino acid sequence between His-178 and Gly-191 plays an active role in L1-L1 homophilic binding.

F. Side-Chain Requirements in the L1 Homophilic Binding

To investigate the relative contribution of the amino acids in the peptide L1-A in L1-L1 homophilic interaction, analogues of peptide L1-A containing different amino acid

substitutions (Figure IV.7) were tested for their ability to compete for L1 binding. In the cell aggregation assay, L1-LR73 cells were reassociated in the presence of either 1 mg/ml or 2 mg/ml of peptide (Figure IV.10). Among the five peptide analogues, peptide L1-AIII was the least effective and it inhibited cell reassociation by <10%. Peptide L1-AIII contained an Arg-184 to Ala substitution, thus highlighting again the importance Arg-184 in homophilic interaction. When the charged residues Lys-180, Asp-182 and Glu-183 were substituted with alanine in L1-AII, the inhibitory effects of this peptide analogue was reduced to 15%. When the hydrophobic residues Val-185 and Met-187 were substituted with alanine in peptide L1-AIV, a similar loss of inhibitory activity was observed. In contrast, when Ile-179 and Gly-188 were substituted with serine, in peptide L1-AI and peptide L1-AV, respectively, both peptide analogues retained the full inhibitory effect identical to that of peptide L1-A (Figure IV.10).

The inhibitory effects of these peptide analogues were also tested using the Covasphere-to-substratum binding assay. The results were similar to those obtained with the cell aggregation assay (Figure IV.11). Substitution of either the charged residues or the hydrophobic residues in peptide analogues L1-AII, L1-AIII, and L1-AIV led to the complete loss of inhibitory effects in the Covasphere-to-substratum attachment assay (Figure IV.11). These results confirm the importance of the hydrophilic and hydrophobic side-chains adjacent to Arg-184 in L1 homophilic binding. On the other hand, the peptide analogues L1-AI and L1-AV were fully active. At 1 mg/ml of peptide, they inhibited Covasphere attachment to a level similar to that of peptide L1-A (Figure IV.11).

G. Loss of Neuritogenic Activity in L1 Δ2

To investigate the relationship between homophilic binding and the neuritogenic activity of L1, we first examined the ability of LR73 cells to promote neurite outgrowth from neural retinal cells. Retinal cells were labeled with DiI and cultured overnight on a monolayer of LR73 transfectants expressing either wildtype L1 or L1 Δ2 and neurite lengths extending from neurons were measured (Figure IV.12A). As a negative control, retinal cells were cultured on a monolayer of LR73 cells transfected with the antisense L1 construct. LR73 cells expressing intact L1 promoted neurite outgrowth from retinal cells, which gave a mean neurite length of ~50 μm (Figure IV.12B). However, when Ig2 was deleted, the mutant form of L1 failed to promote neurite outgrowth from retinal neurons. Retinal cells cultured on L1 Δ2-LR73 cells had a mean neurite length of ~20 μm, similar to that obtained with retinal cells seeded on the antisense L1- transfectants.

H. Inhibition of L1-dependent Neurite Outgrowth by Synthetic Peptides

The above results suggest that the neuritogenic activity of Ig2 might be linked to the homophilic binding activity of L1. It is conceivable that protein-protein interactions at the homophilic binding site may trigger a signaling cascade leading to neurite outgrowth. To test this hypothesis, synthetic peptides were used to inhibit L1 homophilic interaction and their effects on neurite outgrowth was examined. Retinal cells were cultured on substrate-coated GST-Ig2 in the presence of different synthetic peptides. In the presence of 1 mg/ml of peptide L1-A, relatively short neurites were observed and only 15% of the neurites were longer than 30 μm (Figure IV.13A). In contrast, ~70% of

neurites longer than 30 μm when retinal cells were cultured either in the absence of peptide or in the presence of the control peptide P7 (Figure IV.13A). Inhibition of homophilic interaction thus led to the abrogation of the neuritogenic activity associated with Ig2.

Next, we examined whether there was any correlation between the effects of peptide analogues on neurite outgrowth and their effects on cell-cell adhesion. Interestingly, peptide that inhibited cell-cell adhesion were also potent inhibitors of neurite outgrowth from retinal cells (Figure IV.13B). Substitutions that resulted in the loss of inhibitory effects on L1 homophilic binding were also inefficient in inhibiting neurite outgrowth. A strong correlation, therefore, exists between the homophilic binding activity and the neuritogenic activity of L1 Ig2.

IV. DISCUSSION

In this study, we have confirmed the pivotal role played by Ig2 in L1 homophilic binding and mapped the homophilic binding site to a 14 amino acid sequence. Among all the extracellular domains of L1, Ig2 has the greatest interspecies sequence conservation (Hlavin et al., 1991). The conservation of the Ig2 is likely related to its importance in L1 function. Experiments using L1-transfected LR73 cells demonstrate that Ig2 is involved in *trans*-interactions between L1 molecules on apposing cells. Weak interactions between Ig1 and fragments containing Ig3 have also been observed (Holm et al., 1995; Zhao and Siu, 1995). It is conceivable that initial interactions centering at Ig2 may lead to the alignment of two L1 molecules in an anti-parallel manner, followed by binding interactions at secondary sites between Ig1 and Ig3 in a double reciprocal manner. The latter interactions might have a significant contribution to the stability of L1-L1 binding. Weak heterologous interactions between the Ig domain pairs and the fibronectin type III repeats have also been observed (Holm et al., 1995). Since deletion of Ig2 abrogates L1-L1 binding, these interactions may not be strong enough to support homophilic binding. On the other hand, intramolecular interactions may occur and may in turn regulate the availability of L1 domains for intermolecular interactions.

Electron microscopy and computer-assisted modeling suggest that the extracellular domains of L1 are organized linearly into a helical superstructure (Drescher et al., 1996). While the N-terminal Ig-like domains are more extended to form a fibrillar structure, the fibronectin type III repeats are organized into a globular structure. The extracellular region of L1 can adopt several different shapes, suggesting that the molecule

is fairly flexible. This flexibility should facilitate the N-terminal Ig-like domains to seek out the appropriate binding partner on an approaching cell.

In many respects, the homophilic interaction of L1 resembles that of NCAM (Ranheim et al., 1996). NCAM contains five Ig-like domains, followed by a proline-rich hinge region and two fibronectin type III repeats (Cunningham et al., 1987). The homophilic binding site of NCAM has been mapped to Ig3 (Rao et al., 1992). Deletion or point mutations introduced in the homophilic binding site can abrogate NCAM-NCAM interaction (Rao et al., 1993). While only Ig3 is capable of homophilic binding, the other Ig-like domains can undergo interactions with each other, notably Ig1 with Ig5 and Ig2 with Ig4 (Ranheim et al., 1996). Also, deletion of any one of the these four Ig-like domains affects NCAM redistribution to the cell contact region (Sandig et al., 1996) and reduces NCAM-dependent cell aggregation (Rao et al., 1992). Although interactions between Ig1-2 and Ig4-5 alone cannot support NCAM homophilic binding, interactions between these four Ig-like domains may contribute to the overall avidity and stability of NCAM homophilic binding initiated at Ig3. Therefore, both NCAM and L1 appear to adopt a common strategy in homophilic binding.

The synthetic peptide approach has narrowed down the homophilic binding site in L1 Ig2 to the 14 amino-acid sequence, 178-HIKQDERVTMGQNG-191. An alignment of the human L1 homophilic binding site with similar sequences in L1 homologues is shown in Figure IV.14. Four amino acid residues: Ile-179, Arg-184, Val-185, and Gly-191, are conserved among all the vertebrate species examined so far. Arg-184 and Gly-191 are conserved even in tobacco hornworm and fruitfly, suggesting that they may have important structural or functional roles. Significantly, three HASA mutations, I179S, R184Q and R184W, have been found in this sequence (Ruiz et al., 1995; Jouet et al.,

1994; Wong et al., 1995; MacFarlan et al., 1997) and a fourth HSAS mutation Y194C is located in the C-terminal region (Gu et al., 1996). This cluster of HASA mutations thus highlights the functional importance of this region. We have previously observed that substitution of Arg-184 with Gln leads to a drastic decline in homophilic binding capability of L1 Ig2, while the MASA mutation His210Gln has only modest effects (Zhao and Siu, 1996). Similarly, the synthetic peptide L1-A which contains sequences flanking Arg-184 is a potent inhibitor of L1 homophilic binding, whereas peptide L1-B which contains sequences flanking His-210 has relatively minor effects (Figure IV.8). These results suggest that His-210 and its flanking regions may not participate directly in homophilic binding. Since His-210 is situated next to the Cys residue which is probably involved in intramolecular disulfide bond formation, mutation in His-210 may result in conformation changes that are unfavorable to homophilic interaction.

By analogy to the known crystal structures of telokin (Holden et al., 1992), the L1 Ig2 domain has been predicted to adopt a sandwich structure consisting of two β-sheets (Bateman et al., 1996). One β-sheet contains strands A, B, E and D, while the other one contains stands A', F, G, C and C'. The homophilic binding site lies in the C'D region of Ig2 and Arg-184 is predicted to be part of a buried salt bridge. Their predictions suggest that the side-chain of Arg-184 is buried and may contribute of the stability of the conformation of the Ig-fold. However, our results show that when Arg-184 is substituted with Ala in peptide L1-AIII, the inhibitory effects of the peptide is almost completely lost. This would suggest that the side-chain of Arg-184 is exposed and involved in stable binding with Ig2. Further analysis of the side-chain requirements of the L1 homophilic binding site has shown that substitution of either the hydrophobic residues or charged residues flanking Arg-184 with Ala also renders these peptide analogues ineffective in

competing for the L1 homophilic binding site. These results suggest that L1 homophilic binding involves both electrostatic and hydrophobic interactions at this site. The charged residues in the C'D loop may serve an important function in the initial docking mechanism between two apposing L1 molecules. Stability of the binding may be achieved by subsequent hydrogen bond formation and hydrophobic interactions.

It is of interest to note that the NCAM homophilic binding site in Ig3 also maps to the C'D region of the Ig-fold (Rao et al., 1993). The cell adhesion molecule gp80 of *Dictyostelium discoideum* which shows partial structural and sequence resemblance with NCAM and immunoglobulins (Matsunaga and Mori, 1987; Siu and Kamboj, 1990), also has its homophilic binding site residing on a β-strand and its following loop structure (Kamboj et al., 1989; Siu, 1990). It is therefore likely that a common structural motif for homophilic binding has been preserved among distantly related members of the Ig superfamily, even though the binding sequences have diverged. In the case of NCAM and gp80, the homophilic binding sequence is capable of isologous interaction with the same sequence on the apposing molecule (Kamboj et al., 1989; Rao et al., 1994). Similar to L1, both charged and hydrophobic side-chains are crucial to the binding reaction.

In addition to homophilic binding, L1 Ig2 is also capable of promoting neurite outgrowth from primary neuronal cells (Zhao and Siu, 1995). L1 Δ2 presented on the surface of LR73 transfectants are unable to stimulate neurite outgrowth from neural retinal cells, indicating an absolute requirement for Ig2. Consistent with these results, a recombinant protein containing Ig1-2 has been shown to promote neurite outgrowth from small cerebellar neurons (Appel et al. 1993). Peptides that compete for L1 binding also prevent the stimulation of neurite outgrowth on L1 substrate. This result thus suggests that homophilic interactions are required to generate signals that lead to neurite

outgrowth. In contrast to NCAM where binding of peptides to the homophilic binding site can elicit neurite outgrowth (Sandig et al., 1994), binding of peptide pL1-A to L1 has no stimulatory effects, suggestion that L1 clustering and/or L1-dependent adhesion to the substratum may be required.

The relationship between homophilic binding and signal transduction is not well understood. It is possible that homophilic binding events may trigger conformational changes and the signal is then transmitted down the molecule to its cytoplasmic domain. Appel et al. (1995) have found that a monoclonal antibody that recognizes a region between the fibronectin type III repeats 2 and 3 is capable of stimulating neurite outgrowth upon binding small cerebellar neurons. This finding suggests that this region may be a key sensor of signals generated by homophilic binding and be responsible for the activation of the intracellular domain of L1. Also, the clustering of L1 molecules, whether by antibody crosslinking or by cell contact formation, may facilitate the recruitment of signaling molecules to the plasma membrane.

The signaling pathway(s) involved in L1-dependent neurite outgrowth is currently an active area of research. The highly conserved cytoplasmic domain of L1 is believed to be involved in signal transduction. The cytoplasmic domain of L1 is phosphorylated primarily on its serine sites and is associated with casein kinase II (Wong et al., 1996a). L1 is also associated with and phosphorylated by $p90^{rsk}$, suggesting L1 signaling may involve activation of the MAP kinase pathway (Wong et al., 1996b). Also, several reports have shown a functional association of L1 with the fibroblast growth factor receptor 1 (Williams et al., 1994a; Doherty et al., 1995). Recently, a dominant negative form of the fibroblast growth factor receptor has been shown to inhibit neurite outgrowth on substrate-coated L1 and other cell adhesion molecules (Saffell et al., 1997). Ignelzi et al.

(1994) showed that L1-stimulated neurite outgrowth is dependent on the presence of the nonreceptor tyrosine kinase $pp60^{c\text{-}src}$, implicating tyrosine phosphorylation in the signaling cascade. Pharmacological studies have also confirmed a role for tyrosine phosphorylation in L1 signaling (Williams et al., 1994b). More recently, tyrosine phosphorylation of L1 cytoplasmic domain by the Eph kinase Cek5 has been demonstrated in both *in vitro* and *in vivo* studies (Zisch et al., 1997), suggesting that L1 and cek5 may undergo cis-interaction. Finally, the association of ankyrin with members of the L1 family is regulated by tyrosine phosphorylation/dephosphorylation of a highly conserved cytoplasmic site (Garver et al., 1997). Interactions with ankyrin would anchor L1 to focal contact sites and facilitate cell-cell adhesion and neurite outgrowth (Davis and Bennett, 1994). It is therefore evident that protein phosphorylation and dephosphorylation are important regulatory events in the signaling cascade triggered by L1. Further work is now required to determine how the different kinases, phosphatases and the various downstream components and linked in this signaling pathway.

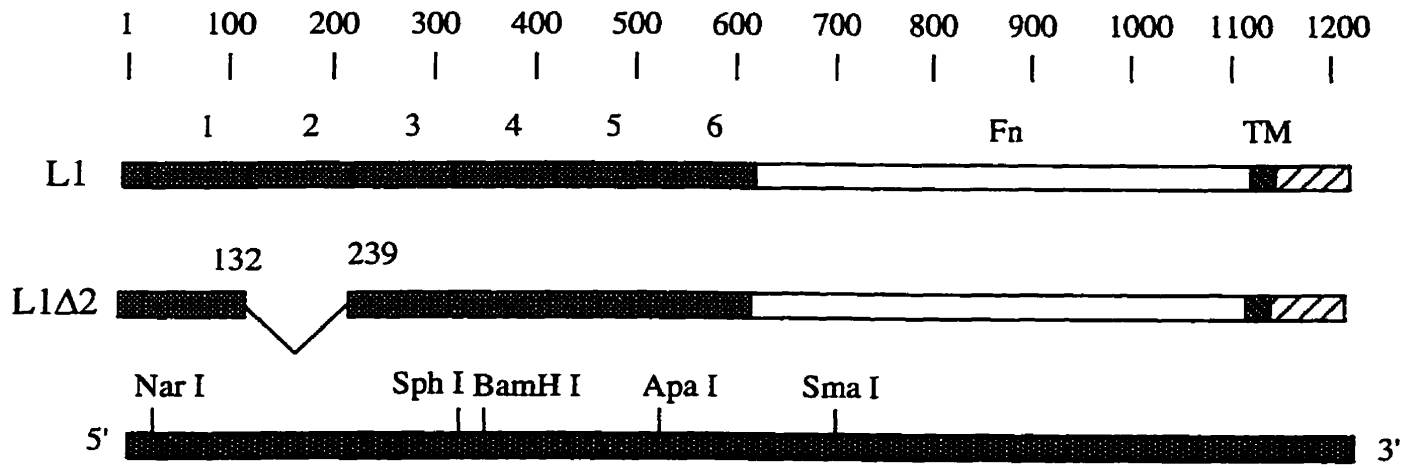


Figure IV.1 Construction of wildtype and mutant L1 in LR73 cells. Schematic drawings of L1 cDNA constructs subcloned into the expression vector of pRc/CMV.

Figure IV.2 Confocal micrographs of LR73 transfectants. LR73 cells stably transfected with L1 or L1Δ2 cDNA constructs were fixed and immunostained with monoclonal antibody 74-5H7. (a) An X/Y image of cells expressing intact L1; (b) an X/Z image of the cell pair shown in (a). (c) An X/Y image of cells expressing L1Δ2; (d) an X/Z image of the cells shown in (c). The lines in (a) and (c) indicate the location of the X/Z scans shown in (b) and (d), respectively. Bars = 10 μm.

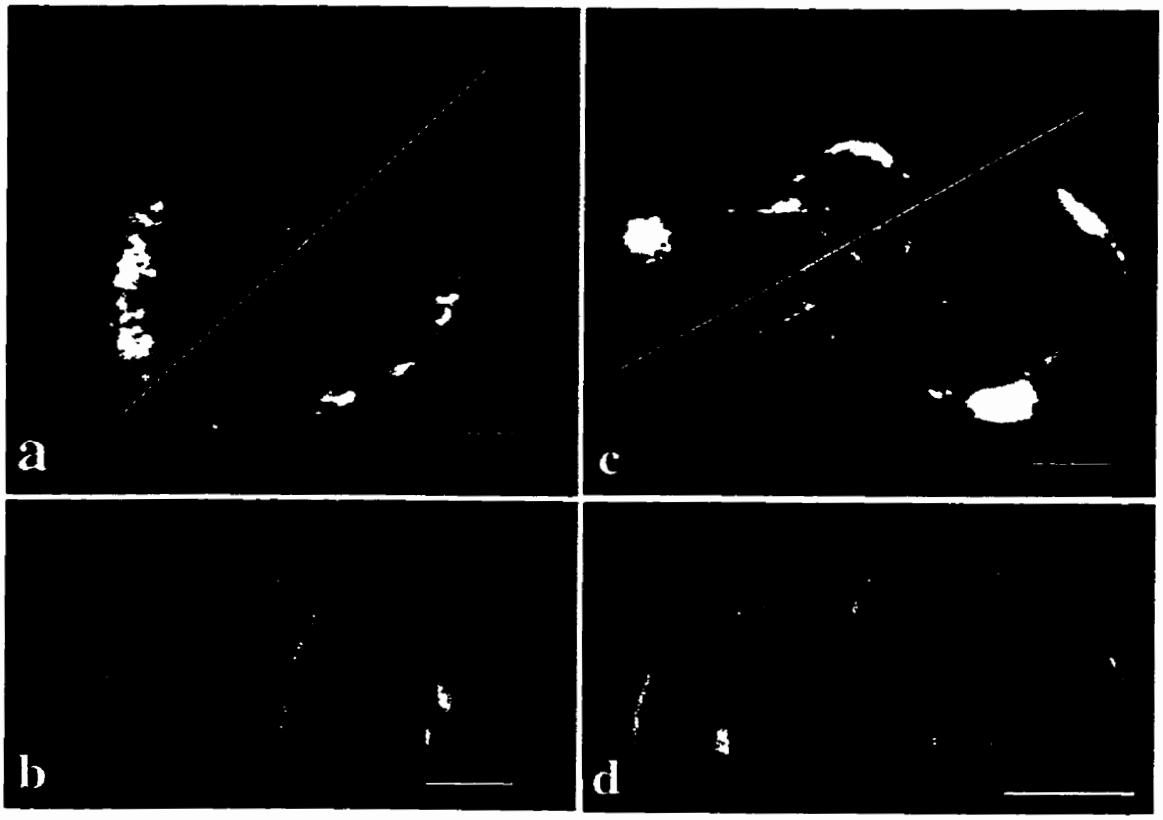
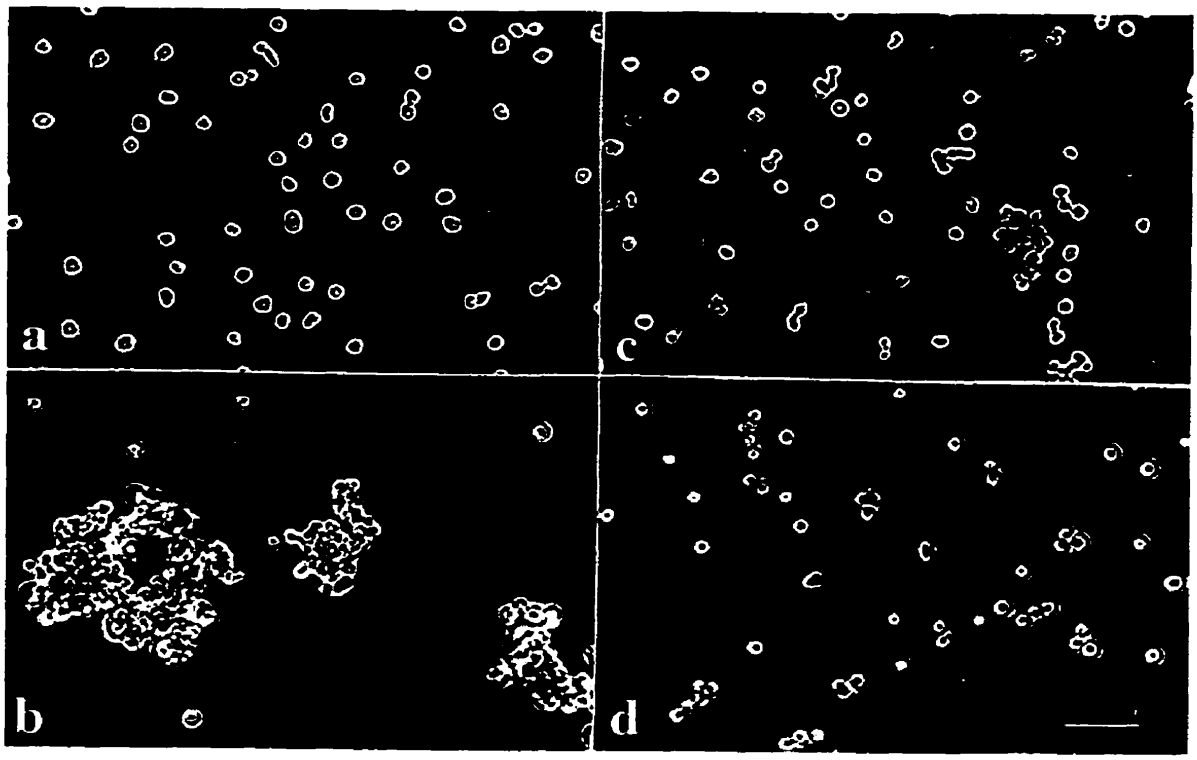

a
c
b
d

Figure IV.3. Phase constrast micrographs of cell aggregates. LR73 transfectants were dissociated by EDTA and resuspended in α-MEM at 5 x 10^6 cells/ml. Cell reassociation was carried out on a platform shaker rotating at 200 rpm and samples were examined at different time intervals. (a) L1-LR73 cells at 0 min of the assay; (b) L1-LR73 cells at 45 min; (c) L1-LR73 cells after rotating in the present of 0.5 mg/ml of soluble GST-Ig2 for 45 min; (d) L1Δ2-LR73 cells at 60 min. Bar = 50 μm.


a
c
b
d

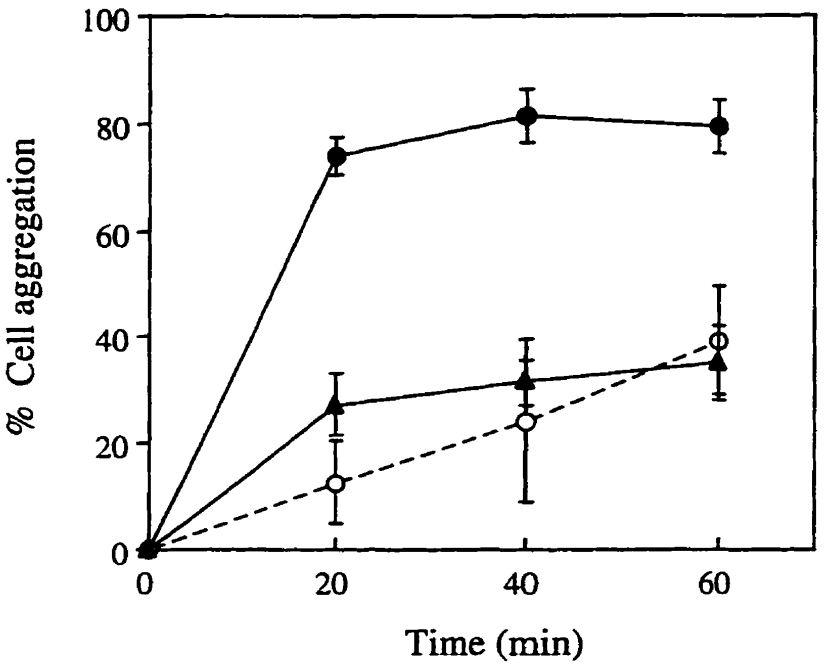


Figure IV.4. Aggregation of L1-transfected LR73 cells. LR73 cells with or without L1-expression were disassociated by 2 mM EDTA and transferred to α-MEM at $5x10^6$cell concentration. Cells were shaked at 37°C at 200 rpm. Numbers of single cells were counted at different time points and the percentage of cell aggregation was calculated. (●) L1/LR73 cells, (O) antisense L1/LR73 cells, and (▲) L1Δ2/LR73 cells were used. Data represents the mean of three experiments.

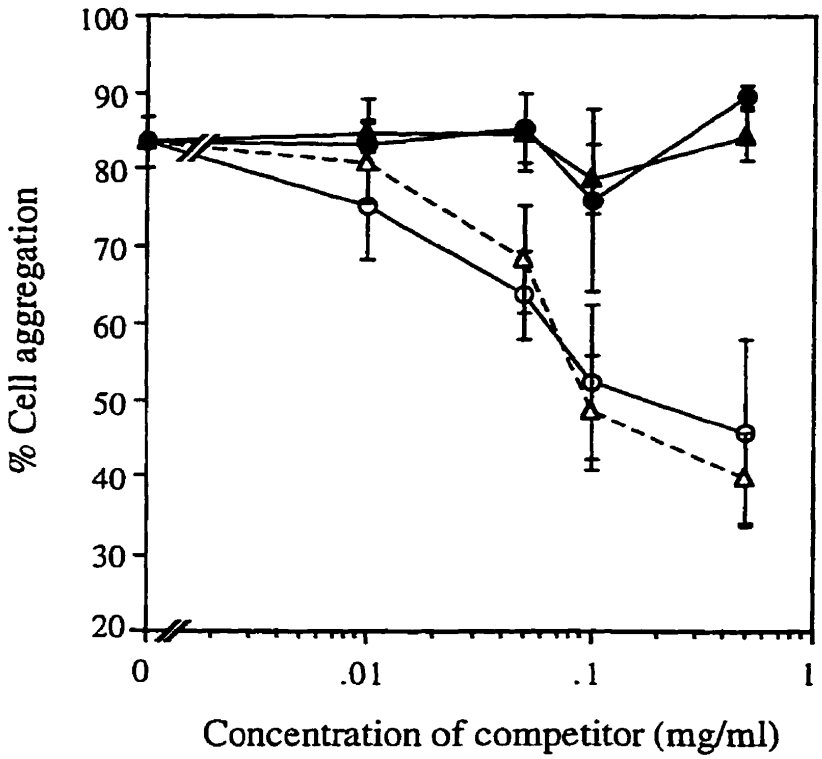


Figure IV.5. Inhibition of cell reassociation by fusion proteins and antibodies. L1-LR73 cells were dissociated with EDTA and resuspended in α-MEM at 5 x 10^6 cells/ml. Cells were first incubated on ice for 10 min with different concentrations of competitors: GST-Ig2 (○), GST (●), anti-L1-Ig1-2-3 Fab (Δ), and anti-L1-Ig4-5-6 Fab (▲). Cell reassociation was carried out at 37°C on a platform shaker rotating at 200 rpm. The percentage of cell aggregation was estimated after 45 min. Data represent the mean ± S.D. of three experiments.

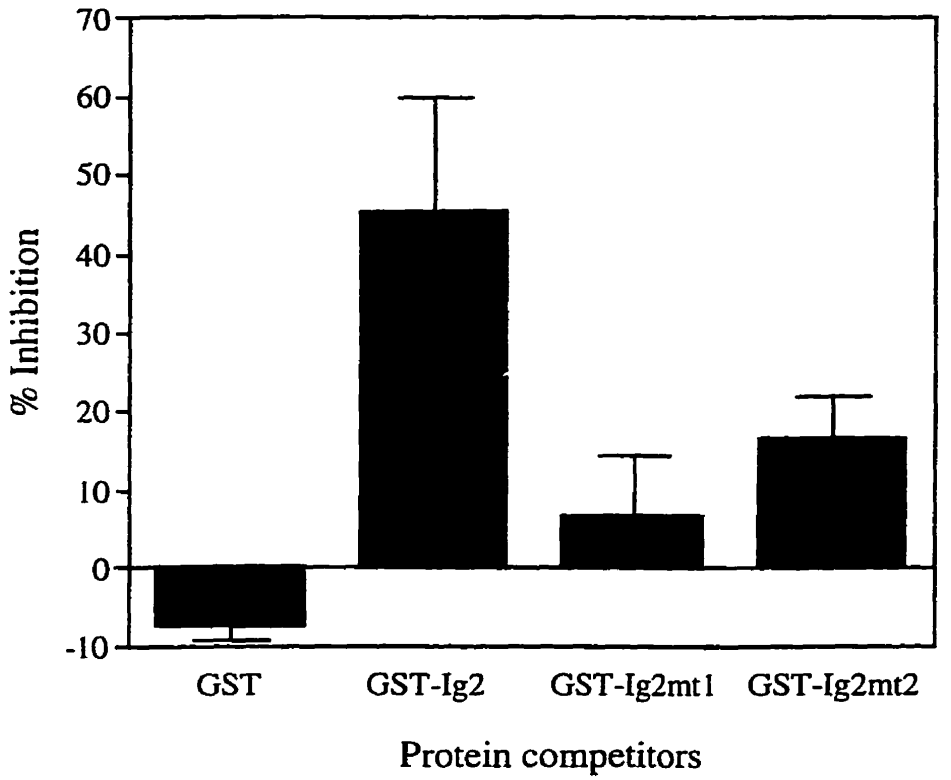


Figure IV.6. Effects of fusion proteins carrying HASA and MASA mutations on cell aggregation. L1-LR73 cells were dissociated with EDTA and resuspended at 5 x 10^6 cells/ml in α-MEM in the presence of 0.5 mg/ml of the competitor. The percentage of cell aggregation was estimated after 45 min of reassociation and the percentage of inhibition was calculated relative to the control. Data represent the mean ± S.D. of three experiments.

Figure IV.7. List of synthetic peptides used in competition experiments. The L1 Ig2 sequence between Cys-158 and Lys-222 is listed, with Arg-184 (HASA mutation position) and His-210 (MASA mutation position) shown in boldface type. Peptide L1-A contains the flanking sequences of Arg-184 and peptide L1-B contains the flanking sequences of His-210. Substitutions in the L1-A analogues are shown in boldface type and underlined.

158-CNPPPSAEPLRIYWMNSKILHIKQDE**R**VTMGQNGNLYFANVLTSDNHSDYIC**H**AHFPGTRTIIQK-222

Peptides:

L1-A	HIKQDERVTMGQNG
L1-AI	H**S**KQDERVTMGQNG
L1-AII	HI**A**Q**AA**RVTMGQNG
L1-AIII	HIKQDE**A**VTMGQNG
L1-AIV	HIKQDER**A**T**A**GQNG
L1-AV	HIKQDERVTM**S**QNG
L1-B	DNHSDYICHAHFPG
P7	TKDGEPIEQEDNEEK

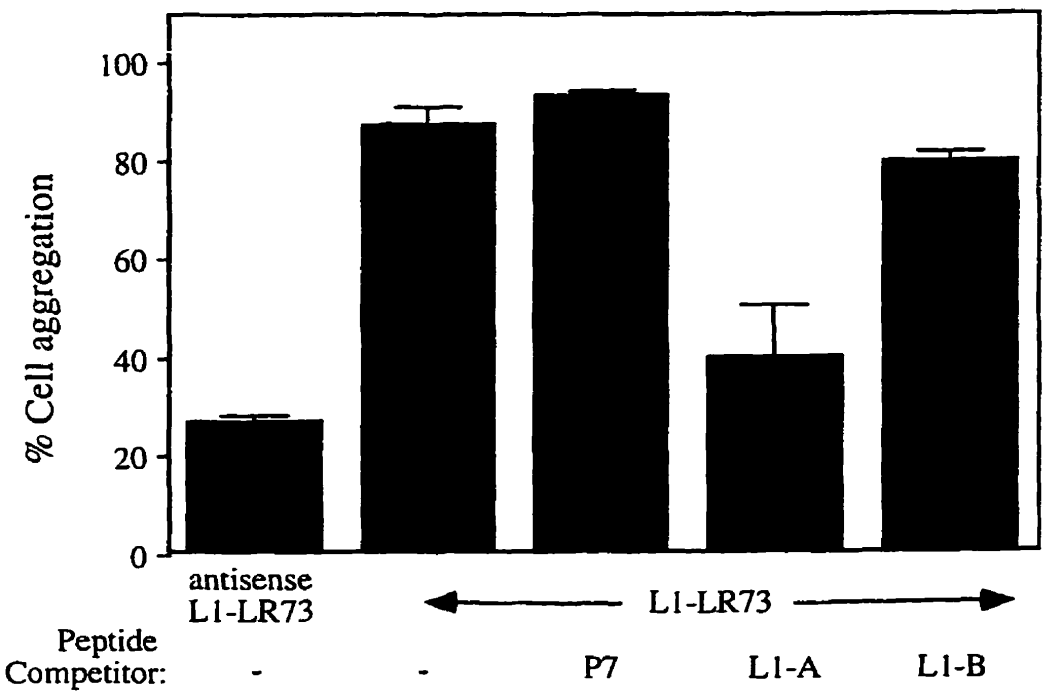


Figure IV.8. Effects of synthetic peptides on the aggregation of L1-LR73 cells. Reassociation of L1-LR73 cells was carried out in the presence of 2 mg/ml of peptide competitors. The percentage of cell aggregation was determined after 45 min. LR73 cells transfected with the antisense-L1 cDNA construct were used as the negative control, while L1-LR73 cells assayed in the absence of peptide competitor were used as the positive control.

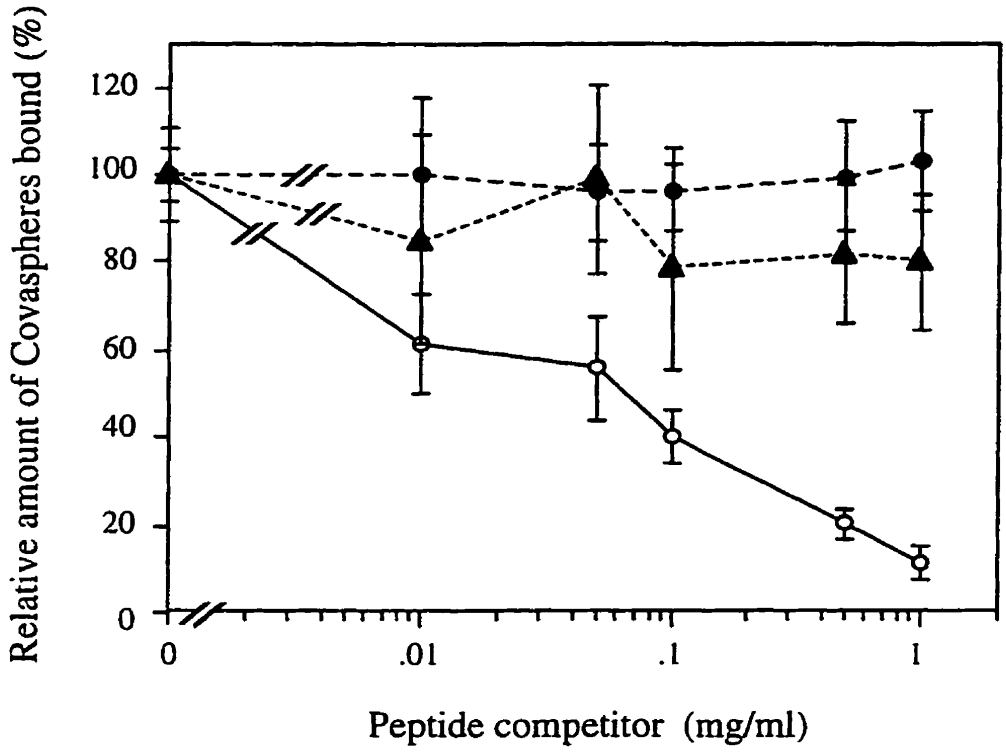


Figure IV.9. Effects of synthetic peptides on the binding of GST-Ig2-conjugated Covaspheres to substrate-coated GST-Ig2. Dose-dependent effects of peptides on the attachment of Covaspheres to substratum. Spots on Petri dish were coated with 5 µl of 3 µM GST-Ig2. GST-Ig2-conjugated Covaspheres were mixed with different concentrations of peptides: L1-A (○), L1-B (▲), or the control peptide P7 (●), and then placed on GST-Ig2-coated spots. After 30 min of incubation with gentle shaking at room temperature, the relative amounts of Covaspheres bound per unit area were estimated relative to the minus-peptide control.

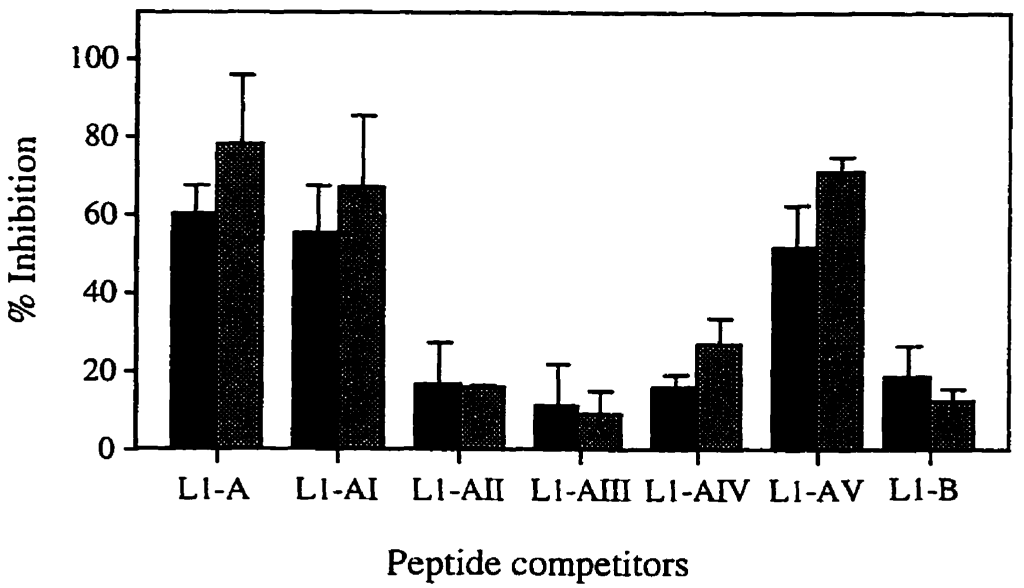


Figure IV.10. Effects of peptide analogues on cell aggregation. Reassociation of L1-LR73 cells was carried out in the presence of 1 mg/ml (solid bars) or 2 mg/ml (dotted bars) of peptide analogues. Percentage of cell aggregation was determined at the 45-min time point. The percentage of inhibition was calculated relative to the difference between the positive and negative controls. Data represent the mean ± S.D. of three experiments.

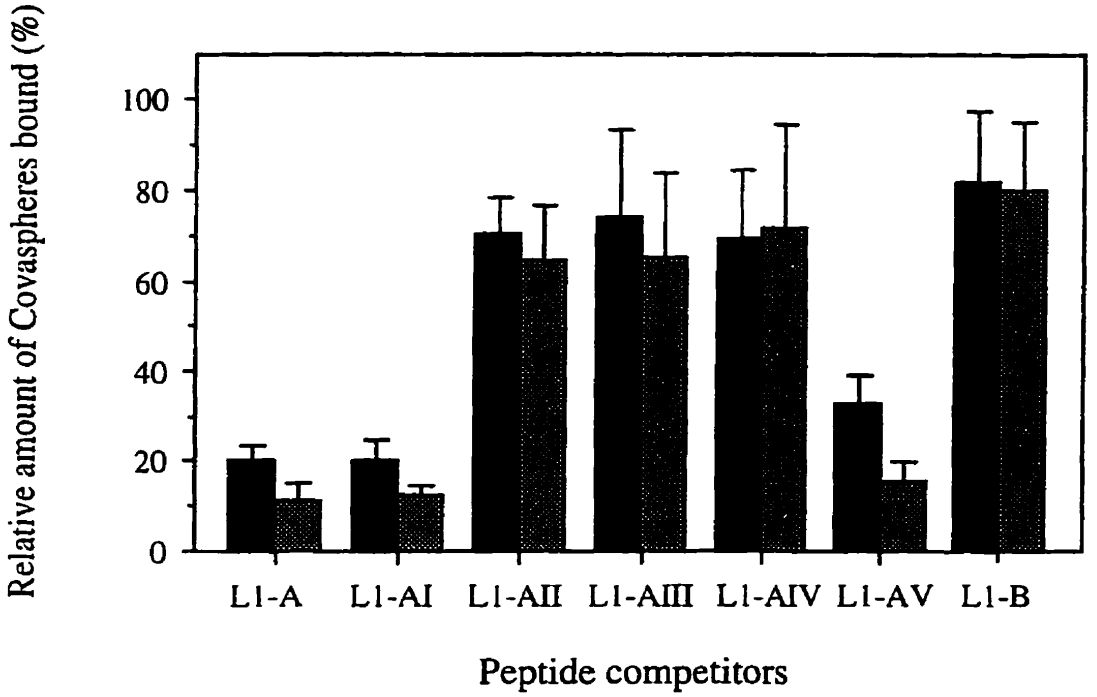
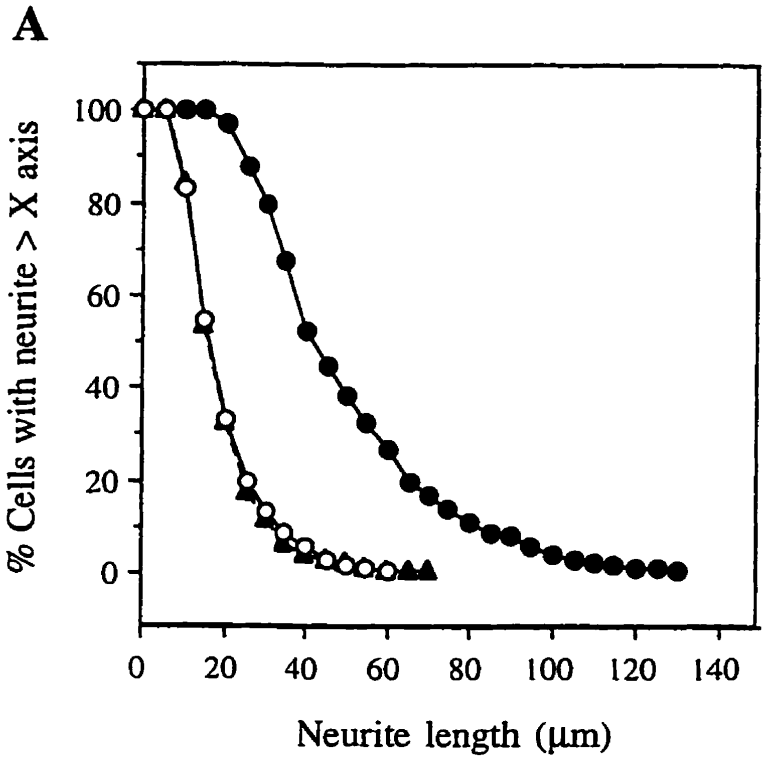


Figure IV.11. Effects of peptide analogues on the binding of Covaspheres to substratum. GST-Ig2-conjugated Covaspheres were mixed with 0.5 mg/ml (solid bars) or 1 mg/ml (stippled bars) of peptide analogues before seeding on top of GST-Ig2-coated spots on Petri dishes. Unbound beads were removed after 30 min of incubation at room temperature, and the number of Covaspheres bound was determined. Data represent the mean ± S.D. (n = 9).

Figure IV.12. Neurite outgrowth from retinal cells on L1-LR73 cells. Retinal cells were isolated from day 5 chick embryos and labeled with DiI. Retinal cells were then seeded on a monolayer of LR73 transfectants. After 18 h of coculture, cells were fixed and neurites extending from retinal neurons were measured. (A) Size distribution of neurites extending from retinal neurons cultured on different monolayers: L1-LR73 cells (●), L1 Δ2-LR73 cells (▲), and LR73 antisense-L1 transfectants (○). (B) Mean neurite lengths extending from retinal neurons. Data represent the mean ± S.D. of three experiments.


A
% Cells with neurite > X axis
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Neurite length (μm)

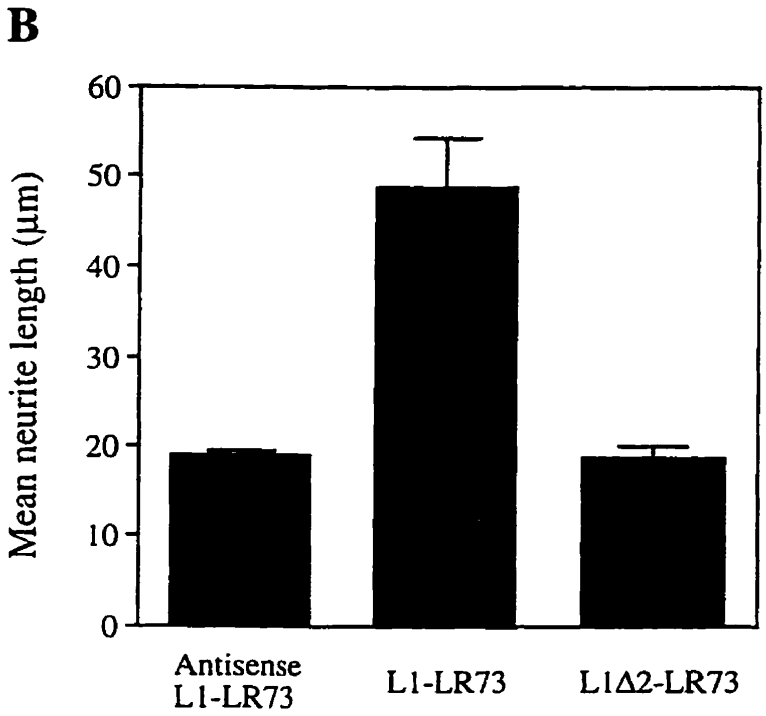
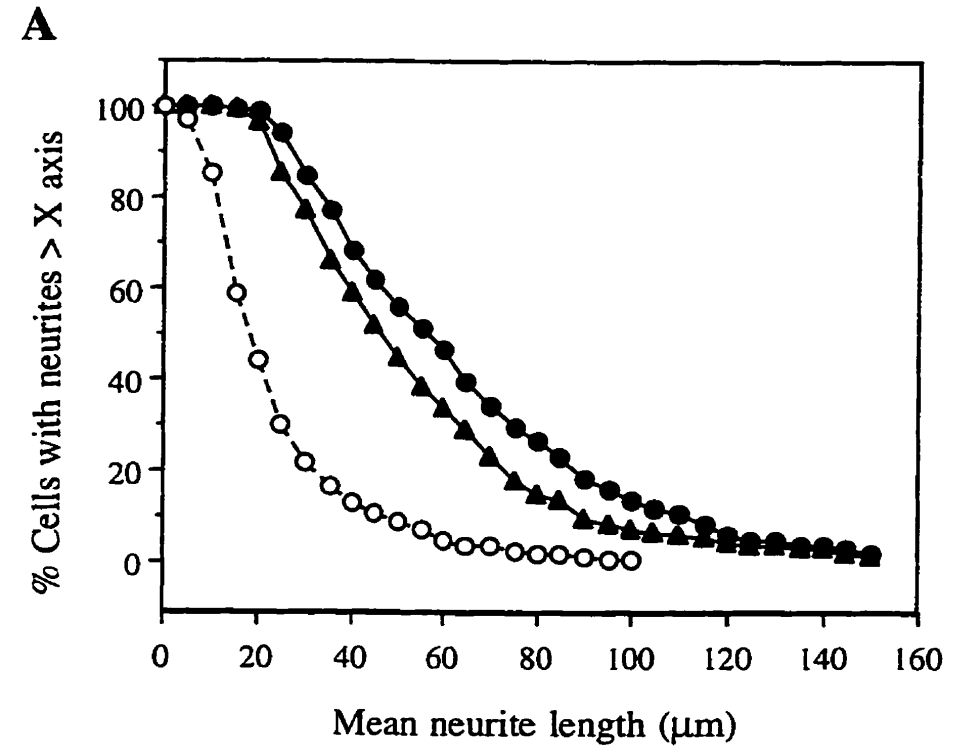
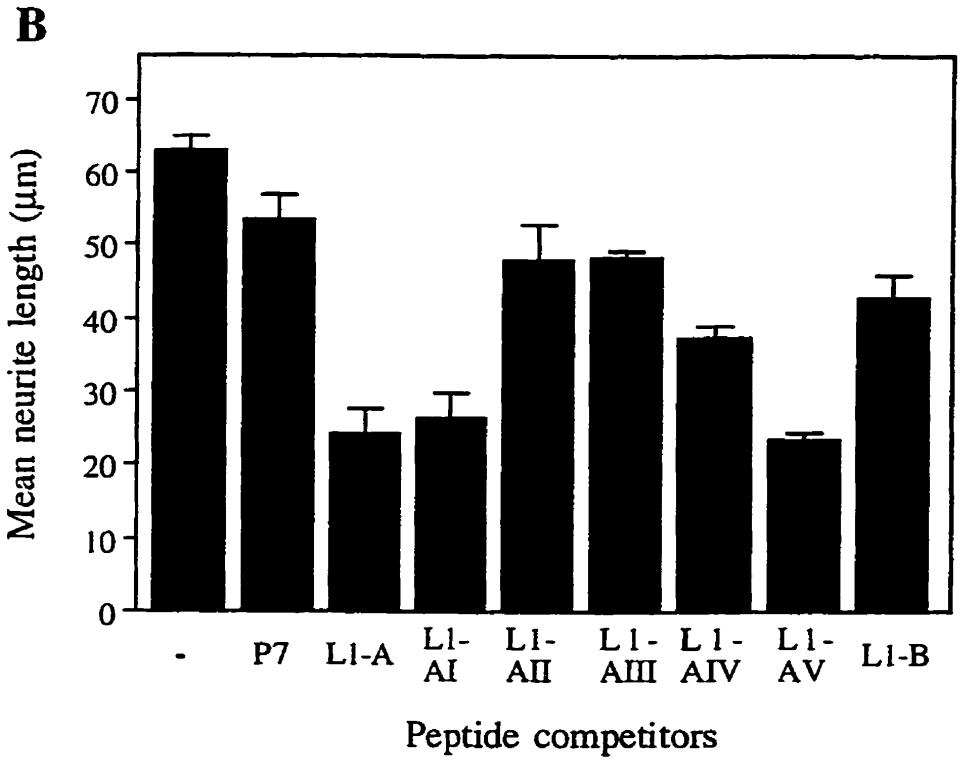

B
Mean neurite length (μm)
60
50
40
30
20
10
0
Antisense L1-LR73
L1-LR73
L1Δ2-LR73

Figure IV.13. Effects of synthetic peptides on neurite outgrowth from retinal cells cultured on substrate-coated GST-Ig2. Coverslips were coated with 0.01% poly-L-lysine, followed by 1 μM GST-Ig2. Retinal cells were seeded on these coverslips and cultured in N2 medium for 18 h at 37° C. (A) Size distribution of neurites extending from retinal neurons cultured in the presence of 1 mg/ml of peptide L1-A (○) or control peptide P7 (▲) and in the absence of peptide competitor (●). (B) Mean neurite length of retinal neurons cultured in the presence of different peptide competitors or analogues of peptide L1-A. Peptides were added at the beginning of the assay at a final concentration of 1 mg/ml. Data represent the mean ± S.D. of three experiments.


A
% Cells with neurites > X axis
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Mean neurite length (µm)


B
Mean neurite length (µm)
70
60
50
40
30
20
10
0
-
P7
L1-A
L1-AI
L1-AII
L1-AIII
L1-AIV
L1-AV
L1-B
Peptide competitors

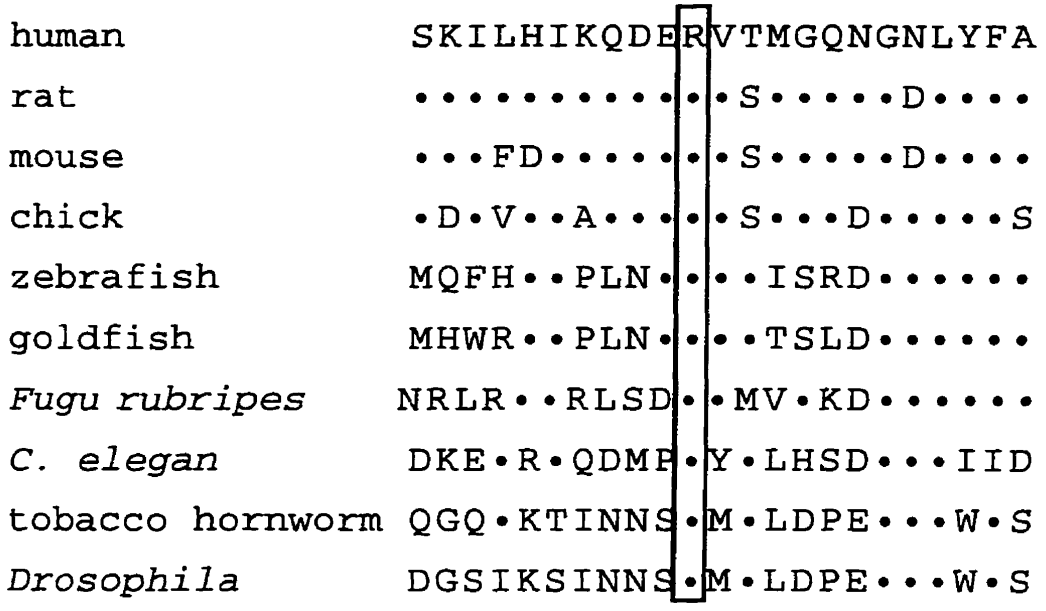


Figure IV.14. Alignment of the human L1 homophilic binding site with sequences of other species. The sequence between Ser-174 and Ala-196 within the second Ig-like domain of human L1 is aligned with homologous sequences from mouse L1 (Moos et al., 1988), rat NILE/L1 (Miura et al., 1991; Prince et al., 1991), chick Ng-CAM (Burgoon et al., 1991), zebrafish L1 (Tongiorgi et al., 1995), goldfish L1 (Giordano et al., unpublished), *C. elegan* L1-like sequence (Wilson et al., 1994), *Fugu rubripes* L1 (Coutelle et al., unpublished) and tobacco hornworm neuroglian (Chen et al., 1997), and *Drosophila* neuroglian (Bieber et al., 1989).

CHAPTER FIVE

CONCLUSIONS AND FUTURE PERSPECTIVES

The focus of my thesis project is to map the homophilic binding site and the neuritogenic site in the neural cell adhesion molecule L1. When I started to work on my thesis project in 1992, there was little known about the mechanism of L1-mediated cell-cell adhesion and the relationship between L1 homophilic binding and neurite outgrowth. Results presented in this thesis have provided important insights into these two intrinsic properties of L1. The primary approach that I have taken is the expression of L1 fragments as fusion proteins in bacteria. The recombinant proteins were subjected to the Covasphere binding assay and the neurite outgrowth assay. In addition, both wildtype and mutant L1 constructs were transfected in LR73 cells and their homophilic and neuritogenic activities were assayed. My results have demonstrated that the second Ig-like domain of L1 harbors both homophilic binding and neurite outgrowth promotion activities.

Several HSAS/MASA mutations have been localized to L1 Ig2. To investigate the effect of these mutations on L1 homophilic binding and neurite outgrowth promotion activity, GST-fusion proteins containing these two mutations were expressed and purified. The HSAS mutation R184Q was found to completely abolish these two intrinsic activities of L1. However, the MASA mutation H210Q has only partial effects. My results therefore demonstrate a close correspondence between the *in vitro* activities of the mutant proteins and the pathological phenotype caused by these two mutations.

These results also highlight the importance of Arg-184 in L1 homophilic binding. Indeed, synthetic peptides flanking Arg-184 are able to inhibit L1-mediated cell-cell aggregation and Ig2-mediated Covasphere binding to Ig2-coated substrates, whereas the peptide containing the flanking sequences of His-210 has only minor effects. These results thus help to narrow down the homophilic binding site to the 14 amino-acid

sequence (HIKQDERVTMGQNG) within L1 Ig2. Further studies using peptide homologues suggest that the side-chains of both charged residues and hydrophobic residues flanking Arg-184 participate directly in homophilic interactions.

To search for potential L1 interactors, the yeast two-hybrid system was employed. Using L1 cytoplasmic domain and FNIII repeats as baits, I have screened a human fetal brain cDNA library. The non-integrin laminin receptor, laminin binding protein (LBP32/67), has been found to interact with the L1 cytoplasmic domain in the two hybrid system. This interaction suggests the possible involvement of L1 in cell-to-substratum adhesion.

Several outstanding questions remain to be answered. Although my thesis work has identified the polypeptide sequence HIKQDERVTMGQNG in L1 Ig2 as the homophilic binding site, it is not clear whether this sequence interacts isologously with the same site or heterlogously with a different region on Ig2 of an apposing L1 molecule. According to the outline structure proposed by Bateman et al., (1996), the homophilic binding site of L1 is localized in the C'D region of Ig2. The charged residues K180, D182 and E183 are present at the C'D loop and facing outwards, while the hydrophobic residues V185 and M187 are present on the D strand and the side-chains are buried inside the Ig-fold. The exposed charged amino acids are most likely involved in recognition and salt bridge formation with other molecules, while the buried hydrophobic residues are more likely involved in the stabilization of the Ig-fold. Interestingly, the C'D loop is located at the edge of the β-barrel structure of the Ig-fold, which forms a perfect interacting pocket for an apposing Ig-structure. The reciprocal salt bridge formation between the positively charged residues and the negatively charged residues may enable the anti-parallel alignment of these two Ig-domains. This prediction is consistent with

our peptide analogue analysis which shows that substitutions of the charged residues would eliminate the ability of peptide L1-A to compete for the L1 homophilic binding site (see chapter IV).

Further experiments are required to test this model. One approach will involve mutagenesis of the homophilic binding site of L1. Transfectants expressing mutant L1 can be tested in microsphere binding or cell attachment assays using either wildtype or mutant Ig2 recombinant proteins. Alternatively, direct protein-protein interaction can be examined in filter binding assays using different combinations of synthetic peptides and recombinant proteins. Since the mutation Y194C also results in hydrocephalus, the contribution of flanking sequences to homophilic binding should be further evaluated. In addition to biochemical studies, structural analysis using NMR and X-ray crystallography should lead to a better understanding of the molecular interactions during L1-L1 *trans*-interaction.

Much evidence has accumulated that L1 not only mediates cell adhesion, but also transduces signals into the cell, thus allowing the cell to respond to its extracellular environment (Schuch et al., 1989; Asou, 1992; Williams et al., 1992). How are signals initiated by L1? What is the nature of these signals? Unlike cadherins and integrins, L1 does not need to bind to calcium to maintain its structure. Electron microscopic studies of L1 reveal that the fibronectin domains of L1 are clustered to form a globular base, whereas the Ig-like domains of L1 form a fibrillar structure (Drescher et al., 1996). As discussed in Chapter IV, homophilic binding of L1 mediated by Ig2 likely aligns two apposing L1 molecule in an anti-parallel manner, allowing weak interactions between other L1 domains to further stabilize the binding between two molecules. These interactions may lock the Ig-like domains into a binding conformation, resulting in

further conformational changes down through the FNIII repeats and eventually the cytoplasmic domain, in a manner similar to that of integrin, which transduces the ligation signal to the cytosol by structural changes (Dedhar and Hannigan, 1996). To test this hypothesis, one approach would be the development of monoclonal antibodies that recognize conformational epitopes on L1. Cells expressing L1 can be challenged with recombinant Ig2 or peptide and conformational changes can be monitored using these antibodies. Since the highly conserved cytoplasmic domain of L1 has been reported to associate with different protein kinases (Williams et al., 1994a; Wong et al., 1996a, and Wong et al., 1996b) and cytoskeleton components (Davis and Bennett, 1994), another approach is to determine the phosphorylation state of the cytoplasmic tail before and after binding with recombinant Ig2 or synthetic peptide. Its association with ankyrin and other cellular components can be analyzed by co-immunoprecipitation and double immunofluorescence labeling.

L1 signaling may depend on L1 dimer formation since cross-linking of surface L1 with antibodies or soluble L1-Fc chimeric protein triggers intracellular signaling events similar to those induced by L1 binding (Schuch et al., 1989; Atashi et al., 1992; Doherty et al., 1995). L1 is also known to undergo *cis*-interaction with other membrane components, such as axonin-1, F3/F11, and FGFR (Kuhn et al., 1991; Felsenfeld et al., 1994; Brummendorf et al., 1993; Williams et al., 1994a). Therefore, heterodimerization between L1 and these proteins may be involved in the early steps of L1-induced neurite outgrowth and govern cell-type and stage-specific responses of L1. However, direct biochemical demonstration of these heterophilic interactions is still lacking. The formation of L1 *cis*-homodimers or cis-heterodimers can be tested by chemical cross-linking, followed by immunoprecipitation.

Other than its involvement in neurogenesis, L1 has been proposed to play an important role in cancer metastasis and immune response. L1 is synthesized and secreted by melanoma cells, and L1 deposited on the extracellular matrix may facilitate tumor cell attachment and migration (Montgomery et al., 1996). The recent finding that L1 interactions with integrins $\alpha v\beta 3$, $\alpha v\beta 1$ and $\alpha 5\beta 1$ suggests that L1 is involved in both vascular and thrombogenic processes (Felding-Habermann et al., 1997). Our recent finding that L1 interacts with the laminin binding protein LBP67/32 further suggests a role for L1 in the cellular response to the extracellular matrix. LBP was first found as a cancer marker and is up-regulated during metastasis (Yow et al., 1988; Wewer et al., 1986). The association of LBP with L1 suggests that L1 might play an role in cancer metastasis by modulating the adhesive properties of cancer cells. On the other hand, being a laminin binding protein, LBP associated with L1 may result in cross-talk between signaling pathways triggered by different cell and substrate adhesion molecules. Future studies of L1-LBP heterophilic interaction and its effect on the cellular response to laminin should provide new insights into L1 functions other than those in the nervous system.

APPENDIX ONE

TWO-HYBRID SCREENING FOR PROTEINS INTERACTING WITH THE CELL ADHESION MOLECULE L1

I. INTRODUCTION

To investigate the mechanism of L1 functions, we have devoted a lot of effort to the mapping of functional domains in L1. The second Ig-like domain of L1 has been found to be responsible for both homophilic binding and L1-induced neurite outgrowth (Zhao and Siu 1995). The RGD sequence in the sixth Ig-like domain of L1 is involved in L1-integrin interaction (Ruppert et al., 1995; Montgomery et al., 1996; Ebeling et al., 1996; Yip et al., 1998). Interactions of L1 with other molecules in the same membrane plane may also facilitate or augment L1 functions. While the roles of L1 in cell adhesion and promotion of neurite outgrowth have been well characterized, the mechanism by which intracellular signaling events are triggered by extracellular binding signals is less well understood.

Although several protein kinases have been implicated in L1 signal transduction (see Discussion in Chapter IV), there may be components other than protein kinases that associate with the L1 cytoplasmic domain. I have therefore used the yeast two-hybrid system (Fields and Song, 1989) to search for new proteins that interact with the cytoplasmic domain of L1. The yeast two-hybrid system has been widely used to clone cDNAs coding for protein interactors that bind known protein sequences. This approach uses the transcription of yeast reporter genes as a synthetic phenotype to detect protein-protein interaction. Many eukaryotic transcription activators have at least two distinct functional domains, one that binds directly to specific DNA sequences and one that activates transcription (Keegan et al., 1986; Hope et al., 1986). Taking advantage of the modular domain structure of these transcription factors, the two-hybrid system involves a known protein fused to the DNA-binding domain of a transcription factor and a cDNA

library fused to an activation domain. Transcription of a reporter gene will be activated in yeast containing an activation-tagged cDNA-encoded protein that interacts with the known protein (Fields and Song, 1989).

In this appendix, I describe the cloning of the laminin binding protein (LBP67/32), which was found to be positive in L1-two-hybrid screening. LBP is a non-integrin laminin receptor which was first identified as a cancer cell marker (Yow et al., 1988; Wewer et al., 1986). Its expression level is up-regulated upon metastasis. I have found that both LBP and L1 are expressed in the human melanoma cell line WM239. Double immunofluorescence labeling experiments show that LBP and L1 co-cap on the surface of WM239 cells, suggesting that interaction between these two proteins under physiological conditions. Direct interaction between recombinant L1 cytoplasmic domain and LBP has also been examined.

II. EXPERIMENTAL PROCEDURES

A. Materials:

Plasmids pSH 18-34, pEG202, pJK101, the yeast strain EGY48, and the human fetal brain cDNA library in pJG4-5 were generously gifts of Dr. Roger Brent, Harvard Medical School, MA, USA. Amino acids were purchased from Sigma-Aldrich Canada (Oakville, ON). IPTG and X-Gal were purchased from Vector BIOSYSTEMS (Toronto, ON). Anti-LBP antiserum 149 was kindly provided by Dr. Haydon Kleinman, NIH, Bethesda, MD. (Davis et al., 1991). This antiserum was raised agains a 17-mer synthetic peptide from the N-terminal region (residues 24-41) of mouse LBP32, and it recognizes only the 45 kDa and 32 kDa LBP in Western blots. This 17 amino acid is 100% conserved in human LBP32.

B. Construction of Baits

phL1/Blue that contains the entire coding region of human L1 cDNA in pBluescript was kindly provided by Dr. V. Lemmon (Case Western Reserve University, Cleveland, OH). To construct the cytoplasmic domain bait pEG202-Cyto, a PCR product was generated using the forward primer (5'-GCGAATTCAAGCGCAGCAAGGGCGGC-3') and the reverse primer (5'-GGGAATTCTAAGCTTAGTATTCTAGGGCCACGGC-3') to amplify from phL1/Blue template. They were cut with *Eco* RI and subcloned into the *Eco* RI site of pBluescript vector (Cyto/SK), and verified by double-stranded DNA sequencing using a T7 Sequencing™ kit (Pharmacia). This 0.35 kb *Eco* RI fragment was

subsequently released from Cyto/SK and subcloned into the *Eco* RI site of pEG202. The correct orientation was confirmed with internal restriction enzymes.

To construct the pEG202-Fn vector, a cDNA fragment coding for all five FNIII repeats was released from the pGEX-3X-Fn vector that was constructed in a previous study (see Experimental Procedures in Chapter II) using *Bam* HI and *Bgl* II. This fragment was subcloned into the *Bam* HI site of pEG202. The correct orientation of the insert was confirmed using internal restriction enzyme cut sites.

C. Expression of Baits

Yeast cells transformed with bait plasmids were grown in histidine drop out medium (2% galactose, 1% raffinose, 1.7 g/L yeast nitrogen base, 5 g/L amonin sulfate, 2 g/L Histedine drop out amino acid mixture) till OD_{600} = ~0.5. One-ml culture was collected by centrifuging at 10,000 rpm for 30 seconds. Cells were then resuspended in 50 µl of 2x SDS sample buffer and broken by freezing on dry ice for 5 min, followed by boiling for 5 min. Fifteen µl of the supernatant was loaded on SDS-PAGE and a Western blot approach was used to check the expression of bait proteins.

D. Immunofluorescent Cell Staining

Human melanoma WM239 cells were seeded sparsely on glass coverslips 24 h before staining. Cells were washed once with PBS and then fixed with 100% methanol at -20°C for 3 min. Coverslips were incubated for 5 min at room temperature with a

blocking solution containing 1% BSA in PBS. Coverslips were then incubated at room temperature for 1 h with 100 µl of anti-L1 mAb 74-5H7 supernatant or 100 µl of anti-LBP antiserum 149 at 1:200 dilution. Cells were then washed three times with PBS before incubation with FITC-conjugated goat anti-rabbit or Texas Red-conjugated goat anti-mouse IgG for 1 h. Cells were mounted with 75% glycerol and observed using a Zeiss confocal microscope.

E. Co-capping of L1 and LBP

Human melanoma WM239 cells were seeded sparsely on glass coverslips one day before assay. Cells were washed once with PBS followed by blocking with 1% BSA in PBS at 37° C for 5 min. Polyclonal anti-LBP antiserum (100 µl) was added at 1:100 dilution in PBS after removing the blocking reagent. Coverslips were incubated at 37° C for 15 min. After washing with PBS three times, FITC-conjugated goat anti-rabbit IgG was added at 1:100 dilution, and incubated at 37° C for 10 min. After removing the secondary antibody, cells were fixed with methanol at -20° C for 3 min and then stained with anti-L1 mAb 74-5H7 for L1 distribution.

F. Construction and Expression of GST-Fusion Protein of L1 Cytoplasmic Domain

The L1 cytoplasmic domain cDNA sequence was released from Cyto/SK by *Eco* RI and subcloned into the *Eco* RI site of pGEX-1T. IPTG inducible GST-Cyto expression

was checked by SDS-PAGE, followed by Western blot analysis using mAb 74-5H7 that recognized the cytoplasmic domain of L1.

To produce GST-Cyto fusion protein or GST protein, transformed *E. coli* cells were grown at 30° C in 1 L of LB medium with 100 μg/ml ampicillin. Protein synthesis was induced by addition of 0.5 mM isopropyl ß-D-thiogalactopyranoside (IPTG) when OD_{600} of the culture reached 0.6 to 0.8. Cells were collected 3 h after induction and resuspended in 20 ml of sonication buffer (10% glycerol, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, and 0.5 M NaCl in PBS). Cells were lysed by sonication, and the supernatant was collected by centrifugation at 10,000 rpm for 15 min. Triton X-100 was added to the supernatant to a final concentration of 1%. The supernatant was allowed to pass through a pre-washed glutathione-Sepharose 4B column (0.5 ml bed volume). The column was washed with 20X bed volume of PBS containing 1 M NaCl. The glutathione-Sepharose 4B beads with bound fusion protein were used to re-pack mini columns for affinity binding assays.

G. Affinity Column Binding Assay

40 μl of GST-Cyto or GST glutathione beads was packed into a mini-column. The column was equilibrated by passing through 20X bed volume if PBS containing 1% Triton X-100. Melanoma WM239 cells ($5x10^6$) were collected from the dish and centrifuged at 500 rpm for 10 min at 4° C. Cells were lysed with 600 μl lysis buffer (1% triton X-100 and 1 mM PMSF in PBS) and placed on ice for 30 min. 300 μl of whole cell lysate from WM239 melanoma cells was passed through each affinity column. Columns

were washed with 400 µl of washing buffer (PBS + 0.1 M NaCl). Bound protein was eluted with 50 µl of high salt buffer (PBS + 1 M NaCl), followed by 50 µl of 1% SDS. 20 µl of the eluate was analyzed by SDS-PAGE, followed by Western blot analysis using the anti-LBP antibody at 1:1000 dilution.

III. RESULT

A. Construction and Expression of L1 Baits.

The interaction trap, which is an implementation of the two-hybrid system developed by Gyuris et al. (1993) was employed in this study (Figure A.1). Two reporter genes under LexA operator control were used. One of them was the integrated LEU2 gene and the other one was a lacZ gene reporter residing on a plasmid. The cytoplasmic domain of L1 molecule and the fibronectin type III repeats were fused to the DNA binding protein LexA by subcloning L1 cDNA into the pEG202 vector. This vector contained the selectable marker gene HIS3 as well as the full-length LexA coding region flanked by the yeast ADH1 promoter and terminator. Transformants selected from histidine lacking media constitutively expressed the protein of interest with LexA at its amino terminus. The expression of L1 baits in yeast was confirmed by Western blot analysis (Figure A.2). In addition, the ability of L1 bait transformants to activate the lacZ and the LEU2 reporter genes was checked to rule out the possibility of transcription activation by the cytoplasmic domain or fibronectin repeats of L1 alone (Table A.1).

Although neither the cytoplasmic domain nor the fibronectin repeats of L1 contained a yeast nuclear localization signal, LexA and most LexA fusion proteins are known to enter the nucleus (Brent and Ptashne, 1985; Zervos et al., 1993). To confirm this, the repression assay was used to demonstrate that the bait was indeed capable of entering the yeast nucleus and binding to LexA operators. In this assay, cells were co-transfected with the reporter plasmid pJK101 and the bait plasmids. Unlike the plasmid (pSH18-34) used for testing activation, pJK101 contained most of the GAL1 upstream

activating sequence, UASg. The LexA fusion protein in the yeast nucleus would then bind to the LexA operator positioned between UASg and the TATA box and repress lacZ expression. When growing on X-Gal plates, yeast cells co-transformed with either pLexA-Fn or pLexA-cyto together with pJK101 showed >10-fold decrease in blue color intensity, confirming that these baits were able to enter yeast nucleus and bind to the LexA operators.

B. Two-hybrid Screening for L1 Interactors.

The human fetal brain cDNA fusion library generated by Dimitri Krainc (Wang et al., 1996; Chao et al., 1996) was used to screen for proteins that could interact with L1. This library was constructed by inserting cDNA into the EcoR I and Xho I site in the pJG4-5 vector. This insertion site places the cDNA downstream of the GAL1 promoter, the ATG initiation codon, a sequence encoding the SV40 nuclear localization signal, a hemagglutinin epitope tag, and the B42 transcription activation domain. A selectable marker TRP1 was also present in the pJG4-5 vector. The activation-tagged cDNA-encoded proteins were then expressed in yeast cells grown on galactose but not on glucose. The double positive results obtained with both lacZ and LEU2 reporters in the presence of galactose would indicate that the protein encoded by the library cDNA interacted with the L1 fusion protein. More than $1x10^6$ clones were screened for each bait, seven positive clones were isolated from pLexA-cyto screening and ten were obtained from LexA-Fn screening.

The plasmids containing the activation-tagged protein were isolated from the positive clones and retransformed into yeast containing the other bait plasmid to confirm

the specificity of the protein-protein interaction. Four out of seven clones from pLexA-cyto screening remained positive after this selection (Table A.1). The four positive clones were subjected to sequencing, and one of these four clones encoded the full length laminin binding protein LBP 67/32 (Yow et al., 1988; Wewer et al., 1986).

C. L1 and LBP Expression in the Melanoma Cell Line WM 239

Since LBP was identified as a cancer cell marker, we examined the expression of LBP in the melanoma cell line WM239. Immunofluorescence labeling experiments showed that WM239 cells expressed LBP, which was present in both the cytoplasm and the plasma membrane (Figure A.3a). WM239 cells also expressed L1 which had a punctate staining pattern, with a higher staining intensity in the cell-cell contact regions (Figure A.3b).

D. L1 and LBP Co-capped on the Cell Surface.

To demonstrate that L1 and LBP are interacting with one another under physiological conditions, co-capping experiment was carried out. LBP on the WM 239 cell surface was capped by incubating live cells with the anti-LBP antiserum 149 at 37° C for 15 min, followed by incubation with FITC-conjugated secondary for 10 min. Cells were then fixed by methanol, and subsequently stained with anti-L1 mAb 74-5H7. Redistribution of LBP as well as L1 was examined using confocal microscopy.

LBP molecules were capped and formed a crescent on the cell surface due to the cross-linking effects of the divalent IgG and secondary antibodies (Figure A.4). L1

molecules were found to colocalize with LBP (Figure A.4). These results suggest that L1 and LBP are interacting with each other under physiological conditions.

E. Binding of LBP to GST-cyto L1 Fusion Protein

The interaction between LBP and the cytoplasmic domain of L1 was investigated by affinity column assays. The L1 cytoplasmic domain was fused to GST (GST-cyto) and expressed in bacteria. GST-cyto was expressed and purified as a soluble protein (Figure A.5). The fusion protein was recognized by the anti-L1 mAb 74-5H7. The GST-cyto protein was bound on a glutathione column and WM 239 cell lysate was allowed to pass through the column. The bound material was eluted and subjected to Western blot analysis using the anti-LBP antiserum 149. The result showed that both 32 kDa and 45 kDa LBP bands were detected in the eluate of the GST-cyto column (Figure A.6). These bands were observed when the column was eluted with 1% SDS, suggesting high affinity interaction between L1 and LBP. However, LBP was not detected in the eluate of the GST control column.

IV. DISCUSSION

The interactions of L1 with extracellular matrix components or membrane components on adjacent cells are known to generate signals that can regulate biological processes, such as cell migration and neurite outgrowth. The fact that several hydrocephalus mutations have been found on the cytoplasmic domain of L1 suggests that the cytoplasmic domain may play an important role in signal transduction. Using the yeast two-hydrid system, we have identified that LBP32/67 as a binding partner of the L1 cytoplasmic domain.

LBP was first identified as a cancer cell marker on human colon carcinoma cells (Yow et al., 1988; Wewer et al., 1986). The up-regulation of LBP protein correlates closely with cancer cell metastasis (Yow et al., 1988). LBP has an apparent molecular weight of 67 kDa. However, the LBP gene encodes a protein of only 32 kDa. Antibodies raised against LBP also recognize a 45 kDa protein. It has been suggested that the 32 kDa LBP undergoes posttranslational modification to form a covalently linked homodimer which migrates as a 67 kDa band on an SDS gel under reduced conditions (Landowski et al., 1995a). Furthermore, LBP is acylated and the covalently bound fatty acid(s) may provide a mechanism for membrane association (Landowski et al., 1995a). Interestingly, LBP is found both inside the cell and on the surface, although it does not contain a leader peptide sequence (Landowski et al., 1995). The mechanism of LBP67 translocation and its exact orientation of on the cell surface are still not known.

LBP has been reported to be a high affinity receptor of laminin (Wewer et al., 1986). Its binding site has been mapped to the residues 205 -229 of LBP, which interact with the peptide sequence YIGSR on the ß chain of laminin 1 (Landowski et al., 1995b).

However, studies using purified LBP indicate that LBP binds laminin with a low affinity (Landowski et al., 1995a). Therefore, the high affinity laminin binding activity of LBP may be dependent on its association with other membrane components.

The interaction of LBP with L1 implicates a role for L1 in cancer metastasis. Decreased cell-cell adhesion and increased interaction with the extracellular matrix is a crucial control of tumor metastasis. It is conceivable that the up-regulation of LBP may cause a decrease in L1-mediated cell-cell adhesion. The *cis*-interaction of LBP with L1 may lead to the redistribution of L1 from the cell-cell contact regions, resulting in decreased intercellular adhesiveness among tumor cells. Alternatively, their *cis*-interaction may increase the LBP binding affinity to laminin, thus promoting cell-to-substrate attachment and cell motility.

Results obtained with the co-capping experiment support the association of L1 with LBP under physiological conditions. Capping of LBP molecules on the cell surface leads to the redistribution of L1 to the LBP clusters. However, LBP is capable of interacting with the cytoplasmic domain of L1, but not with the five FNIII repeats adjacent to the plasma membrane on the ecto-surface of the membrane. Although binding of LBP with L1 Ig-like domains has not been tested, it is unlikely that they would interact with each other because of the small size of LBP and the physical distance of the Ig-like domains from the plasma membrane. We speculate that the two covalently linked subunits of LBP67 may span the plasma membrane, with one subunit on the extracellular surface and the other on the cytoplasmic surface. LBP interaction with the cytoplasmic domain of L1 would then be mediated by its intracellular subunit. Since L1 is also known to interact with laminin (Hall et al., 1997), the association between L1 and LBP may augment their interactions with laminin. The validity of this model will depend on

further studies on the nature of the dimer structure of LBP and the identification of the LBP site involved in interaction with the cytoplasmic domain of L1.

Table A.1. Summary of two hybrid screens using pEG202-Fn and pEG202-Cyto

Bait	pEG202-Fn	pEG202-Cyto
L1-fragement	Fibronectin domains 1-5	Cytoplasmic domain
Expression (western)	+	+
Nucleus localization (Repression Assay)	+	+
Leu positive	-	-
Lac Z positive	-	-
Clones screened	$>5x10^6$	$>5x10^6$
Double positive	10	7
Bait specific double positive	0	4

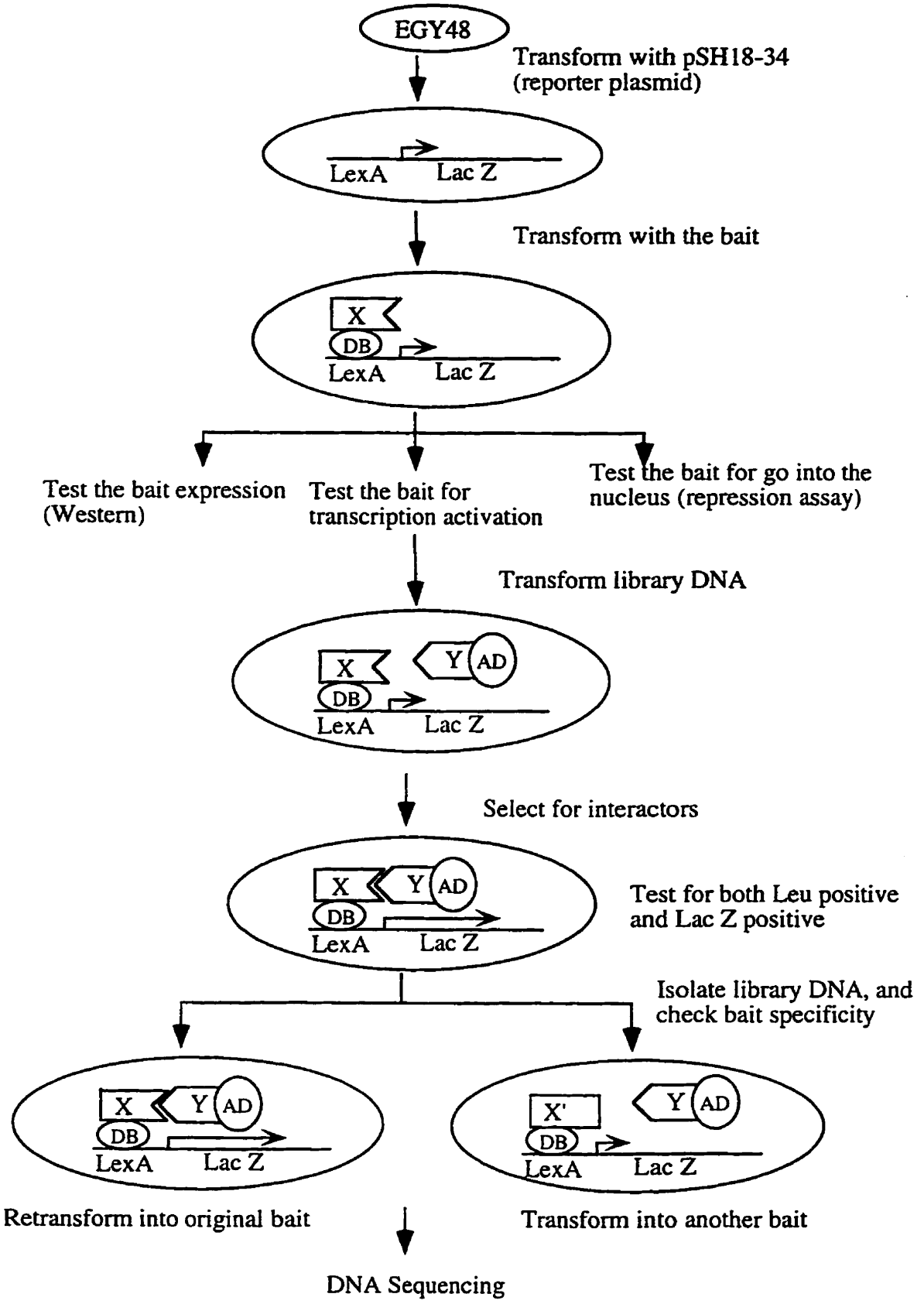
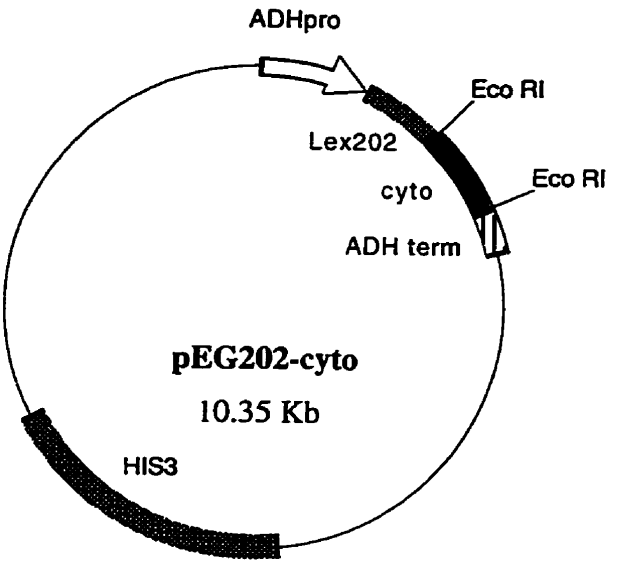


Figure A.1: Schematic representation of the two-hybrid system.

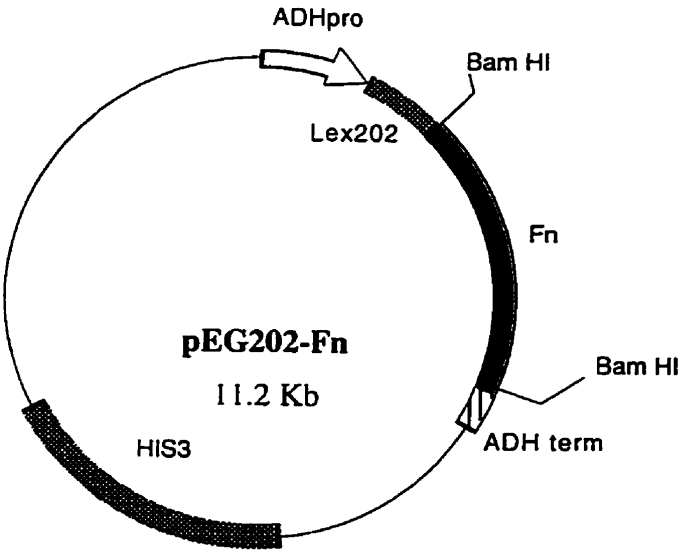
Figure A.2 Construction and expression of L1 baits. (A) Construction and expression of an L1 bait containing the cytoplasmic domain. L1 cDNA encoding its cytoplasmic domain was inserted to the *Eco* RI site of the pEG202 plasmid, resulting in the fusion of L1 cytoplasmic domain to the C-terminus of LexA binding protein. The expression of the L1-cyto bait was confirmed by Western blot analysis using mAb 74-5H7. (B) Construction and expression of an L1 bait containing the fibronectin type III repeats. L1 cDNA encoding all five fibronectin type III repeats were inserted into the *Bam* HI site of the pEG202 plasmid. The expression of the L1-Fn bait was confirmed by Western blot analysis using polyclonal antibodies against L1 fibronectin domains.

A


ADHpro
Eco RI
Lex202
cyto
Eco RI
ADH term
pEG202-cyto
10.35 Kb
HIS3


M_r x 10^{-3}
65
45
29
18
14

B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Lex202
Fn
pEG202-Fn
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Bam HI
HIS3
ADH term

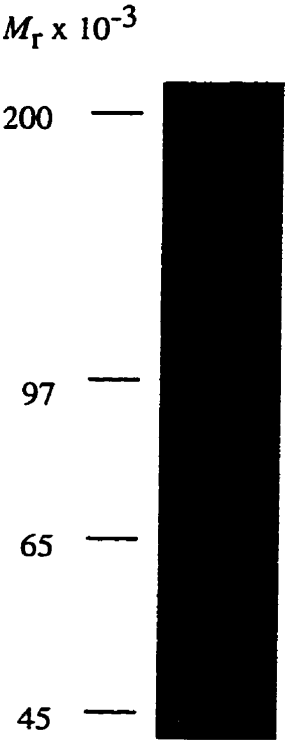

M_r x 10^{-3}
200
97
65
45

Figure A.3 Epifluorescence micrographs of melanoma WM 239 cells. WM 239 cells were cultured on glass coverslips and fixed with methanol. Cells were stained with either (a) rabbit anti-LBP antiserum or (b) mouse anti-L1 mAb 74-5H7. FITC-conjugated goat-anti-rabbit or Texas Red-conjugated goat-anti-mouse secondary antibodies were added after removal of the primary antibody. Cells were examined by confocal microscopy. Bar = 10 μm.

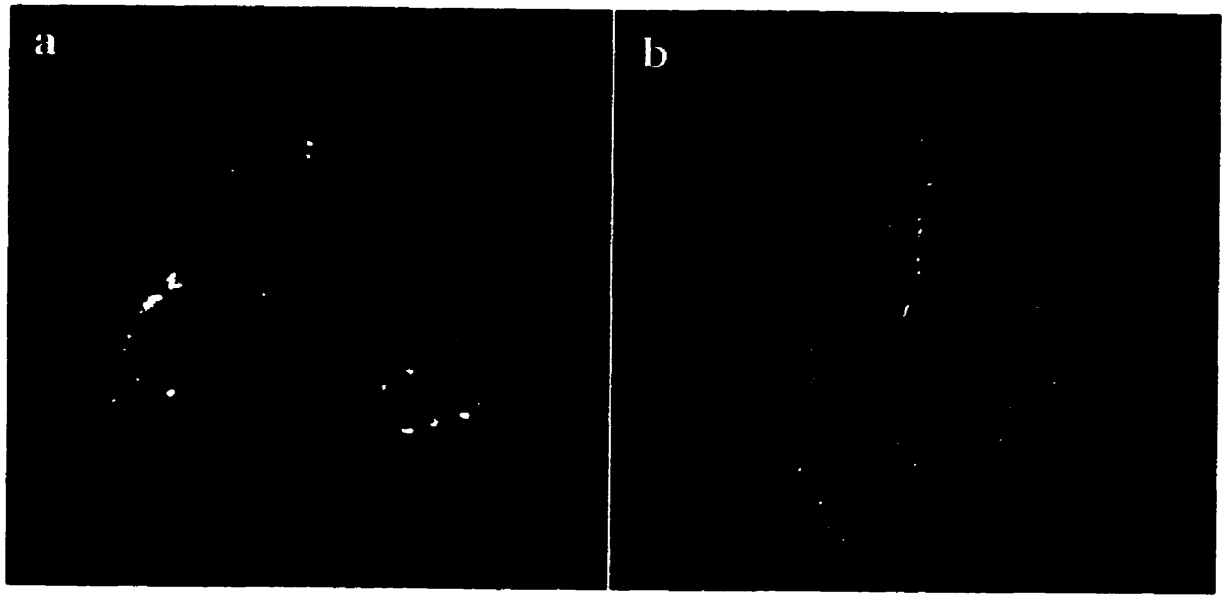

a
b

Figure A.4 Epifluorescence micrographs showing co-capping of LBP and L1 on WM239 cells. WM 239 cells were cultured on glass coverslips. Cells were incubated at 37° C with rabbit anti-LBP serum at 1:100 dilution, followed by FITC-conjugated goat-anti-rabbit IgG. Cells were then fixed with methanol and stained with anti-L1 mAb 74-5H7. Panels a, c, and e show LBP staining, and panels b, d, and f show L1 staining. Bar = 10 μm.

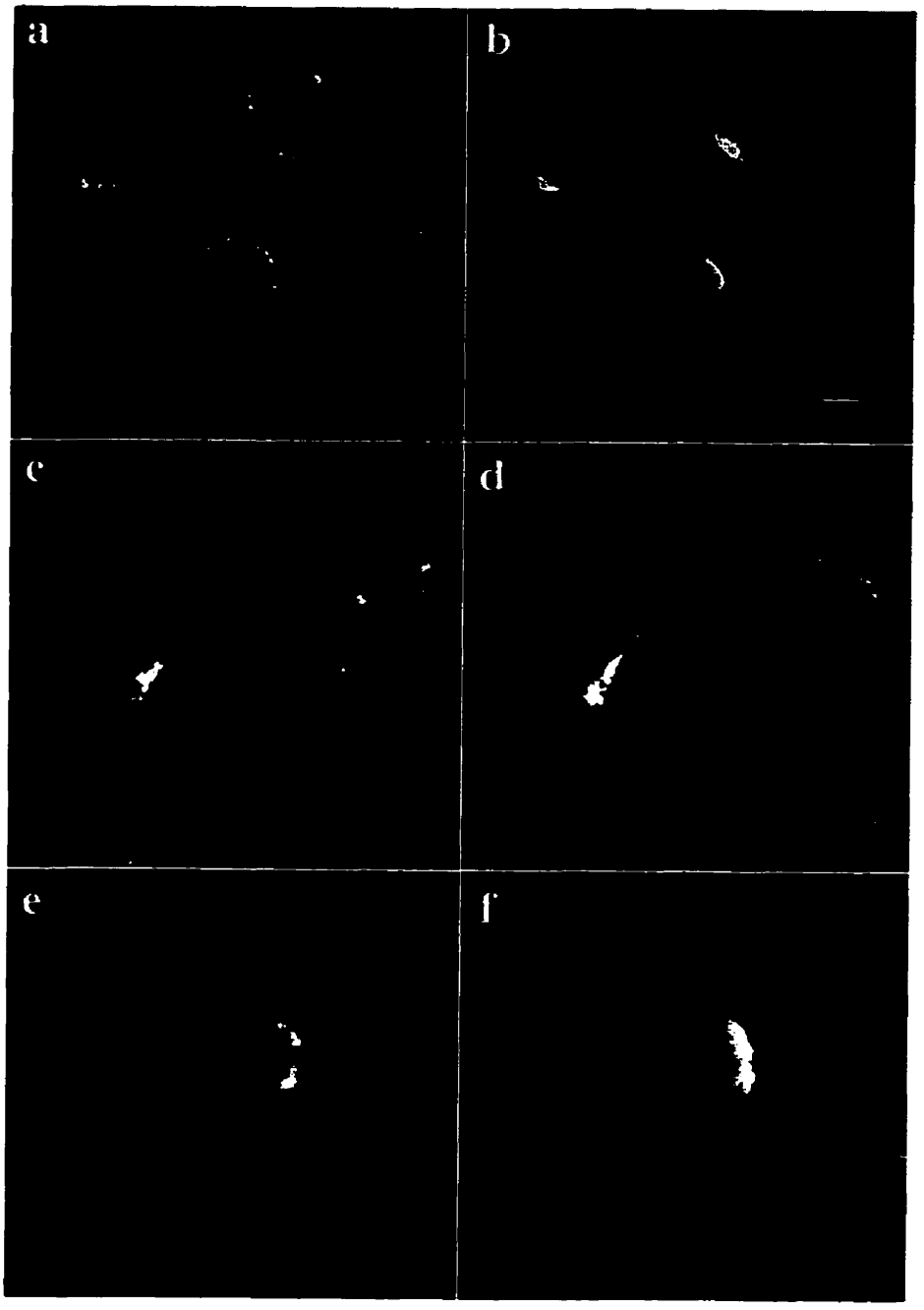
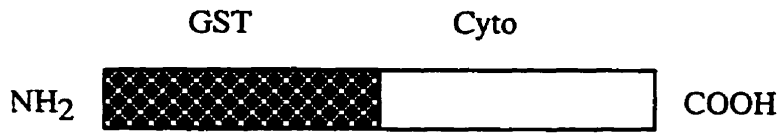

a
b
c
d
e
f

Figure A.5 Construction and expression of L1 cytoplasmic domain fusion protein. (A) Construction L1 cytoplasmic domain GST-fusion protein. (B) Expression of the GST-Cyto fusion protein. GST-Cyto fusion protein was expressed in bacteria and purified as a soluble protein. Lane a: Coomassie blue staining of purified GST-Cyto; lane b: Western blot analysis using mAb 74-5H7 which recognizes the cytoplasmic domain of L1.

A



B

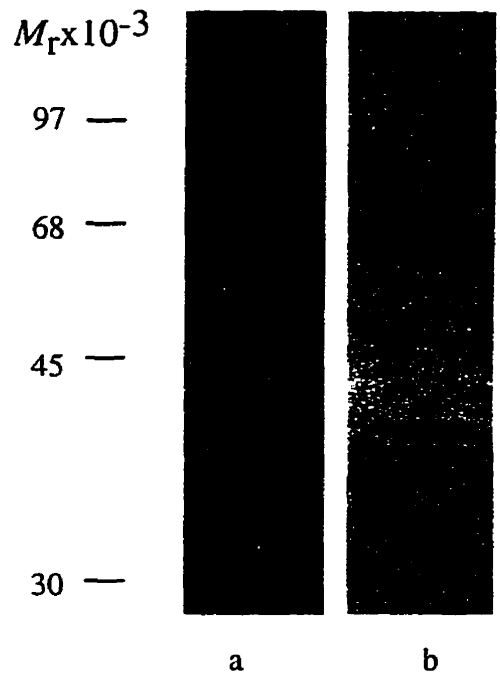


Figure A.6 Binding of LBP to L1 cytoplasmic domain-affinity column. WM 239 cell lysate was passed through either a GST-Cyto column or a GST column. Columns were washed with PBS containing 0.1 M NaCl. Bound material was eluted with 1 M NaCl and followed by 1% SDS. Samples were subjected to SDS-PAGE, and LBP in the eluate was detected by Western blot using rabbit anti-LBP antiserum at 1:1000 dilution. The position of LBP32 is indicated by an arrowhead.

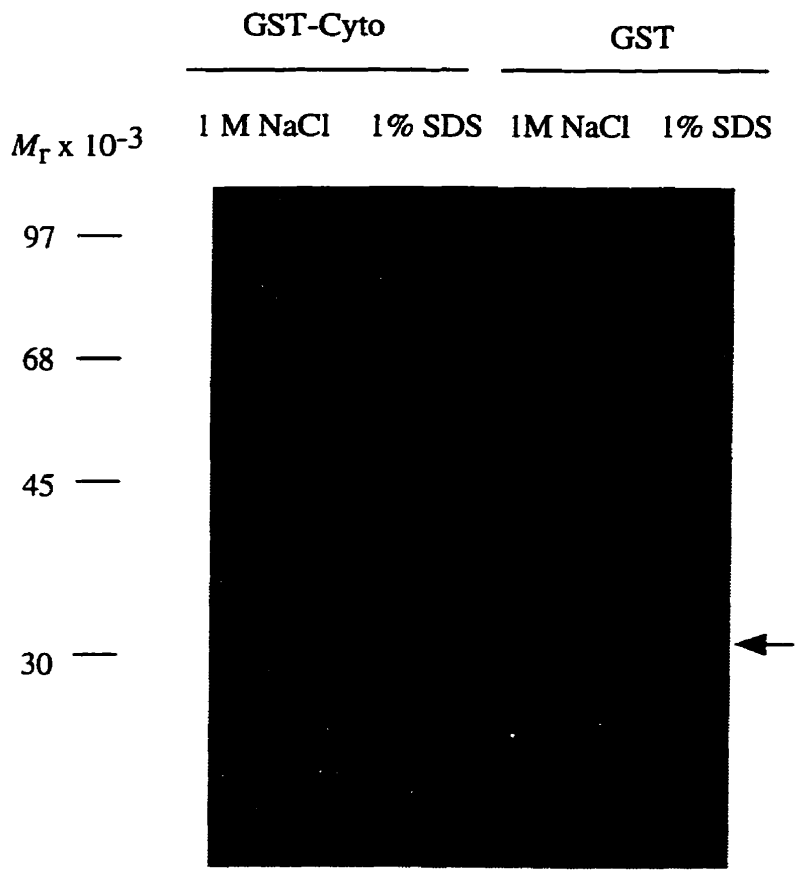

GST-Cyto
GST
1 M NaCl
1% SDS
1M NaCl
1% SDS
M_r x 10^{-3}
97
68
45
30

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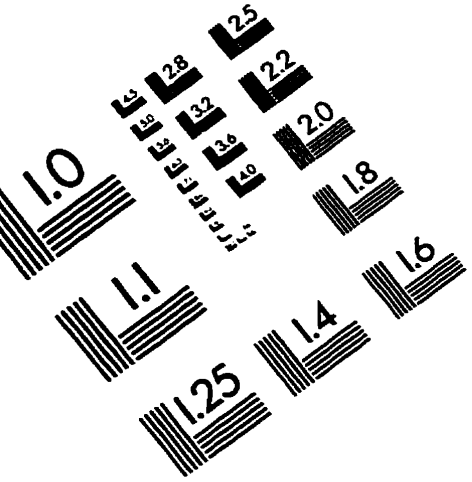
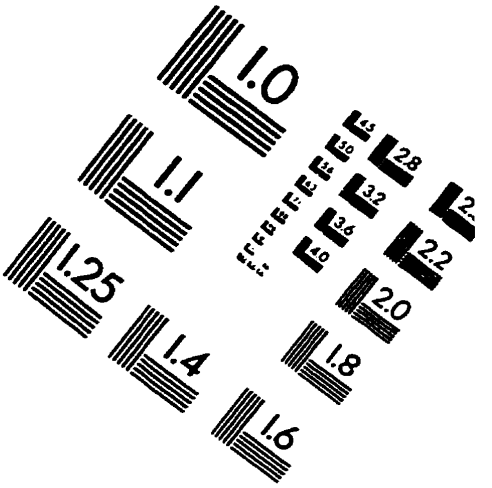
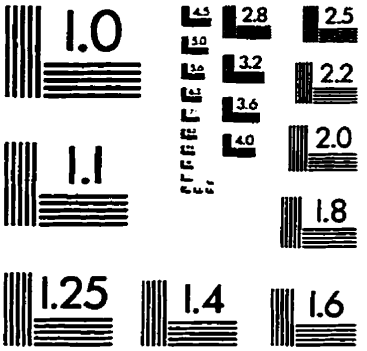
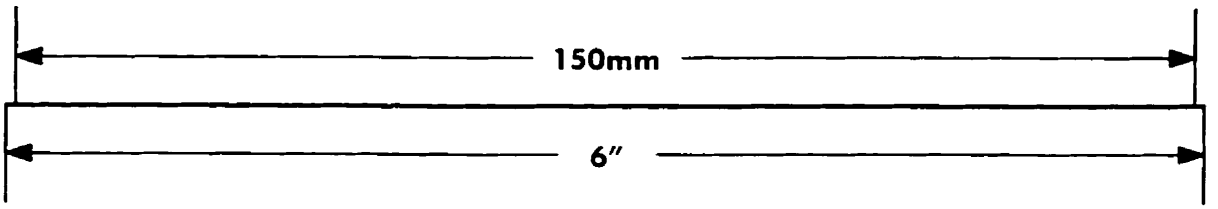
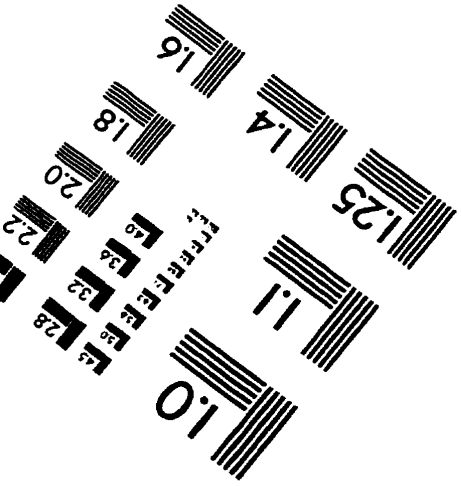
IMAGE EVALUATION
TEST TARGET (QA-3)

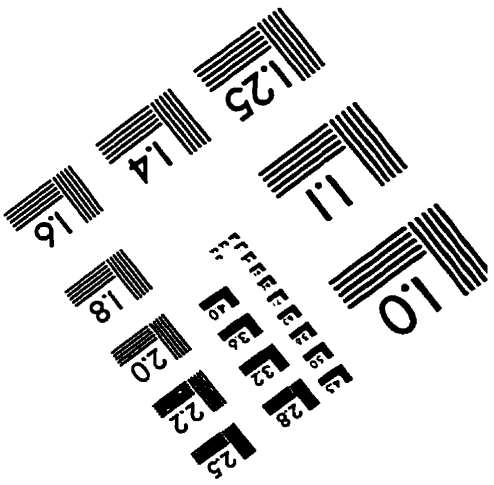












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