Role of Ceramide in Induction of Fos, Jun, and Collagenase in Chondrocytes

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
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ABSTRACT

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Master of Science, 1997

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Arthritis is marked by increased expression of collagenase, which is induced by IL-1. IL-1 is found at increased levels in arthritic joints, and stimulates mRNA expression of c-fos and c-jun, whose products comprise AP-1, a transcription factor necessary for IL-1-induced collagenase expression. Previous data also implicate IL-1 in sphingomyelin hydrolysis to the second messenger ceramide.

IL-1 induced ceramide release in chondrocytes, and ceramide activated c-fos, and c-jun, but not collagenase expression. The induction of c-fos was specific, transient, and transcriptionally and post-transcriptionally regulated. Ceramide potentiated the effect of low IL-1 concentrations, indicating that ceramide is not solely a second messenger. Ceramide induction of c-fos was not accompanied by increased SRE binding, nor did it involve MAPK activation, or ROS as signalling intermediates, but did involve increased SRE transcriptional activity. Ceramide did not induce collagenase expression, but did increase AP-1 transcriptional activity. At high concentrations of ceramide, MMP induction was inhibited. Thus, ceramide appears to function separately from IL-1 through a MAPK-independent pathway that is sufficient to induce c-fos expression.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Tony Cruz, and my committee members, Dr. Rita Kandel, Dr. Harry Elsholtz, Dr. Jack Hay, and Dr. Cathy Prody, for their intellectual contributions to this study, the members of the lab for their advice and help with technical aspects, and for "showing me the ropes," and my family and friends for their support. I would like to thank Dr. H. Elsholtz and Dr. P. Lemieux for the SRE and AP-1 luciferase plasmid constructs, respectively. Thank you to Dr. C. Prody for the use of the luminometer, and to Dr. R. Kandel for the use of the fluorescent spectophotometer. I would also like to express my appreciation for the financing of this work from the Simcoe Special Fellowship and the Ontario Graduate Scholarship.

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A-SMase	acidic sphingomyelinase
AP-1	activator protein-1
AP-1-luc	AP-1-luciferase reporter construct
ARE	AU-rich element
C2-cer	C2-ceramide (*N*-acetyl-D-sphingosine)
C6-cer	C6-ceramide (*N*-hexanoyl-sphingosine)
cAMP	cyclic adenosine monophosphate
CAPK	ceramide-activated protein kinase
CAPP	ceramide-activated protein phosphatase
CKII	casein kinase II
CRE	cAMP response element
DAG	diacylglycerol
DEPC	diethyl pyrocarbonate
DHC	dihydroceramide
DPI	diphenyleneiodonium
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediamine-N,N,N′,N′-tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	O,O′-Bis(2-aminoethyl)ethyleneglycol-N,N,N′,N′-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FB1	fumonisin B_1
FBS	fetal bovine serum

Fc	crystalline fragment of an antibody
Fra	fos-related antigen
FRK	fos-regulating kinase
GAPDH	glyceraldehyde phosphate dehydrogenase
GDP	guanosine 5'-diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
Grb2	growth factor receptor-bound protein 2
GTP	guanosine 5'-triphosphate
HIV	human immune deficiency virus
IFN-γ	interferon-gamma
IgG	immunoglobulin G
IgM	immunoglobulin M
I-κB	inhibitor of kappa B
IL-1	interleukin-1
JAK	just another, or janus, kinase
JNK	jun N-terminal kinase
MAPK	mitogen activated protein kinase
MBP	myelin basic protein
MMP	matrix metalloproteinase
MOPS	morpholinopropanesulfonic acid buffer
mRNA	messenger ribonucleic acid
N-SMase	neutral sphingomyelinase
NAC	*N*-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NF–κB	nuclear factor kappa B
NGF	nerve growth factor
OA	osteoarthritis

PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PKA	protein kinase A
PNPP	p-nitrophenyl phosphate tris buffer salt
RA	rheumatoid arthritis
ROS	reactive oxygen species
RSRF	related to serum response factor
SAPK	stress-activated protein kinase
SIE	*sis*-inducible element
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate
PMSF	phenylmethylsulfonyl fluoride
SIF	*sis*-inducible factor
SM	sphingomyelin
SMase	sphingomyelinase
SRE	serum response element
SRE-Luc	SRE-luciferase reporter construct
SRF	serum response factor
Stat	signal transducer and activator of transcription
TBE	tris borate EDTA buffer
TCF	ternary complex factor
TGF-β	transforming growth factor-beta
TIMP-1	tissue inhibitor of matrix metalloproteinases-1
TLC	thin layer chromatography

TNF-α	tumour necrosis factor-alpha
TPA	12-o-tetradecanoyl phorbol-12-acetate
TRE	TPA-response element
uPA	urokinase plasminogen activator
UTR	untranslated region

CHAPTER 1 INTRODUCTION

1.1 ARTHRITIS

The destruction of articular cartilage that occurs in joint diseases, such as arthritis, results in considerable pain and loss of mobility in the affected joints. Billions of dollars are spent annually on the treatment of arthritis and for lost days of work. About 80 to 90% of men and women over the age of sixty-five have evidence of osteoarthritis, while rheumatoid arthritis afflicts about 1% of the world's population, with women sufferers outnumbering men by three- to five-fold (Cotran, 1994). To begin identifying the causative agents of the processes occurring in diseased joints, it is necessary to have an understanding of the components of normal joint tissue and their interactions with each other. In both health and disease, the character and functional capacity of cartilage are determined by the metabolic balance between the components of the extracellular matrix and by the structural relationship between these components (Muir, 1990).

1.1.1 Normal Joint Structure and Articular Cartilage

Normal joints provide both movement and mechanical support, and their anatomy is directly related to their function (Cotran, 1994). Two types of joints exist in the human body - nonsynovial and synovial joints. Nonsynovial, or solid, joints, are represented by the cranial sutures and the bonds between teeth roots and jaw bones, as well as the manubriosternalis and pubic symphyses. They lack a joint space, providing structural integrity with little movement. Synovial joints, however, have a joint space and allow a wide range of motion. They are situated between the ends of bones formed by enchondral ossification, for example in the knee, and are strengthened by a dense fibrous capsule and ligaments and muscles. The synovial membrane is anchored to the capsule and marks the boundary of the joint space. This membrane is lined by synovial cells, which are of two

distinct lineages: Type A macrophage-like cells and Type B fibroblast-like cells. Lacking a basement membrane, the synovial lining merges with the underlying loose vascular connective tissue stroma and allows for exchange between blood and synovial fluid. Synovial fluid is a filtrate of plasma containing hyaluronic acid that acts as a lubricant and nurtures the articular hyaline cartilage.

Articular cartilage is an avascular, alymphatic, and aneural connective tissue (reviewed in Sandberg, 1991; Kuettner et al, 1991). It serves as an elastic shock absorber and wear-resistant surface. Hyaline cartilage is composed of collagen fibres, mostly Type II, accounting for up to 25% of the wet weight; water, which makes up to 80% of the weight; proteoglycans, making up 10% of the wet weight; and chondrocytes, which compose less than 1 to 2% of the total volume of cartilage. During joint movement, the cartilage can be compressed as much as 20% of its original 6 mm height. Nutrient-rich fluid from the synovial cavity moves in and out of the cartilage, similar to water being squeezed in and out of a sponge (Buchanan and Smith, 1995). For this reason, a totally immobile joint will result in cartilage death. The four components of hyaline cartilage also have specific functions in maintaining cartilage integrity. The collagen fibres are arranged in arches so that they are horizontal to the surface, allowing the cartilage to resist tensile stresses and transmit vertical loads. This three-dimensional network of collagen fibres determines the maximal volume of the tissue by preventing the hydrophilic proteoglycans from taking up too much water (Muir, 1990). Proteoglycans are negatively charged and can bind up to fifty times their weight in water. This combination provides turgor and elasticity and helps to limit friction. The chondrocytes, the cells of cartilage, synthesize and enzymatically digest the extracellular matrix (proteoglycans and collagens) of cartilage. In normal, healthy cartilage these processes are in equilibrium. Matrix turnover, ranging from a half-life of weeks, for proteoglycans, to years, for type II collagen, is carefully controlled. Chondrocytes secrete the degradative enzymes, called matrix metalloproteinases (MMPs), in an inactive form and also supply the matrix with enzyme

inhibitors (reviewed in Mauviel, 1993). Diseases that destroy articular cartilage activate the catabolic enzymes and decrease the production of inhibitors. Chondrocytes react by increasing the production of matrix components to compensate for the acceleration in matrix breakdown. Chondrocytes, as well as synoviocytes, fibroblasts, and inflammatory cells, can also secrete cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor (TNF), which trigger the degradative process. The destruction of articular cartilage by joint cells is an important mechanism in many joint diseases.

1.1.2 Osteoarthritis

Osteoarthritis (OA), or degenerative arthritis, is the most common type of joint disease (Block and Schnitzer, 1997). It is characterized by a dynamic, but slowly progressive, erosion of articular cartilage and other joint tissues. It is a non-inflammatory degeneration, but intermittent inflammatory episodes can occur as secondary phenomena. OA is classified into two types: idiopathic, or primary, OA, which occurs as a normal part of the aging process, and secondary OA, which can occur in younger individuals having a predisposing condition, such as joint trauma, congenital developmental deformity, or a systemic disease like diabetes. Women are affected most often in their hands and knees, while men are usually afflicted in their hips (Cotran, 1994).

Although age-related changes in cartilage occur, OA is more than a disease of cartilage wear-and-tear. Chondrocytes play a major role in the development of OA, for example, by producing IL-1, which is involved in matrix breakdown. Other mediators, such as prostaglandin derivatives, TNF, and TGFβ, induce the release of matrix metalloproteinases from chondrocytes while inhibiting matrix synthesis (Cotran, 1994).

In the early stages of OA, chondrocytes proliferate, while the water content of the matrix increases and the concentration of proteoglycans decreases (Cotran, 1994). The superficial layers of the cartilage are degraded, and the matrix cracks. As the cartilage is degraded, the subchondral bone plate becomes the new articular surface, resulting in bone

thickening and damage to the underlying cancellous bone. Synovial fluid can enter the subchondral area through gaps in the bone. The synovium is congested and fibrotic and may contain inflammatory cells.

OA is insidious, with patients afflicted with the primary disease remaining asymptomatic until their late fifties. Symptoms include loss of joint movement and pain that increases with joint use. The most common joints involved are the hips, knees, vertebrae, knuckles, and toes. The wrists, elbows, and shoulders are usually not affected. OA cannot be prevented or halted, and is second only to cardiovascular disease in causing long-term disability (Cotran, 1994).

1.1.3 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that mainly affects the joints, but can also affect other tissues and organs, such as skin, blood vessels, heart, lung, and muscles. It produces a nonsuppurative proliferative synovitis that often progresses to destruction of the articular cartilage and loss of joint movement caused by a fusing of the bony surfaces (ankylosis) (Cotran, 1994; Schiller, 1994). The peak incidence of affliction is in the third and fifth decades of life, but no age is immune. Evidence points to autoimmunity as playing a pivotal role in the chronicity and progression of RA.

It is believed that RA is triggered by an immune response to a microbial agent that is capable of producing arthritis. An acute arthritis is initiated, but it is the continuing autoimmune reaction, mainly by T cells, and the release of inflammatory mediators and lytic cytokines that leads to joint destruction (Weissman, 1982; Harris, 1990). Involved in the causation of RA is a genetic susceptibility, an arthritogenic antigen, an autoimmune reaction within the synovium, and mediators of joint damage. Rheumatoid factors are anti-idiotypic antibodies (principally IgM) directed against the Fc fragment of IgG (Egeland et al, 1982; Randen et al, 1993) which are found at a high titre in severe forms of RA.

In the progression of the disease, the synovium becomes swollen, thickened, and hyperplastic, and creeps over the articular surface forming a pannus and causing erosion of the underlying cartilage. A dense inflammatory infiltrate composed of CD4+ helper T cells, plasma cells, and macrophages fills the synovial stroma. Mediators released by the inflammatory cells and synoviocytes cause osteoclastic activity, allowing the pannus to penetrate into the bone.

Inflammation and tissue destruction are thought to be initiated by interactions between antigen-presenting cells and CD4+ T cells, leading to the activation of macrophages. These activated cells secrete proinflammatory cytokines, such as IL-1 and TNF (Arend, 1997). TNF and IL-1 have been found to induce resorption of cartilage and bone by the release of collagenases from synovial cells; up-regulate expression of adhesion molecules, leading to an increase in white cells in the synovium; inhibit proteoglycan synthesis; and stimulate proliferation of fibroblasts (Cotran, 1994).

The course of RA is variable, with the disease beginning slowly and insidiously in more than half the patients. It generally begins with malaise, fatigue, and muscle pain, but the joints do not become involved until several weeks to months later. The affected joints are swollen, warm, and are stiff following inactivity. Progressive joint involvement occurs over months to years, with the greatest damage occurring in the first four to five years. Afflicted joints include the small joints of the hands and feet, wrists, elbows, knees, and ankles. Destruction of joint structure leads to deformed joints which lack stability and have limited motion. Most patients have a progressive disease for life, with life expectancy being reduced by an average of three to seven years (Cotran, 1994).

1.2 CYTOKINES AND ARTHRITIS

1.2.1 Interleukin-1

This cytokine has been identified as an important mediator in both OA and RA. In RA, the inflamed synovium proliferates, producing erosive changes in the articular

cartilage. In OA, however, the cartilage erodes with little or no accompanying synovial proliferation (Muir, 1990). It has been found that in the joint IL-1 can be produced by macrophages, synovial cells, and chondrocytes. IL-1 promotes cartilage destruction, both by its ability to suppress synthesis of collagen and proteoglycans in cartilage (Goldring et al, 1988), and by its ability to induce synthesis of MMPs (Frisch et al, 1987).

IL-1 is a pleiotrophic cytokine that plays a central role in regulating inflammatory and immune responses. It is secreted in two genetically distinct polypeptide forms, α and β. Although they are only 26% identical in sequence, both species bind to the same receptor on cell surfaces. (Kuno and Matsushima, 1994). Two IL-1 receptors have been found; however, only the type I receptor mediates the biological responses of IL-1 (Sims et al, 1993). The mechanism of IL-1 action after binding to its receptor and the signal transduction cascades induced have not been fully characterized; however, it is known that a myriad of intracellular signals involving the expression of early, mid, and late gene responses are invoked following IL-1. These signals include the release of arachidonic acid through activation of phospholipase A_2 (Conquer et al, 1992); activation of protein kinase C by diacylglycerol (Schutze et al, 1994); signal transduction by G-proteins (O'Neill, 1992); and activation of sphingomyelinases (Kolesnick and Golde, 1994). As well, IL-1 has been shown to stimulate tyrosine kinase-dependent calcium release (Arora et al, 1995), the release of phosphatidic acid species (Bursten and Harris, 1994), and the production of reactive oxygen species (ROS) (Rathakrishnan et al, 1992). As reviewed in Kuno and Matsushima (1994), IL-1 induces phosphorylation of a number of cellular proteins, and induces several protein kinases, including protein kinase C, a 30 kDa serine protein kinase in the cytosol of peripheral blood mononuclear cells, a 85 kDa nuclear kinase, heat shock protein 27-casein kinase, MAP kinase (p42/p44, p38, and p54/Jun kinase), and a ceramide-activated kinase.

(Unless otherwise noted, herein the term IL-1 refers to the effects of IL-1β.)

1.2.2 Tumour Necrosis Factor

TNF is also an important cytokine mediator of both RA and OA, and initiates similar effects as IL-1 in these diseases. A major hallmark of TNF and IL-1 is their redundancy of function, displaying an overlapping range of biological activities.

TNF is a pleiotrophic cytokine that exerts multiple biological functions depending on cell type, including cell proliferation, differentiation, cytocidal activity, and modulation of gene transcription (for review see Heller and Kronke, 1994). It is involved in inflammation, infection, and cancer, and induces apoptosis and differentiation of cultured cells. Like IL-1, it is expressed in α and β forms that are both recognized by the same cell surface receptors and are associated with similar biological effects. The actions of TNF are initiated by high affinity binding to specific receptors, which are expressed on the cell surface of virtually all somatic cell types (Wiegmann et al, 1992). Two distinct types of TNF receptors, the 55 kDa TR55 and the 75 kDa TR75 have been molecularly cloned from different cell types (summarized in Wiegmann et al, 1992). These two receptors bear no similarity to each other at the primary amino acid sequence, indicating that they may be involved in different signalling (Lewis et al, 1991). Stable transfections of human TR55 in murine cells, which lack binding sites for and are non-responsive to human TNF, revealed the cellular responses of binding to TR55 (Kruppa et al, 1992; Brakebusch et al, 1992).

The cellular responses following TNF binding to TR55 (summarized in Wiegmann et al, 1992) include induction of nuclear factor κB (NF-κB), accumulation of the mRNA of *c-fos*, IL-6, and manganous superoxide dismutase, prostaglandin E_2 synthesis, expression of the IL-2 receptor and HLA class I and class II cell surface antigens, growth inhibition and cytotoxicity. Wiegmann et al. (1992) found that the TNF signalling cascade, including stimulation of protein kinase C (PKC), sphingomyelinase, and phospholipase A_2 (PLA_2), and production of the second messengers diacylglycerol (DAG) and ceramide, occurs completely through exclusive binding of TNF to TR55. In contrast, TR75 has been linked to proliferation of thymocytes and a cytotoxic T cell line (Tartaglia et al, 1991).

Neither TR55 nor TR75 have intrinsic kinase activity, but many second messenger systems and membrane-associated enzymes have been found to be involved in TNF signalling (Heller and Kronke, 1994). TNF induces the activation of PLA_2 (Dayer et al, 1985; Neale et al, 1988) and phosphatidylcholine-specific phospholipase C (PC-PLC) (Schutze et al, 1991). PLA_2 activates release of arachidonic acid (AA), which is further metabolized to the pro-inflammatory mediators leukotrienes and prostaglandin E_2 (Heller and Kronke, 1994). AA metabolites have also been shown to regulate gene activity, such as *c-fos* (Haliday et al, 1991). Activation of PC-PLC leads to the production of DAG, an activator of PKC. This enzyme then mediates the activation of several genes, including *c-myc*, *c-fos*, and *c-jun* (Brenner et al, 1989; Lin and Vilcek, 1987). TNF has been shown to activate both PKC (Schutze et al, 1990) and PKA (Zhang et al, 1988), two major enzymes involved in ligand-receptor signalling. The activation of NF-κB by TNF may be mediated by TNF induction of a sphingomyelinase and release of ceramide (Schutze et al, 1992; Candela et al, 1991; Dressler et al, 1992).

(Unless otherwise noted, herein the term TNF refers to the effects of TNFα.)

1.3 GENE ACTIVATION IN ARTHRITIS

1.3.1 *c-fos*

The *c-fos* gene is a cellular immediate early gene that is rapidly and transiently induced upon stimulation of quiescent cells with cytokines, growth factors, or serum (Lau and Nathans, 1985; Almendral et al, 1988). It can also be induced by non-mitogenic factors, such as UV light (Buscher et al, 1988). Immediate early genes couple biochemical changes in the cytoplasm, arising from the binding of agonists to cell-surface receptors, to mediate specific cell responses. They do not require *de novo* protein synthesis following stimulation, instead their protein products become activated and regulate the expression of downstream effector genes, which then control the tissue- or stimulus-specific functional response of the cell (Foletta, 1996). The *c-fos* gene is the cellular homologue of *v-fos*, a

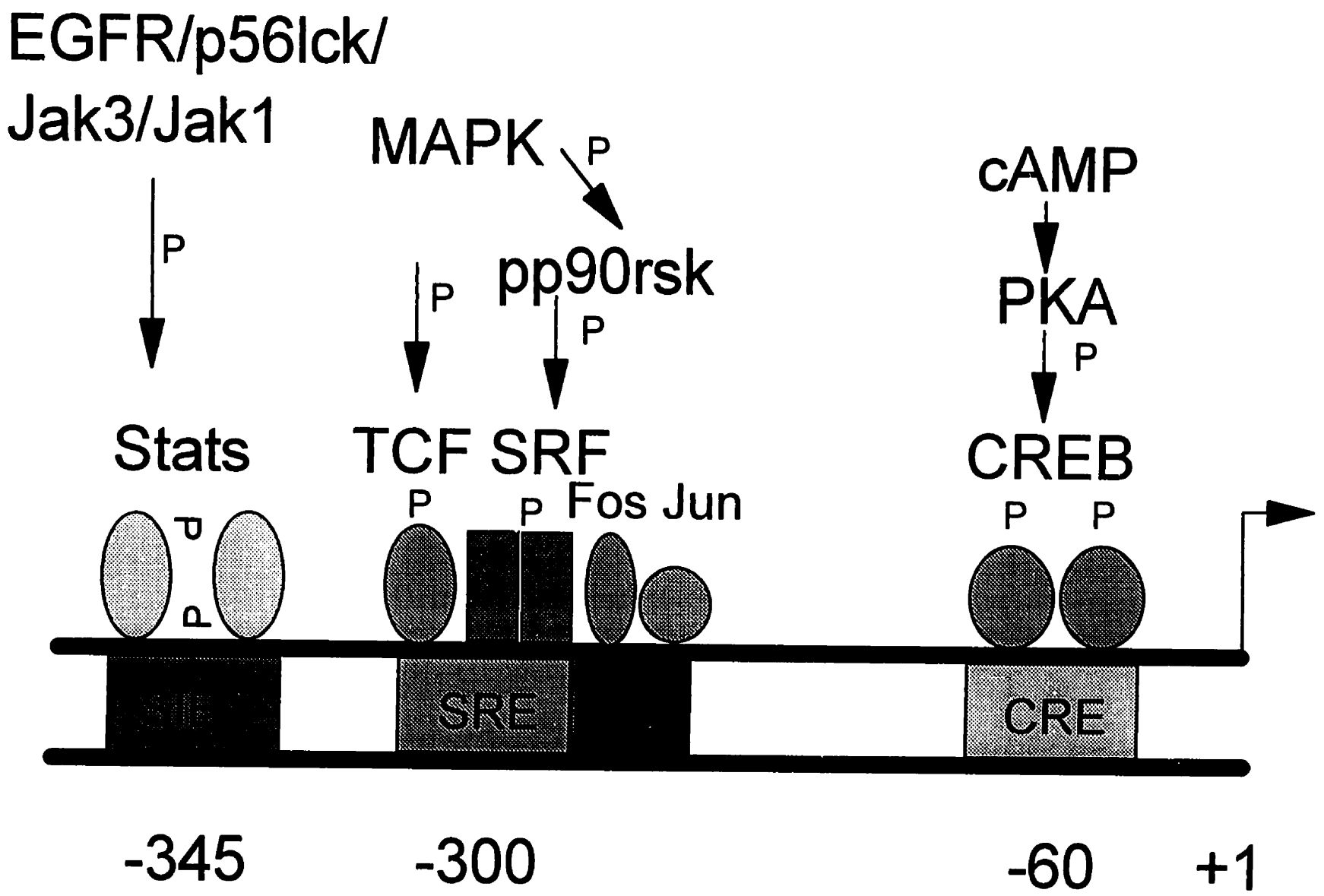
viral oncogene found in the Finkel-Biskis-Jenkins and the Finkel-Biskis-Reilly murine osteosarcoma viruses (Finkel and Biskis, 1966; Finkel and Biskis, 1968), and encodes the protein c-Fos, a basic region-leucine zipper transcription factor that requires dimerization with a member of the Jun family of transcription factors for stable DNA binding (Janknecht et al, 1995). Its induction is independent of *de novo* protein synthesis, indicating that the components are already present for the signalling cascades that target the *c-fos* promoter (Janknecht et al, 1995). There are three proximal sites within the *c-fos* promoter (see Figure I) that have been identified as major targets for cell signals: the *sis* inducible element (SIE), the serum response element (SRE), and the cyclic AMP (cAMP) response element (CRE) (Janknecht et al, 1995).

In *v-sis* transformed cells, the SIE has been shown to confer *c-fos* induction from conditioned medium. SIE is involved in stimulation of *c-fos* by platelet-derived growth factor (PDGF; the B-chain gene of PDGF is the cellular homologue of *v-sis*) (Hayes et al, 1987; Wagner et al, 1990), epidermal growth factor (EGF) (Sadowski et al, 1993), and interleukin-2 and -6 (Zhong et al, 1994). PDGF, EGF, and IL-2 can also target SRE, indicating that the two promoter elements might collaborate (Hatakeyama et al, 1992). Growth factors and cytokines that activate the JAK (Janus kinase, or 'just another kinase') group of protein kinases are involved in the induction of *c-fos* by the SIE (Darnell et al, 1994). The SIE is inducibly bound by the protein aggregate *sis* inducible factor (SIF) (Hayes et al, 1987; Sadowski, 1993). Two members of the Stat (signal transducer and activator of transcription) family form the SIF complexes (Zhong et al, 1994). Stat proteins are latent monomeric cytoplasmic transcription factors that dimerize and enter the cell nucleus upon tyrosine phosphorylation by JAKS (Janknecht, 1995).

Another important element of the *c-fos* promoter is the CRE, a binding site for cAMP response element binding protein (CREB). The CRE mediates *c-fos* induction in response to neurotransmitters and polypeptide hormones, stimuli that use either cAMP or Ca^{2+} as second messengers to activate protein kinase A (PKA) or calmodulin-dependent

Figure I. **The *c-fos* Gene Promoter**

Regulatory sequence elements within the human *c-fos* promoter. Examples of signals directed towards distinct sequence elements are given.

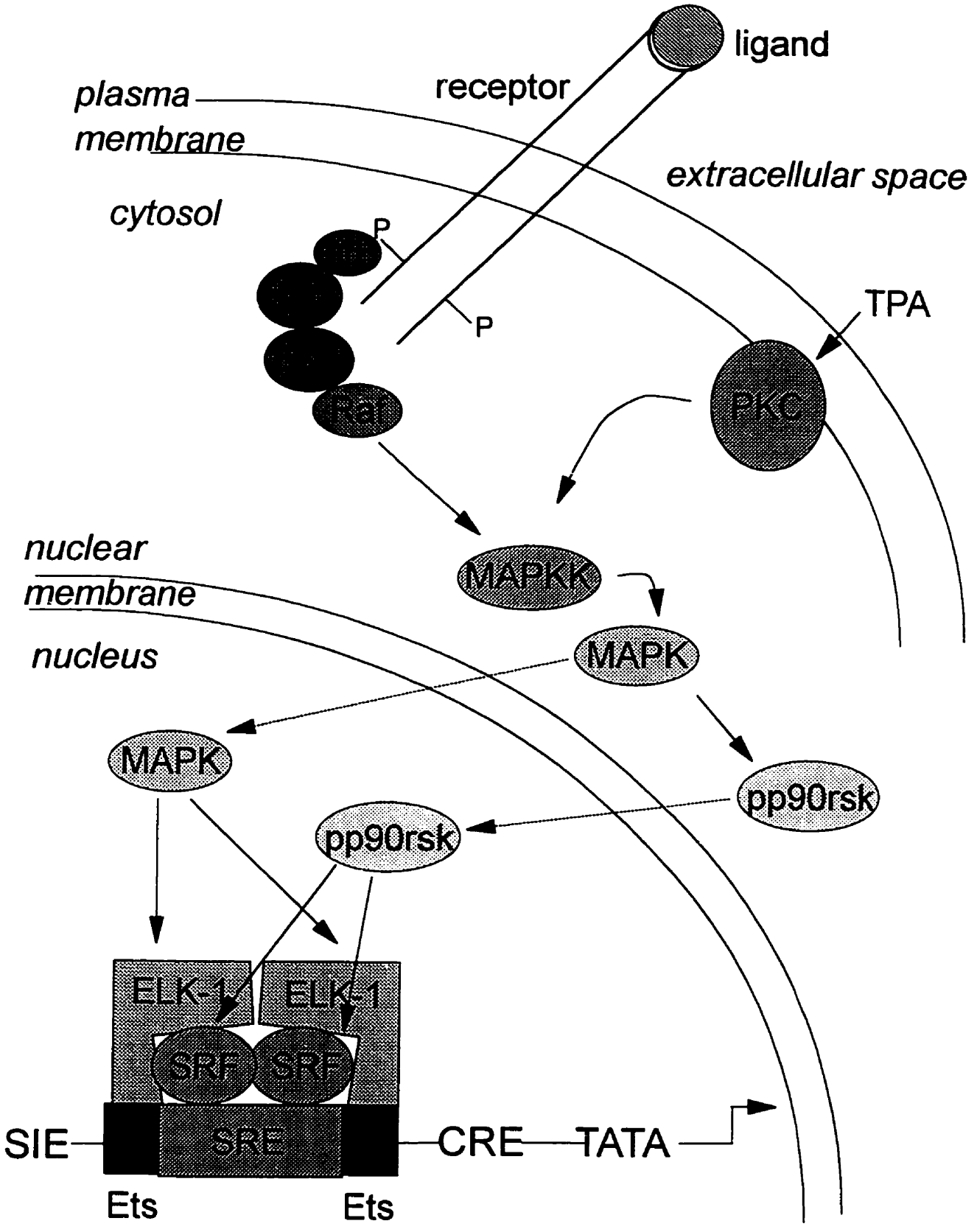

EGFR/p56lck/
Jak3/Jak1
MAPK
P
cAMP
P
pp90rsk
PKA
P
P
P
Stats
TCF SRF
CREB
P
P
Fos Jun
P
P
SRE
CRE
-345
-300
-60
+1

kinases (CaMKs), respectively. CREB contains a basic region-leucine zipper motif that is responsible for dimerization and DNA binding (Dwarki et al, 1990). Phosphorylation of CREB allows it to interact with CREB binding protein (CBP) and thereby establish contact with the transcriptional machinery (Kwok et al, 1994). PKA phosphorylates CREB upon activation by cAMP (Lalli and Sassone-Corsi, 1994), with G proteins transducing the signal from the plasma membrane receptor.

The SRE was originally identified through its induction of *c-fos* by serum, a complex mixture of growth factors and mitogens (Treisman, 1986). Serum, growth factors, and cytokines, and other stimuli that activate mitogen-activated protein kinases (MAPKs) stimulate *c-fos* through the SRE (Treisman, 1992). Stimulation of *c-fos* upon binding of a growth factor to its receptor usually starts with dimerization of growth factor receptor subunits and their subsequent autophosphorylation on intracellular tyrosine residues (see Figure II). Growth factor receptor-bound protein-2 (Grb2) is an adapter molecule that binds via Src homology 2 (SH2) domains to the phosphotyrosyl peptide on the receptor, as well as to Sos, a guanine nucleotide exchange factor that catalyzes the transformation of inactive GDP-Ras to active GTP-Ras. Active Ras recruits Raf-1 to the plasma membrane, leading to its activation and the start of a MAPK pathway. Raf-1 phosphorylates MAPK kinases (MEKs), which phosphorylate a threonine and a tyrosine residue in MAPKs. Several MAPKs exist, with the best studied in mammalian cells being the extracellular signal-regulated protein kinases (ERKs). Once stimulated, MAPK enters the nucleus and phosphorylates serine and threonine residues on substrates such as ternary complex factors (TCFs), for example Elk-1, activating their ability to stimulate transcription. When TCFs are in conjunction with the serum response factor (SRF) they can interact with the *c-fos* SRE (Shaw, 1989), which appears to be constitutively occupied *in vivo* (Treisman, 1992). The SRE was also shown to be the target of another signalling pathway activated by mitogens such as lysophosphatidic acid (LPA), which is not activated by MAPKs (Hill et al, 1995). The transcription factor that mediates this response

Figure II. **Signalling to the SRE of the *c-fos* Gene Promoter**

Example of a signal transduction pathway via the *c-fos* SRE. The MAPK pathway following the binding of a representative growth factor to its receptor is illustrated.


ligand
receptor
plasma membrane
extracellular space
cytosol
P
P
TPA
Raf
PKC
nuclear membrane
nucleus
MAPKK
MAPK
MAPK
pp90rsk
pp90rsk
ELK-1
ELK-1
SRF
SRF
SIE
SRE
CRE
TATA
Ets
Ets

is most likely another SRF interacting protein, different from the known TCFs (Hill et al, 1995).

Following the rapid stimulation of *c-fos* by mitogens, it is rapidly downregulated through dephosphorylation. The attenuation of *c-fos* expression occurs coincidentally with the inactivation of TCF. Addition of phosphatase inhibitors blocks both the dephosphorylation of Elk-1 and the downregulation of *c-fos* following serum stimulation (Zinck et al, 1993; 1995). Phosphorylation of serine 383 in the C-terminal domain of Elk-1 is directly related to its ability to form a quaternary complex of two Elk-1 molecules and two SRF molecules assembled on the SRE (Gille et al, 1996). Although the phosphorylation of TCFs, such as Elk-1, has been shown to promote and to be necessary for activation of *c-fos*, the phosphorylation of SRF by casein kinase II (CKII) does not appear to affect the gene expression of *c-fos* (Janknecht et al, 1992; Manak and Prywes, 1993). In senescent human diploid fibroblasts, hyperphosphorylation of the SRF inhibits its binding to the SRE (Atadja et al, 1994).

The N-terminal domain of the human SRF has a significant role in imparting DNA-binding activity (Sharrocks et al, 1993). The constitutive phosphorylation of N-terminal residues by CKII is not thought to be significant in mediating transcriptional induction in response to stimuli (Janknecht et al, 1992; Manak and Prywes, 1993), but growth factor-regulated phosphorylation by $pp90^{rsk}$ (ribosomal S6 kinase) affects the affinity and rate of SRF association with its binding site (Rivera et al, 1993).

The C-terminal domain of the SRF functions as the transcriptional activation domain, and maximal response to TCF-dependent and -independent pathways of *c-fos* transcriptional induction require its function. Interaction of this domain with the RAP74 subunit of the TFIIF portion of the transcriptional machinery has been reported to be required for transcriptional activation by SRF (Joliot et al, 1995).

Many of the signals targeting one site on the *c-fos* promoter also act on another of the promoter sites as well. Most signals stimulating the SIE also stimulate the SRE, and

several of the signals activating the CRE also act on the SRE (Janknecht et al, 1995). This dual activation may ensure that even weak signals are able to activate *c-fos*, and it also appears that signalling to the *c-fos* promoter nearly always involves the SRE (Janknecht et al, 1995).

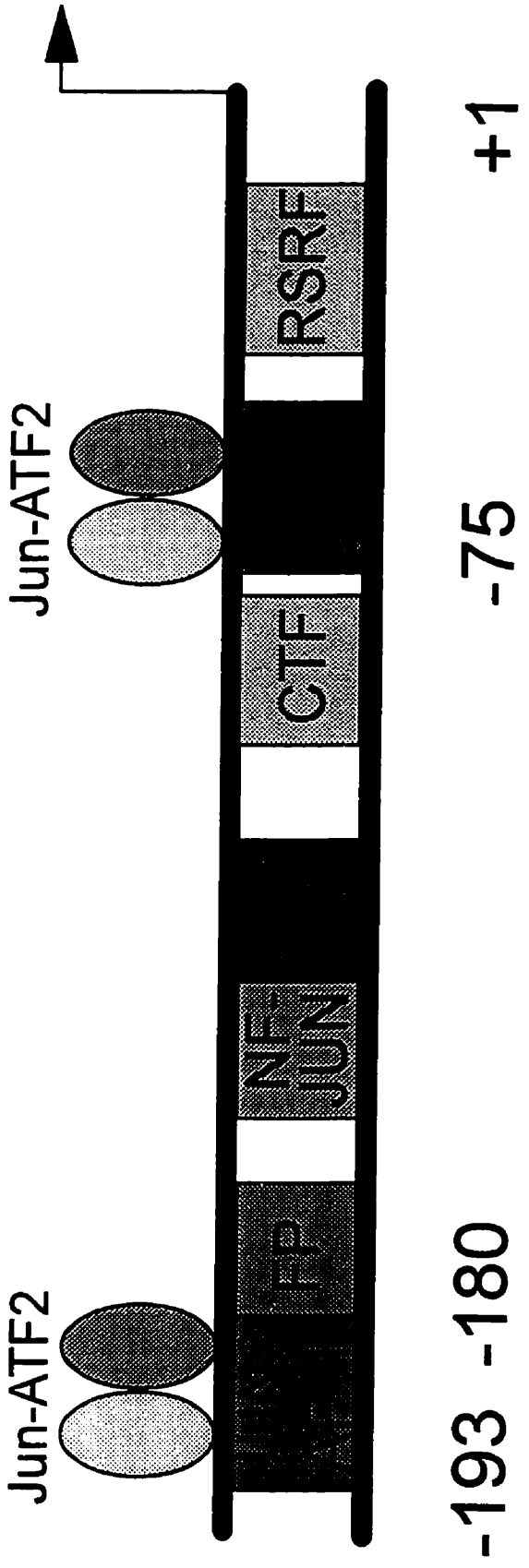
The c-Fos protein has been linked to cell proliferation and transformation (Curran and Vogt, 1992); however, *c-fos*-deficient cells retain the ability to proliferate *in vitro*, and homozygous *c-fos* knock-out mice still mature to adulthood, indicating that *c-fos* is not absolutely necessary for survival (Johnson et al, 1992; Wang et al, 1992).

1.3.2 *c-jun*

The *c-jun* gene, like *c-fos*, is a cellular immediate early gene. It is the cellular homologue of *v-jun*, carried by the avian sarcoma virus 17 (Maki et al, 1987). *c-jun* encodes a DNA-binding domain of the bZIP class (meaning that it consists of a basic DNA binding domain that is *N*-terminal to a hydrophobic amphipathic α-helix), located near its carboxy-terminus, and a *trans*-activation domain near its amino-terminus (Angel et al, 1988; Smeal et al, 1989). Its transcription is induced by genotoxic agents, such as ultraviolet (UV) light and cellular stress. Enhancement of *c-jun* transcription is mediated by the JUN1 and JUN2 regulatory elements on the *c-jun* gene promoter (Angel et al, 1988; Devary et al, 1991; Stein et al, 1992) (see Figure III). These elements are divergent AP-1 sites (differing from the consensus sequence by a 1 base pair insertion) that are bound by either c-Jun homodimers (Angel et al, 1988; Deng and Karin, 1993), or c-Jun-ATF heterodimers (van Dam et al, 1993); thus, c-Jun can regulate its own promoter. JunB, however, acts as a negative regulator of c-Jun (Chiu et al, 1989). Since c-Jun autoregulates its own expression and its induction is unaffected by the inhibition of protein synthesis, it is thought that the transcription of *c-jun* is regulated by post-translational modifications of c-Jun protein (Angel et al, 1988). Phosphorylation of C-terminal serine/threonine residues by glycogen synthase kinase 3 or CKII inhibits the

Figure III. **The *c-jun* Gene Promoter**

Regulatory sequence elements within the human *c-jun* promoter. Examples of transcription factors bound to distinct sequence elements are given.


Jun-ATF2
Jun-ATF2
FP
NF-JUN
CTF I
RSRF
-193 -180
-75
+1

ability of c-Jun to bind to DNA (Boyle et al, 1991). Other protein kinases, including MAPKs, phosphorylate the N-terminus and activate C-Jun. The N-terminal region contains two phosphorylation sites, which are sufficient for transcriptional activation *in vivo* (Franklin et al, 1992). Like the *c-fos* SRE, Jun-AP-1 sites are constitutively occupied *in vivo* (Rozek and Pfeifer, 1993). The activity of these complexes is stimulated by the phosphorylation of c-Jun (Pulverer et al, 1991; Smeal et al, 1992) and possibly ATF-2, a transcription factor of the family ATF/CREB that can form heterodimers with c-Jun (Derijard et al, 1994). This autoregulation is mediated by the JNK/SAPK subgroup of MAPKs, which can phosphorylate both c-Jun and ATF. Phosphorylation also is increased following activation of receptor tyrosine kinases, Src family tyrosine kinases, and Ha-Ras (Smeal et al, 1992). Phosphorylation on serine residues Ser-63 and Ser-73 in the *trans*-activation domain greatly enhances c-Jun transcriptional activity and induction of AP-1 target genes (Pulverer et al, 1991; Smeal et al, 1992). This phosphorylation enhances the transcriptional ability of c-Jun and ATF in part by recruiting coactivator molecules.

The *c-jun* promoter contains other transcription factor binding sites as well. Two SP1 sites most likely function as transcriptional repressor sequences (Unlap et al, 1992). A sequence that binds NF-Jun, which shares some properties with NF-κB, can autoregulate *c-jun* after stimulation by TNF, but appears to be specific for leukemic cells (Brach et al, 1992). A sequence that binds RSRF (related to serum response factor) contributes to *c-jun* induction by EGF and serum (Han et al, 1992).

1.3.3 Activator protein-1 and the Induction of Matrix Metalloproteinases

Activator protein-1 (AP-1) is involved in coupling signals at the cell periphery to changes in gene expression in the nucleus (Wyke et al, 1996). It is a collection of transcriptional activators composed of different members of the Fos and Jun families of proto-oncogenes. These genes are immediate early genes, and their protein products

belong to the bZIP superfamily of DNA binding proteins, which bind to a common DNA recognition site (5′-TGA$^{G}/_{C}$TCA-3′) as a variety of homo- and heterodimers (Lee et al, 1987a). The interaction between Fos and Jun appears to be brought about by the interaction of the leucine zipper structures in the proteins (Lucibello et al, 1989). The leucine residues, which occur in heptad repeats in this domain, are exposed on one side of an α helix and allow the leucine side groups (δ methyl groups) of one protein to align laterally and interact by hydrophobic bonding with the corresponding leucine side chain of the other protein. It is also possible, however, that other amino acids play a crucial role in the interaction between Fos and Jun.

AP-1 was first identified by its role in human metallothionein II_A gene regulation (Lee et al, 1987b), and was also found as a transcription factor that mediates gene induction from the phorbol ester tumour promoter 12-o-tetradecanoyl phorbol-12-acetate (TPA) (Angel et al, 1987). The recognition site for AP-1 is thus also known as the TPA response element (TRE). AP-1 has also been found to be induced by other stimuli, including growth factors, cytokines, T cell activators, neurotransmitters, and UV irradiation (Angel and Karin, 1991). The induction of AP-1 activity can be mediated by the increase in abundance of AP-1 components, or by the modulation of their activity (Karin, 1996).

Although AP-1 binding activity can be measured easily, changes in DNA binding activity do not mirror the regulation of its transcriptional activity. Several proteins form complexes that bind to AP-1 sites, but these proteins differ considerably in their ability to activate transcription of target genes. Optimal binding to the AP-1 consensus sequence (5′-TGA$^{G}/_{C}$TCA-3′), is obtained with a heterodimer of c-Fos and c-Jun due to the increased binding ability and thermostability of this complex as compared with other Jun-Fos heterodimers (Ryseck and Bravo, 1991). It has also been shown that Jun B and Jun D can interact with c-Fos (Nakabeppu et al, 1988), and that Fra-1 and Fos B can interact with c-Jun, with Fos B also being able to interact with Jun B (Cohen et al, 1989;

Zerial et al, 1989). Jun homodimers have also been found to bind to AP-1 consensus sequences, but the binding is weaker than that of a Jun-Fos heterodimer (Ryseck and Bravo, 1991). The half-lives of Jun-Fos-DNA complexes are longer than those of Jun-Jun-DNA, and the most stable complexes are formed with Fos B, followed by Fra-1, and c-Fos (Ryseck and Bravo, 1991). Phosphorylation of specific sites on these proteins enhances their transactivating potential without changing their DNA binding activities or dimerization abilities. Results demonstrate that the binding affinities of Jun family members decrease in the order c-Jun > Jun D > Jun B (Ryseck and Bravo, 1991). These Jun proteins also bind with different affinities to different oligonucleotides containing an identical AP-1 site, indicating that the sequences adjacent to the AP-1 site influence the stability of the Jun-DNA complexes (Ryseck and Bravo, 1991).

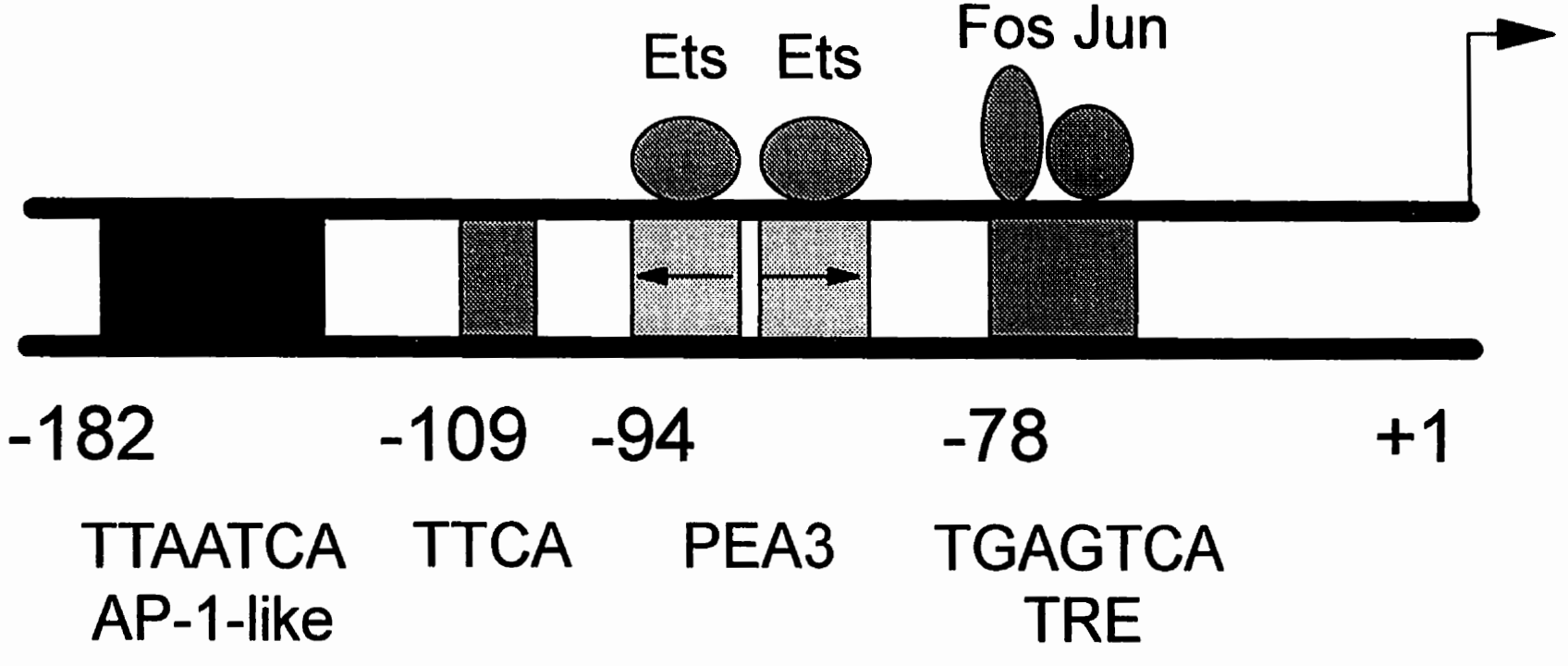
AP-1 activation is accomplished by the induction of *c-fos* and *c-jun*. ERK activation leads to elevated AP-1 activity via *c-fos* induction (Karin. 1996). Synthesis of c-Fos is increased, which then translocates to the nucleus where it combines with pre-existing c-Jun, forming stable AP-1 dimers. Increased dimer stability results in higher levels of AP-1 DNA binding activity, as dimers are required for DNA binding. Phosphorylation of c-Jun and ATF-2 stimulates their transcriptional ability, leading to *c-jun* induction (Karin, 1996). The regulation of AP-1 may occur at the transcriptional, post-transcriptional, and post-translational levels via various signal transduction pathways. At the transcriptional level, *fos* and *jun* genes are regulated by the binding of other transcription factors to regulatory elements within their promoters. Post-transcriptional regulation involves the transient induction of expression by the short-lived stability of the mRNA transcript, for example with *c-fos*, or by alternate splicing, as found with ΔFosB (Foletta, 1996). One mechanism of post-translational control is through regulation of protein transport back to the nucleus; however, the major regulatory mechanism of AP-1 proteins is phosphorylation. The activities of both pre-existing and newly formed AP-1 components are mediated through their phosphorylation.

AP-1 is an important regulatory site in several genes encoding proteins important in the progression of arthritic diseases. The matrix metalloproteinases collagenase (see Figure IV) and stromelysin both contain an AP-1 binding site in their promoters, and this site in necessary for their induction by IL-1 (Angel et al, 1987; Zafarullah et al, 1992). MMPs are members of a family of zinc-binding endopeptidases that are responsible for degrading the components of the extracellular matrix (ECM). MMPs have different substrate specificity, with interstitial collagenase I (MMP-1) being able to degrade collagen types I, II, and III, and stromelysin-1 (MMP-3) degrading collagen types III and IV, proteoglycans, fibronectin, and laminin (Sato and Seiki, 1992). The combined action of various MMPs is essential for efficient turnover of the ECM; however in pathological conditions like arthritis, the expression of MMPs is no longer tightly controlled. Since the AP-1 site is thought to be the common element that mediates the induction of MMP gene activity by cytokines, an understanding of the regulation of AP-1 and its components, c-Fos and c-Jun, plays an important role in the understanding of the mechanisms of damage in arthritis.

Although the induction of AP-1 is necessary for cytokine induction of MMPs, it is not sufficient. Most of the MMP promoters also contain polyoma enhancer activator (PEA3) sites, which bind the Ets family of oncoproteins. Functional cooperativity between AP-1 and PEA3 sits is well documented (Crawford and Matrisian, 1996). Mutation of either the AP-1 site or both of the PEA3 sites of the human interstitial *collagenase* (MMP-1) promoter reduce TPA- and oncogene-inducibility of this MMP by more than 50% (Gutman and Wasylyk, 1990; Wasylyk et al, 1990), indicating a synergistic relationship between transactivators binding to these sites. *c-fos* expression is not sufficient for *collagenase* or *stromelysin* induction, and conditions that allow for *collagenase* expression are not sufficient to induce *stromelysin-1* expression (Gack et al, 1994). Work using *c-fos* null mice indicates that *c-fos* is necessary for *collagenase* expression and is required to enhance *stromelysin-1* expression under certain conditions

Figure IV. **The *collagenase* Gene Promoter**

Regulatory sequence elements within the human *collagenase* promoter. Examples of transcription factors bound to distinct sequence elements are given.


Ets
Ets
Fos Jun
-182
-109
-94
-78
+1
TTAATCA
AP-1-like
TTCA
PEA3
TGAGTCA
TRE

(Hu et al, 1994). In *c-jun*-deficient fibrosarcoma cells, *c-jun* has been shown to be essential for *collagenase*, but not *stromelysin-1*, transcription (Marshall-Heyman et al, 1994).

Fos and Jun family members also act as transcriptional repressors of MMP expression. JunB inhibits *c-jun*-induced collagenase expression in human fibroblasts (Chiu et al, 1989), and TGFβ represses *collagenase* expression through a *junB*-dependent mechanism in dermal fibroblasts (Mauviel, 1995).

Other regulators, such as NF-κB and PEA3 sites, function cooperatively with AP-1 sites in MMP transcription (Sato and Seiki, 1993). Fos and Jun proteins interact with NF-κB (Stein et al, 1993), leading to functional cooperativity between NF-κB and the AP-1 site under conditions that induce *fos* and *jun* (Sato and Seiki, 1993). AP-1 binding to the AP-1 site can facilitate binding at the weak PEA3 site in *collagenase*, with the functional cooperativity mediated by protein-protein interactions independent of promoter binding (Wasylyk et al, 1990). Ets family members c-Ets-1 and c-Ets-2 are neither necessary nor sufficient for *collagenase* transcription (Buttice and Kurkinen, 1993). Additional elements may act to regulate AP-1 and Ets binding, such as the urokinase plasminogen activator (uPA) enhancer, where the PEA3/AP-1 site cooperates with a downstream TRE in a manner dependent on proteins binding to intervening sequences (Nerlov et al, 1992). Other regulatory elements, such as NF-κB or the TGFβ-inhibitory element (TIE), as well as DNA methylation, chromatin structure, potential enhancers outside of the oncogene-responsive units, or post-translational modification of *trans*-acting factors, are important in MMP transcriptional regulation (Sato and Seiki, 1993). However, productive occupation of the TRE is likely to be a precondition of MMP transcription. Regulation of the factors that bind to the PEA3 and AP-1 sites may define conditions as permissive or restrictive for MMP expression in different cell types and in response to various stimuli (Crawford and Matrisian, 1996).

1.4 SPHINGOLIPIDS AND SIGNAL TRANSDUCTION

Previous research has demonstrated the role of ceramide in the activation of AP-1 (Sawai et al, 1995). Ceramide is a putative second messenger released from sphingomyelin by the action of a sphingomyelinase, which is induced by IL-1 (Spiegel et al, 1996). Since IL-1 is a prominent cytokine in arthritic joints, perhaps ceramide could also be elevated in arthritis. The role of the sphingomyelin pathway and ceramide was examined in chondrocytes in this thesis.

1.4.1 The Sphingomyelin Pathway

The current paradigm for the action of sphingolipids is that complex sphingolipids, such as gangliosides, interact with growth factor receptors, the extracellular matrix, and neighbouring cells; whereas the backbones of sphingolipids, including sphingosine and other long chain sphingoid bases, ceramides, and sphingosine 1-phosphate, activate or inhibit protein kinases and phosphatases, ion transporters, and other regulatory machinery (Spiegel and Merrill Jr., 1996).

The sphingomyelin pathway is a ubiquitous, evolutionarily conserved signalling pathway analogous to the cAMP and phosphoinositide pathways (Spiegel et al, 1996). Sphingomyelin (N-acylsphingosine-1-phosphocholine; SM) is a phosphosphingolipid concentrated preferentially in the outer leaflet of the plasma membrane of most mammalian cells. It is composed of a long chain sphingoid base backbone, a fatty acid (ceramide), and a phosphocholine head group (Kolesnick and Golde, 1994). The sphingolipid biosynthetic pathway (see Figure V) begins with serine palmitoyl transferase catalyzing the condensation reaction of serine with palmitoylCoA to form 3-ketodihydrosphingosine or 3-ketosphinganine (Spiegel et al, 1996). An NADPH-dependent reductase reduces 3-ketodihydrosphingosine to dihydrosphingosine

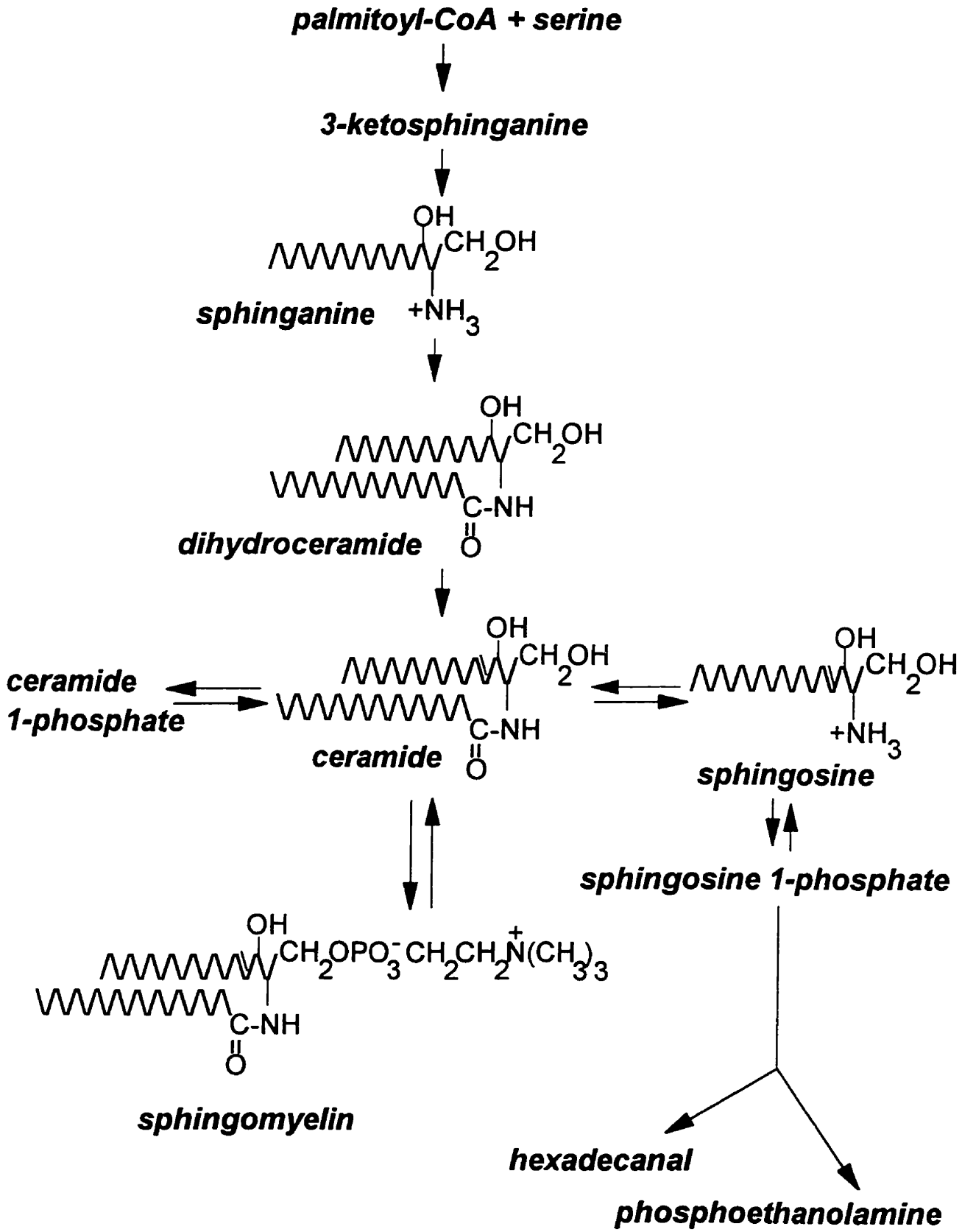
(sphinganine). Dihydroceramide is produced by acylation with fatty acyl CoA. Ceramide, the precursor of all complex sphingolipids, is produced from dihydroceramide by the addition of a *trans*-4,5 double bond (Merrill Jr. and Jones, 1990). The data on the specificity of actions of the various portions of SM indicate that homologues lacking this *trans*-unsaturated bond are inactive (Divecha and Irvine, 1995). SM synthase catalyzes the transfer of a phosphocholine head group from phosphatidylcholine to ceramide, yielding SM.

SM is hydrolyzed to ceramide by the induction of a sphingomyelinase (SMase), a sphingomyelin-specific phospholipase C. SMases are induced by signals generated from a variety of cell-surface receptors. These receptors include those of IL-1, interferon-γ, TNF, nerve growth factor (NGF), and CD95 (Fas). As well, ceramide release has been found to occur in response to cellular stresses, such as nutrition withdrawal and irradiation, and as a downstream event in the responses of the Vitamin D3, progesterone, and CD28 receptors (Testi et al, 1996; Spiegel et al ,1996). Different pools of SM are available to SMases during membrane biogenesis and degradation (Testi, 1996). SM is transported along the exocytic pathway from the Golgi apparatus, where it is synthesized, to the external cell membrane where it is exposed (Rosenwald and Pagano, 1993). Although little sphingolipid "flip-flop" occurs, a discrete inner membrane leaflet SM pool has been proposed by Linardic and Hannun (1994). SMases must access SM at the external cell surface, along the endocytic pathway, and within the lysosomal compartment (Testi, 1996). The activation requirements of different SMases might be responsible for SM hydrolysis in different cellular compartments (Testi, 1996).

Several distinct SMases have been identified, including the acidic enzyme (pH optimum approximately pH 5.0), which might require diacylglycerol to be activated (Kolesnick, 1987), and which resides in caveolae, endosomes, and lysosomes (Liu and Anderson, 1995); the neutral pH optimum, Mg^{2+}- or Mn^{2+}-dependent enzyme, possibly

Figure V. **The Sphingolipid Biosynthetic Pathway**

Metabolism of sphingolipids. Production of ceramide by *de novo* synthesis, which occurs separately from the generation of ceramide by sphingomyelinases. The structure of several sphingolipids is shown.


palmitoyl-CoA + serine
3-ketosphinganine
sphinganine
dihydroceramide
ceramide
1-phosphate
ceramide
sphingosine
sphingosine 1-phosphate
sphingomyelin
hexadecanal
phosphoethanolamine

located on the outer leaflet of cellular membranes; and the Mg^{2+}-independent neutral enzyme, found in the cytosol (Okazaki et al, 1994) and activated by arachidonic acid (Jayadev et al, 1995), which might hydrolyze the inner leaflet SM pool. The genes for the neutral and acidic SMases are distinct, as confirmed by genetic targeting of the acidic SMase in mice, causing loss of acidic SMase activity, but leaving neutral SMase activity intact (Horinouchi et al, 1995; Otterbach and Stoffel, 1995). Mutations in the acidic SMase are linked to the inherited childhood disorder Niemann-Pick disease (Kolesnick and Fuks, 1995).

Originally it was believed that signalling involved only the neutral form of SMase, whereas the acidic SMase was used for membrane turnover in the lysosomal compartment (Kolesnick and Fuks, 1995). Stimuli linked to activation of the neutral SMase include TNF, IL-1, Vitamin D3, NGF, ionizing radiation, and Fas (Spiegel et al, 1996). The acidic SMase is activated by TNF, Fas, and CD28 (reviewed in Merrill Jr. and Jones, 1990). The activity of the cytosolic Mg^{2+}-independent enzyme is enhanced within 1 h of Vitamin D3 stimulation in HL-60 cells (Okazaki et al, 1989); however purification of this enzyme and determination of its amino acid sequence is necessary for further understanding of its activation and action. Ceramide generated from neutral SMase (N-SMase) by the binding of TNF to its receptor has been shown to activate proline-directed serine/threonine protein kinase(s) and phospholipase A_2 (PLA_2); the acidic SMase (A-SMase) triggers NF-κB activation (Wiegmann, 1994). When SM is hydrolyzed, the phosphocholine head group is released into the aqueous environment, while ceramide diffuses within membranes (Testi, 1996). The diverse SM pools and their differing availability to distinct SMases may account for the specificity of the signalling pathways mediated by ceramide (Testi, 1996).

Ceramide acts as the intracellular second messenger of the sphingomyelin pathway. It exists in mammalian cells at a concentration of 1-2% with respect to total phospholipid concentration (Hannun, 1994; Kolesnick and Golde, 1994). Ceramide exists in both a structural pool and a signalling pool of lipids (Spiegel et al, 1996). As a component of

phospholipids, ceramide is found in bound and free forms distributed throughout all cellular membranes. In response to signals from cell surface receptors, the levels of free ceramide increases 1.4-2.0-fold over control levels (Hannun, 1994; Kolesnick and Golde, 1994). This value greatly underestimates the increase in the signalling pools, which are believed to contain little or no second messenger in the absence of stimulus (Spiegel et al, 1996). As well, signals are amplified following their generation, which helps to explain how small transient signals at the cell surface are able to exert pleiotrophic biological effects.

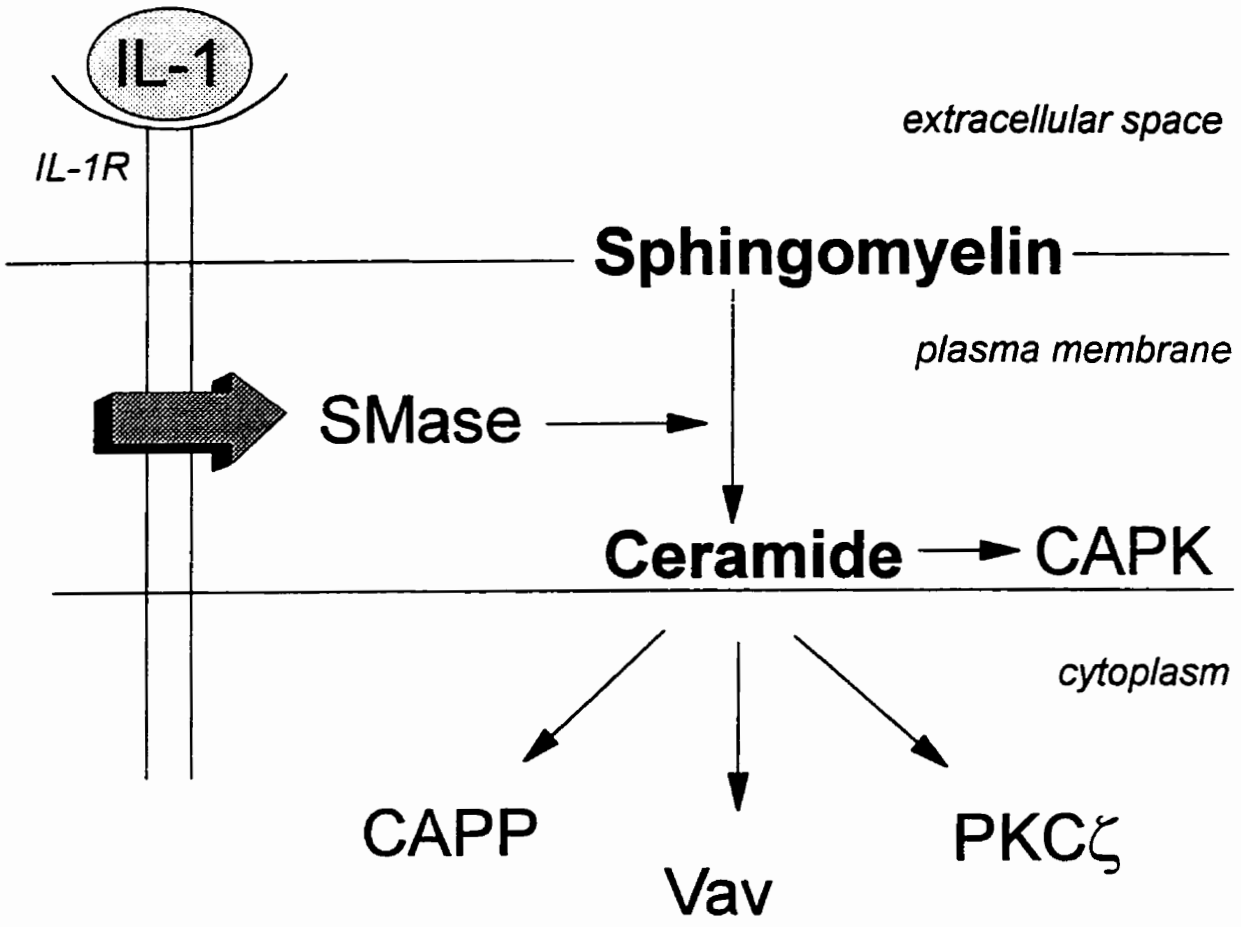
The generation of ceramide has been linked to inflammation (Joseph et al, 1994; Wright and Kolesnick, 1995), differentiation (Okazaki et al, 1989), proliferation (Boucher et al, 1995), and apoptosis (Obeid et al, 1993) in various cell types. Ceramide elicits a number of actions, including the induction of protein kinases and protein phosphatases, regulation of the levels of *c-myc* proto-oncogene and of the translocation of NF-κB, as well as modulation of the activity of phospholipase A_2 and of the release of prostaglandin (Hannun et al, 1994). Direct targets of ceramide action (as reviewed in Spiegel et al, 1996) include a ceramide-activated serine/threonine protein kinase (CAPK), a ceramide-activated heterotrimeric protein phosphatase 2A (CAPP), the guanine-nucleotide exchange factor Vav, and the protein kinase C ζ isoform (see Figure VI). Intracellular signalling molecules that respond to elevation of cellular ceramide levels include Raf-1, MAPK, stress-activated/Jun protein kinase, arachidonic acid, *c-myc*, the retinoblastoma gene product, the inhibitor of κB (I-κB), AP-1, Bcl-XL, and fodrin.

1.4.1 Sphingomyelin and Phosphoinositide

Similarities have been observed between the sphingomyelin signal transduction pathway and that of the phosphoinositide pathway. Ceramide and DAG, the central lipids in these pathways, are both rapidly released by receptor-induced activation of membrane-bound phospholipases (Kolesnick, 1991). They are both small molecules of

Figure VI. **Putative Targets for Ceramide**

Direct targets for ceramide action include a ceramide-activated protein kinase (CAPK), a ceramide-activated protein phosphatase (CAPP), the guanine-nucleotide exchange factor Vav, and the protein kinase C zeta isoform (PKCζ) (Spiegel et al, 1996).


IL-1
IL-1R
extracellular space
Sphingomyelin
plasma membrane
SMase
Ceramide
CAPK
cytoplasm
CAPP
Vav
PKCζ

approximately 600 Da, are very hydrophobic, and readily redistribute across the membrane bilayer (Kolesnick and Fuks, 1995). Ceramide and DAG are both substrates of the bacterial enzyme diacylglycerol kinase, implying some structural similarity (Kolesnick and Golde, 1994). SM synthase, the mammalian enzyme responsible for the generation of SM, transfers the head group from DAG to ceramide to form SM from phosphatidylcholine (Kolesnick and Fuks, 1995). The phosphorylated forms of these molecules, ceramide 1-phosphate and phosphoinositide, respectively, are also structurally similar (Kolesnick and Golde, 1994). The neutral form of SMase is a phospholipase C, just as the main enzyme in the phosphoinositide pathway is phospholipase C (PLC). DAG uses PKC for signalling and, although ceramide does not activate PKC, it does activate its own kinase.

Although DAG and ceramide appear to be quite similar, some differences do exist (Barenholz and Thompson, 1980). Natural DAG contains two long-chain fatty acids of approximately equal length (C16-C20), while natural ceramide contains a 24-carbon fatty acid. Ceramide is more saturated than DAG and contains a significantly more polar region than the complementary region of DAG. Ceramide has a greater interaction with water and increased hydrogen bonding, allowing for greater stabilization of the lipid bilayer. These differences are recognized by mammalian cells and may be responsible for determining specificity of function.

Isoforms of PKC that are stimulated by DAG are not stimulated by ceramide, and PKCζ, which is recognized by ceramide, is not affected by DAG. The targets of ceramide, other than Vav, are not targets of DAG. Ceramide is able to activate CAPK, NF-κB translocation to the nucleus, HIV replication, and apoptosis in HL-60 cells, as well as IL-2 secretion in EL4 cells, although none of these effects are observed with DAG or other potential lipid second messengers (Kolesnick and Fuks, 1995).

Studies comparing the effects of activators of PKC were the first to show that SM is not only a structural element, but that it is actively metabolized and participates in signal transduction as well (Kolesnick, 1987). Kolesnick found that treatment with DAG caused

rapid SM hydrolysis to ceramide in GH_3 rat pituitary tumour cells. Phorbol esters, the other class of PKC activator, did not cause SM hydrolysis. This DAG-induced SM hydrolysis was not affected by the down-modulation of PKC. It was later found that the ceramide generated was subsequently deacylated to free sphingoid bases (Kolesnick and Clegg, 1988), which are potential inhibitors of PKC (Hannun et al, 1986; Merrill et al, 1986). DAG might activate PKC directly, and then inhibit it indirectly, through the liberation of ceramide. Since the physiologic DAGs, but not the tumour-promoting phorbol esters, stimulated SM hydrolysis, the differences between the physiologic activation of PKC and tumour promotion might involve sphingolipids. The proposed inhibitory pathway of PKC involving SM hydrolysis to ceramide and deacylation to sphingoid bases was appealing, however the amount of sphingoid bases generated was minimal (Slife et al, 1989). Since Schneider and Kennedy (1973) had shown that commercial preparations of *Eschericia coli* DAG kinase efficiently utilized ceramide as a substrate, ceramide 1-phosphate was used as a probe to determine whether mammalian cells contained an endogenous supply of this phosphorylated compound (Dressler and Kolesnick, 1990). Human leukemia-60 (HL-60) cells contained ceramide 1-phosphate and its level increased with the generation of ceramide. This conversion of ceramide to its phosphorylated form was specific for ceramide derived from SM, as ceramide from glycosphingolipids was not phosphorylated.

The mammalian enzyme responsible for phosphorylating ceramide is a calcium-dependent kinase distinct from DAG kinase (Kolesnick and Hemer, 1990). It is incapable of using DAG as a substrate, and mammalian DAG kinase is unable to use ceramide as a substrate (Younes et al, 1992). These studies provide evidence of a pathway from SM to ceramide that bifurcates at ceramide to either ceramide 1-phosphate or sphingoid bases (Kolesnick, 1994).

Cross-talk between the glycerolipid and sphingolipid signalling pathways could meet at the plasma membrane enzyme phosphatidate phosphohydrolase, a relatively

non-specific lipid phosphomonoesterase that is the site of mutual competition between lipid phosphomonoesters (Brindley et al, 1996).

1.4.2 Sphingosine and Sphingosine 1-Phosphate

It was originally believed that the bioeffector molecules of the sphingomyelin pathway were sphingoid bases, such as sphingosine. Sphingoid bases and ceramide are functionally interconvertible by acylation-deacylation. Sphingosine was originally identified as an inhibitor of PKC (Hannun and Bell, 1987), but has also been found to induce phosphorylation of the EGF receptor (EGFR) at Thr-669, independent of PKC inhibition in A431 human epidermis carcinoma cells (Faucher et al, 1988). Ceramide, as well, induced time- and concentration-dependent phosphorylation of EGFR at Thr-669 (Goldkorn et al, 1991). Ceramide concentrations of 0.1 - 1.0 μM induced EGFR phosphorylation, but ceramide was not deacylated to sphingosine. These concentrations of sphingosine also induced EGFR phosphorylation and sphingosine was acylated to ceramide. Only at concentrations of sphingosine above 1 μM, which overwhelmed the capacity of the system to acylate sphingosine, did the levels of free sphingoid bases rise. These concentrations are those found to inhibit PKC in most systems (Merrill and Stevens, 1989). These studies suggested that ceramide had its own direct bioeffector properties, and that some effects of sphingosine might occur through its conversion to ceramide (Kolesnick, 1994).

Sphingosine inhibits PKC and may function as a negative effector of processes involving this enzyme. Sphingosine (as reviewed in Spiegel et al, 1996) inhibits platelet and neutrophil responses, as well as differentiation of HL-60 cells induced by phorbol esters. It modulates receptor functions and has anti-tumour activity. The regulation of ceramidase, which catalyses the conversion of ceramide to sphingosine, may determine through which lipid the signalling pathway acts.

Zhang et al. (1990) found that low concentrations of sphingosine stimulates DNA synthesis and acts synergistically with other growth factors, such as insulin, EGF, TPA (12-O-tetradecanoylphorbol-13-acetate), the B subunit of cholera toxin, and serum, to induce proliferation of quiescent Swiss 3T3 fibroblasts. Sphingosine reverses ISP-1/myriocin-induced growth inhibition in T cells (Miyake et al, 1995), and inhibition of sphingosine and sphingosine 1-phosphate generation reduces PDGF-induced cellular proliferation (Olivera and Spiegel, 1993; Coroneos et al, 1995). Growth factors, such as PDGF, induce a rapid and transient elevation of sphingosine and sphingosine 1-phosphate levels in fibroblasts (Olivera and Spiegel, 1993), arterial smooth muscle cells (Bornfeldt et al, 1995), and glomerular mesangial cells (Coroneos et al, 1995). Sphingosine and sphingosine 1-phosphate stimulate an increase in the levels of phosphatidic acid, a putative mitogenic signal, but ceramide decreases levels of this mitogen by inhibiting phospholipase D (PLD) activation (Gomez et al, 1994). The inhibition of PLD occurs through ceramide-dependent inhibition of G-protein translocation to the membrane fraction (Brindley et al, 1996). C2- and C8-ceramide have been found to displace the G-proteins ARF and Rho from the membrane fraction to the cytosol. ARF is involved in vesicle movement, while Rho is involved in the organization of the cytoskeleton. The effect of ceramide on the association of G-proteins with membranes could affect the regulation of cell activation, cell motility, and vesicle trafficking.

As reviewed in Brindley et al. (1996), ceramides have been shown to stimulate the dephosphorylation of phosphatidate, lysophosphatidate, sphingosine 1-phosphate, and ceramide 1-phosphate when these agonists are added exogenously to cells. The phosphohydrolase that acts on phosphatidate, lysophosphotidate, sphingosine 1-phosphate, and ceramide 1-phosphate is identical to phosphatidate phosphohydrolase and may terminate the signals from these lipids, generating the other signalling molecules DAG, ceramide, and sphingosine.

Both sphingosine and sphingosine 1-phosphate mobilize internal calcium through an inositol-triphosphate-independent pathway (Spiegel et al, 1996). This response to calcium appears to be a receptor-mediated event. The kinase converting sphingosine to sphingosine 1-phosphate is located in the endoplasmic reticulum, and a sphingosinephosphorylcholine-gated calcium channel has been characterized, and may represent the sphingosine 1-phosphate-gated calcium channel.

Other downstream effects of sphingosine 1-phosphate include activation of the MAPK cascade and of AP-1 (Spiegel et al, 1996).

1.4.3 Ceramide and its Targets

It was originally believed that ceramide liberated from SM hydrolysis was rapidly deacylated to sphingoid bases, and that these compounds were the true signalling molecules. The first evidence of signalling through ceramide occurred when Okazaki and coworkers (1989) showed that ceramide itself had bioeffector properties and was able to induce monocytic differentiation in HL-60 cells. They observed agonist-induced SMase activation and ceramide-induced differentiation of HL-60 cells in response to Vitamin D3, IFN-γ, and TNF. Ceramide was identified as a second messenger due to the rapid activation of SMase (within 15-120 min of cellular stimulation with the above agents), and the ability of synthetic cell-permeable ceramide analogues to bypass receptor activation and mimic the agonists' effects.

It is widely accepted that ceramide initiates a select set of intracellular events and acts as a second messenger analogous to DAG in the phosphoinositide pathway and cAMP in the adenylate cyclase system. Stereospecificity is involved in signalling of ceramide, as the D-erythro configuration is required (Wright and Kolesnick, 1995).

Agents that rapidly increase the cellular content of ceramide (as reviewed by Spiegel et al, 1996) include the 55 kDa TNF receptor, the 80 kDa IL-1 receptor, the 75 kDa NGF receptor, Fas, and ionizing radiation.

The precise mechanism of ceramide action on cells is not precisely defined, but at least four protein targets for stimulation by ceramide have been identified: CAPK (Mathias et al, 1991), CAPP (Dobrowsky and Hannun, 1992), PKCζ (Lozano et al, 1994), and the putative guanine nucleotide exchange factor Vav (Gulbins et al, 1994) (see Figure VI).

CAPK is a 97 kDa plasma membrane-bound kinase with a preference for the sequence X-Ser/Thr-Leu-Pro-X, in which the phosphoacceptor site (Ser/Thr) is followed on the carboxyl terminus by a proline residue and X is any amino acid (Joseph et al, 1993). This enzyme is tightly coupled to both the TNF and IL-1 receptors based on reconstitution experiments performed in cell-free systems (Mathias et al, 1993). Substrate phosphorylation by CAPK, as well as its autophosphorylation, is enhanced ten-fold following an increase in cellular ceramide levels by either the addition of ceramide analogues, or the action of TNF (Spiegel et al, 1996), IL-1, or a purified bacterial SMase (Liu et al, 1994). This stimulation appears to be specific for ceramide, as elevated levels of other lipid second messengers, such as DAG, AA, and phosphatidic acid do not enhance CAPK activity (Dressler et al, 1992). Autophosphorylation of CAPK may be required for enhanced kinase activity towards the exogenous substrates the EGF receptor, Raf, and MBP (Wright and Kolesnick, 1995). Although the activity of CAPK is well characterized, its gene has not been cloned.

CAPK was first identified through the similarities between ceramide and DAG. The ceramide analogue N-octanoyl sphingosine (C8-cer), which is structurally analogous to 1,2-dioctanoylglycerol, a hydrophilic form of DAG, was found to induce the phosphorylation of the EGF receptor (EGFR) at Thr-669 (Kolesnick, 1991). Exogenous sphingoid bases also induced phosphorylation at this site (Kolesnick and Golde, 1994). Kinase activity towards a peptide derived from the amino acid sequence surrounding Thr-669 of the EGFR was detected in subcellular fractions of A431 cells (Kolesnick, 1991). This kinase activity was exclusively membrane-bound, and was directed solely towards Thr-669, despite the presence of another potential phosphorylation site. Ceramide

also enhanced kinase activity *in vitro* (Kolesnick, 1991). Kinase activity was found to be linearly dependent on substrate concentration. K*m* values were determined to be 15 μM for ATP and 0.25 mg/mL for EGFR peptide. The maximum reaction velocity was found to be 50-100 pmol of peptide phosphorylated/min/mg protein and the pH optimum was 7.0-7.4. Ceramide enhanced kinase activity in a time- and concentration-dependent (1 nM-1 μM) manner, with an effective $dose_{50}$ (ED_{50}) of approximately 30 nM. These same concentrations are those found effective for EGFR phosphorylation *in vivo*. Similarly, sphingosine induced EGFR phosphorylation with a similar concentration-dependence and to a similar extent; however, palmitic acid, the major fatty acid in natural ceramide (Merrill and Stevens, 1989), did not increase kinase activity, suggesting that the kinase was activated by the sphingosine moiety within ceramide (Kolesnick, 1994).

Examining single site amino acid substitutions within the Pro-Leu-Thr-Pro motif of the EGFR revealed the minimum recognition motif for CAPK. Substitution of Ala for the C-terminal Pro abolished kinase activity, but the same mutation at the N-terminal had no effect (Kennelly and Krebs, 1991; Joseph et al, 1993). Substitution at the Leu reduced activity. The minimal motif required for CAPK recognition is Leu-Thr-Pro, which distinguishes it from other proline-directed kinases, such as MAPKs (Pro-X-Ser/Thr-Pro) and cdc2 kinases (X-Ser/Thr-Pro) (Kennelly and Krebs, 1991). Similar sequences to this motif are found in a variety of potential substrates, such as the previously mentioned EGFR and the proto-oncogene *raf* (Liu et al, 1994).

CAPP is a cation-independent Ser/Thr protein phosphatase with distinct functional domains (Wolff et al, 1994). The C subunit houses the catalytic activity, while binding to ceramide is conferred by the B subunit (Spiegel et al, 1996). An elevation in ceramide levels may trigger CAPP translocation from the cytoplasm (where it is found in resting cells) to membranes, where protein dephosphorylation will occur. CAPP activity has been linked to growth inhibition in numerous systems. CAPP is activated by ceramides with different lengths of acyl groups, and is not activated by sphingomyelin, sphingosine (which

differs from ceramide in that it lacks an acyl group attached to the nitrogen and instead contains a single hydrogen), or dihydroceramide (which differs from ceramide in that it lacks the 4-5 *trans* double bond) (Hannun, 1994).

Other targets of ceramide action include the guanine nucleotide exchange activity of the proto-oncoprotein Vav, as well as the activation of PKCζ, which may link the TNF receptor to NF-κB (Muller et al, 1995). The protein Vav has sequence homology to members of the family of guanine-nucleotide exchange proteins, which activate Ras by enhancing exchange of GDP for GTP (Kolesnick and Fuks, 1995). It is a potential membrane-bound target for ceramide in B and T cells. Vav contains a cysteine-rich motif downstream of its putative catalytic domain that is similar to the PKC lipid-binding domain that recognizes phorbol ester and DAG. Ceramide was found to enhance Vav exchange activity *in vitro* and in Vav preparations immunoprecipitated from ceramide-stimulated cells. The coupling of CAPK and Vav stimulation might allow for coordinate activation of multiple elements of the MAPK cascade within the plasma membrane (Kolesnick and Fuks, 1995).

PKCζ, originally cloned by Nishizuka and coworkers (Ono et al, 1989), contains a lipid-binding domain and is stimulated by phospholipid. Phorbol ester and DAG, although able to activate other members of the PKC family, are unable to activate this isoform. Ceramide has been shown to enhance the activity of purified PKCζ *in vitro* (Lozano et al, 1994). As well, treatment of quiescent NIH-3T3 cells with SMase to elevate cellular ceramide levels enhanced the I-κB phosphorylating activity of PKCζ in an immunoprecipitate. The reason for this difference in binding of PKC isoforms by ceramide and DAG may result from the domain structures of these isoenzymes. All of the isoforms contain similar catalytic domains, but the regulatory domains differ significantly (Walsh et al, 1996). Group A PKCs, which include isoenzymes α, β_I, β_{II}, and γ, require Ca^{2+}, phospholipid, and DAG for activity. PKCζ, a group C PKC, lacks the constant region C2, and exhibits kinase activity that is Ca^{2+}-independent. PKCζ, also contains only one

cysteine-rich zinc butterfly motif within its C1 motif, which causes it to be unaffected by DAG.

As discussed in Wiegmann et al. (1994), downstream signalling events triggered by ceramide involve activation of the MAPK cascade, down-regulation of *c-myc* in HL-60 cells, induction of I–κBα degradation and nuclear translocation of NF-κB, regulation of prostaglandin secretion in IL-1-treated cells, enhancement of IL-2 secretion by T cells, mediation of apoptosis, and differentiation of HL-60 cells to monocytes.

The acute elevation of cellular ceramide content may result in a complex pattern of integrated signals that acts on several potential targets. Targets for ceramide may be differentially expressed in different cell types. The pattern of signal transmission and its outcome may also be regulated by other signalling systems. The response of the system might be controlled both by genetics and by external stimuli (Kolesnick and Fuks, 1995).

1.4.4 Cytokines and Ceramide

The criteria for establishing a pathway as a *bona fide* second messenger system are the rapid kinetics of activation of a signalling system, and the ability of analogues of the proposed second messenger to bypass receptor activation and mimic agonist action, both of which are observed with the sphingomyelin pathway and ceramide (Kolesnick and Golde, 1994). The cascade of SM degradation and CAPK activation occurs early in TNF signalling.

Ceramide appears to be the primary mediator of both the inflammatory and apoptotic responses to TNF (Spiegel et al, 1996). The signalling pathway of the inflammatory response, following the activation of a SMase by the binding of TNF to its receptor, begins with the stimulation of CAPK by ceramide. CAPK phosphorylates Raf1, which then initiates signalling through the MAPK cascade (Yao et al, 1995). Activation of cytosolic phospholipase A_2 and release of arachidonic acid leads to inflammation.

TNF induces release of ceramide in all cells in which it initiates apoptosis (Spiegel et al, 1996). Ceramide can also mediate apoptosis induced by other factors besides cytokines. Ionizing radiation causes immediate hydrolysis of SM to ceramide by directly targeting membranes (Haimovitz-Friedman et al, 1994). Daunorubicin, a chemotherapeutic drug, may initiate apoptosis by stimulating ceramide synthase. Fumonisin B1, an inhibitor of ceramide synthase, blocks daunorubicin-induced ceramide synthesis and apoptosis in P388 cells, indicating that this apoptosis resulted from *de novo* ceramide synthesis and not from SM hydrolysis (Bose et al, 1995). Ceramide may also stimulate Fas-mediated apoptosis (Gulbins et al, 1995).

It was found that Vitamin D3 treatment for 30 min activated a neutral SMase, which decreased SM levels to 77% of control levels after 2 h (Hannun, 1994). The SM level returned to control values by 4 h. Synthetic ceramide analogues (C2- and C6-cer) were able to mimic Vitamin D3 to induce monocytic differentiation in HL-60 cells. Incubation of HL-60 cells with 100 nM-1 μM ceramide for 2 h induced differentiation when measured after 4 d, while sphingosine had no effect. TNF and IFN-γ, which induce monocytic differentiation, also stimulated SM degradation after 15 min of stimulation. Other investigations (Kolesnick, 1994) found that SM hydrolysis to ceramide and stimulation of CAPK occurred within the first minute of TNF stimulation. TNF did not increase DAG or free sphingoid base levels. Other agents capable of differentiating HL-60 cells, including retinoic acid, dibutyryl-cAMP, and phorbol ester, did not induce SM hydrolysis.

Evidence that the SM signalling pathway is tightly coupled to TNF receptor activation is derived from reconstitution of events in a cell-free system. TNF was shown to induce rapid SM degradation in post-nuclear supernatants (Dressler et al, 1992) and microsomal membrane preparations (Yanaga and Watson, 1992) of HL-60 cells. Stimulation of CAPK was found to accompany these events. Kolesnick and coworkers (1994) found that TNF enhanced CAPK activity in a time- and concentration-dependent

manner. Kinase activity increased within 5 min, and remained elevated for 2 h. The ED_{50} for kinase activation was 200 pM TNF, which was similar to the values of 100-300 pM for SM hydrolysis and monocyte differentiation in HL-60 cells (Kim et al, 1991). Incubation of HL-60 cell extracts with bacterial SMase (*Staphylococcus aureus*) reproduced the effect of TNF on CAPK activation. Other bacterial phospholipases (including A_2, C, and D) were ineffective at activating CAPK. It is believed that the 55 kDa isoform of the TNF receptor is involved in coupling TNF stimulation to SM hydrolysis. Wiegmann et al. (1992) showed that cells expressing the 55 kDa TNF receptor responded to TNF by rapidly hydrolysing SM to ceramide, while in cells not expressing this receptor, TNF did not induce SM hydrolysis. Yanaga and Watson (1992) showed that an antibody against the 55 kDa TNF receptor that is able to stimulate the receptor resulted in rapid SM hydrolysis in microsomal membranes from HL-60 cells, whereas an inhibitory antibody against the 75 kDa receptor had no effect on TNF-induced SM degradation.

TNF and IL-1 might involve common pathways in signalling through the SM pathway. These agents both initiate an almost identical pattern of early signalling events, including kinase, transcription factor, and early response gene activation (Wright and Kolesnick, 1995). Some common early events occurring in cells following TNF or IL-1 stimulation include MAPK activation, NF-κB translocation, AP-1 stimulation, PLA_2 activation, and TNF gene expression. Ballou et al. (1992) showed that IL-1 induced SM hydrolysis within 1 h of treatment in human dermal fibroblasts. Synthetic ceramide was found to mimic IL-1 in the induction of cyclooxygenase gene expression. Mathias et al. (1993) found that the SM pathway appeared to mediate signalling for IL-1 in the murine T helper cell line EL4. In this system, IL-1 induced SM hydrolysis within seconds and stimulated CAPK, and these results could be reproduced in a cell-free system. SMase, but not phospholipases A_2, C, or D, recapitulated the effect of IL-1 in stimulating IL-2 secretion. Synthetic ceramide also mimicked IL-1 action.

IL-1 has been shown to induce a rapid decrease in SM and elevation of ceramide in HL-60, EL4, and fibroblast cells (Kim et al, 1991; Ballou et al, 1992; Mathias et al, 1993). Synthetic cell-permeable ceramides and exogenous SMase have been shown to bypass IL-1 receptor activation and mimic the biological effects of IL-1 (Kim et al, 1991; Ballou et al, 1992; Mathias et al, 1993; Obeid et al, 1993; Raines et al, 1993). As well, ceramide has been shown to play a similar role in TNF signalling (Dressler et al, 1992; Kolesnick and Golde, 1994; Schutze et al, 1994). It is believed that IL-1-mediated activation of the sphingomyelin pathway and resultant increase in intracellular ceramide may be important in modulating the effects of IL-1, and that this activation may also represent an additional mechanism for intracellular signal transduction resulting from IL-1 receptor binding (Ballou et al, 1992).

The activation of IL-1 and TNF receptors, SM hydrolysis, and CAPK activation most likely occur within the plasma membrane. Signal processing from cell-surface receptors to the cellular interior may occur through the MAPK cascade. In HL-60 cells, SMase and ceramide treatment mimicked TNF action, inducing tyrosine phosphorylation of $p42^{MAPK}$ and also enhancing MAPK enzymatic activity (Raines et al, 1993). The SM pathway may gain access to signalling molecules such as EGFR, cytoplasmic phospholipase A_2, cyclin b, MAPKAP kinase-2, and S6 kinase, as well as to transcription factors such as c-Myc, NF-IL-6, Elk-1, ATF-2, and c-Jun (Davis, 1993).

Another piece of evidence indicating that the SM pathway is a central early event in TNF signal transduction is the ability of the SM pathway to signal NF-κB translocation. In HL-60 cells, SMase and C8-ceramide, like TNF, increased nuclear translocation of NF-κB within 30 s, but DAG analogues or exogenous PLC had no effect (Yang et al, 1993). Other studies (Finco and Baldwin, 1993) suggest that Raf is involved in coupling TNF signalling at the cell surface to the cytoplasm.

As reviewed in Ballou et al. (1996), the effects of ceramide released by the cytokines IL-1 and TNF vary depending on cell type. In HL-60 cells, the cellular targets of

ceramide are CAPK, CAPP, and *c-myc* expression, inducing monocytic differentiation, inhibition of proliferation, and apoptosis; in Jurkat T cells, ceramide released by the action of TNF activates NF-κB, leading to cellular toxicity; while in U937 monocytic cells, TNF-induced liberation of ceramide targets NF-κB, MAPK, PLA_2, and CAPK, resulting in apoptosis. In EL-4 thymoma cells, ceramide liberated by the action of IL-1 activates CAPK leading to the induction of IL-2 secretion. In RA and OA synoviocytes, IL-1 and TNF induce SM hydrolysis to ceramide, which then targets the Cox and IL-6 promoters, resulting in induction of PGE_2 and IL-6 production.

1.5 HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Ceramide acts as a second messenger in an IL-1 signalling pathway that leads to the induction of *c-fos* and *c-jun* expression, and/or the activation of AP-1.

GENERAL OBJECTIVE

Sphingomyelinase activity and consequent levels of ceramide have been found to be increased in various cell types following binding of IL-1 to its receptor. The general aim of this research will be to determine the involvement of sphingomyelinase activity in IL-1 stimulation of *c-fos*, *c-jun*, and *colllagenase* gene expression in chondrocytes.

SPECIFIC AIMS

1. To determine whether ceramide stimulates *c-fos*, *c-jun*, and *collagenase* gene expression in chondrocytes.

2. To determine the mechanism of action of induction by ceramide in chondrocytes.

3. To determine whether induction by ceramide occurs through a mechanism independent of IL-1 activation, and how ceramide and IL-1 interact in signalling through the sphingomyelin pathway.

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents

Human recombinant IL-1β (IL-1), *N*-acetylsphingosine (C2-ceramide), *N*-hexanoylsphingosine (C6-ceramide), Dihydroceramide, and *N*-Acetylcysteine (NAC) were from Sigma. Antibiotics and fetal bovine serum (FBS) were from Gibco. Collagenase A (from *Clostridium histolyticum*) and pronase (from *Streptomyces griseus*) were from Boehringer Mannheim. Diphenyleneiodonium (DPI) was from Toronto Research Chemicals. γ^{32}P-ATP and α^{32}P-dCTP isotopes were from Dupont NEN. N-(4,4-difluoro-5,7-dimethyl-4-bora-3a-4a-diaza-*s*-indacene-3-dodecanoyl)sphingosyl phosphocholine (Bodipy FL C12-sphingomyelin) was from Molecular Probes. SRE, CRE, SIE, and AP-1 consensus and mutant oligonucleotides, and anti-ERK1 antibodies were from Santa Cruz. Poly(dIdC)-poly(dIdC) was from Pharmacia Biotech. Myelin basic protein (MBP) was from Upstate Biotechnology.

2.2 Cell Culture

Primary cultures of bovine articular chondrocytes were used in the following experiments as no reliable chondrocyte cell lines exist. Chondrocytes must be plated at high density to maintain phenotype; proliferating chondrocytes differentiate to fibroblast-like cells. When plated at high density, chondrocytes do not proliferate, and are thus resistant to apoptosis. Reagents that would normally cause apoptosis in proliferating cell types can be used in chondrocytes without this effect.

Primary cultures of bovine articular chondrocytes were isolated as described (Cruz et al, 1990). Cartilage fragments were aseptically collected from the metacarpalphalangeal joints of fresh calves' legs and placed in Ham's F-12 medium. The fragments were washed three times with Ham's F-12 medium and incubated with 0.5% pronase and 3% antibiotics (100X: 10,000 U/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, 25 μg/mL amphotericin B in 0.85% saline) for 1 h at 37 °C in a humidified atmosphere supplemented

with 5% CO_2. The softened cartilage was washed three times with Ham's F-12 medium and then incubated with 0.25 units/mL collagenase, 3% antibiotics, and 5% FBS overnight at 37 °C in a humidified atmosphere supplemented with 5% CO_2. The next day, the cells were plated at a density of 2 X 10^6 cells/mL in 12 mL of Ham's F-12 medium containing 3% antibiotics and 5% FBS. The cells were allowed to recover for 24 h at 37 °C in a humidified atmosphere supplemented with 5% CO_2. The cells were then deprived of serum for 16 h in Ham's F-12 medium containing 3% antibiotics before treatment. IL-1 or ceramide was added for 1 h to induce *c-fos* or *c-jun* expression, and for 16 h to examine *collagenase* expression.

2.3 Northern Blot Analysis

Total RNA was isolated by the acidified guanidine isothiocyanate method (Chomczynski and Sacchi, 1987) after cell treatment. Cells were washed three times in phosphate buffered saline (PBS) prepared in diethyl pyrocarbonate (DEPC) water and lysed in 4 mL Solution D (5.3 M guanidium isothiocyanate, 30 mM sodium citrate, 0.72% N-laurylsarcosine, 0.72% β-mercaptoethanol). To each sample was added 0.5 mL 2 M sodium acetate (pH 4), 4 mL phenol, and 1 mL chloroform. The solution was mixed well and placed on ice for 15 min. After centrifugation in a fixed angle rotor at 7000 rpm for 40 min at 4 °C, the aqueous layer was removed and mixed with an equal volume of isopropanol. The samples were placed at -20 °C for 1 h and the RNA was pelleted at 7000 rpm for 30 min 4 °C. The pellet was resuspended in 0.5 mL Solution D, mixed with 0.75 mL isopropanol, and placed at -20 °C for 1 h. The RNA was pelleted by spinning in a microcentrifuge at 14000 rpm for 15 min 4 °C, and then washed with 70% ethanol in DEPC water. The final RNA pellet was air-dried and dissolved in DEPC water. Total RNA was estimated by spectrophotometry at 260 nm. Denatured RNA samples (15 μg) were analyzed by gel electrophoresis in a 1% agarose denaturing gel in a morpholinopropanesulfonic (MOPS) acid buffer (pH 7) at 100 V for 2.5 h. The RNA was

transferred to a nylon membrane (Bio-Rad) using 20X SSC (175.3 g/L NaCl, 88.2 g/L Na citrate, pH 7.0) and cross-linked with an ultraviolet cross-linker (Stratagene UV Stratalinker 1800). The blots were hybridized with ^{32}P-labelled rat *c-fos*, rat *c-jun*, or human *collagenase* genes, and subsequently stripped and re-probed with ^{32}P-labelled rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. The membranes were exposed to X-ray films with 2 intensifying screens at room temperature for 1 to 5 days.

2.4 Nuclear Extract Preparation

Extracts were prepared by the method of Adunyah et al. (1991). Approximately 2 X 10^6 cells/mL plated in 12 mL of Ham's F-12 medium containing 3% antibiotics and 5% FBS were treated with IL-1 or C2-ceramide for 1 h to induce SRE binding or 3 h to induce AP-1 binding, following an overnight deprivation of serum. The cells were washed two times in PBS. The final wash was aspirated, and 1 mL of cold Buffer 1 (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 (NP-40), 20 µg/mL leupeptin, 20 µg/mL pepstatin, 20 µg/mL aprotinin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) was added to each plate. The cells were scraped with a cell scraper (Sarstedt), collected in a 1.5 mL tube, and placed on ice for 15 min. The nuclei were collected by centrifugation at 5000 rpm for 10 min at 4 °C, and resuspended in 20 µl of Buffer 2 (20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 300 mM NaCl, 20% glycerol, 20 µg/mL leupeptin, 20 µg/mL pepstatin, 20 µg/mL aprotinin, 0.5 mM PMSF). The tubes were placed on ice for 30 min, centrifuged at 14,000 rpm for 20 min at 4 °C, and the supernatant was used as the nuclear extract. The protein concentration of the extract was determined using the Bio-Rad protein assay.

2.5 Electrophoretic Mobility Shift Assay

Nuclear extract (8 µg) was mixed with 1 µg of poly(dIdC)-poly(dIdC) in a 5X binding buffer (20 mM Hepes, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT) for 20

min on ice. Oligonucleotide (40 ng) was 5'-end-labelled with polynucleotide T4 kinase by incubation in 10X Plus enzyme buffer (Pharmacia Biotech) with 100 μCi of γ^{32}P-ATP at 37 °C for 45 min. The labelled probe (50,000 cpm) was added, and the binding mixture was incubated for 20 min at room temperature. The samples were separated on a 4% native polyacrylamide gel electrophoresis in a 0.5X TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA). The gel was dried under vacuum and subjected to autoradiography overnight at room temperature. Competition studies were performed using 100X unlabelled consensus oligonucleotide before addition of the labelled probe.

2.6 Plasmid Construct

The SRE-luciferase (SRE-Luc) construct was obtained from Dr. H. Elsholtz (University of Toronto), and contains the serum response element of the *c-fos* promoter driving expression of the luciferase reporter gene. The AP-1-luciferase (AP-1-luc) construct was obtained from Pierre Lemieux (Angiotech, Vancouver), and contains 4 copies of the TRE consensus sequence driving expression of the luciferase reporter gene.

2.7 Transient Transfection

Chondrocytes plated at a density of 2 X 10^6 cells/mL in 2.5 mL of Ham's F-12 medium containing 3% antibiotics and 5% FBS in 6-well plates were allowed to recover for 24 h at 37 °C in a humidified atmosphere supplemented with 5% CO_2. The cells were transfected with 10 μg of DNA per well using the calcium phosphate-DNA precipitation method (Ausubel, 1987). Twenty-four hours following transfection, the cells were deprived of serum for at least 16 h in Ham's F-12 medium containing 3% antibiotics before treatment. IL-1 or ceramide was added for 6 h to induce transcriptional activation.

2.8 Luciferase Assay

At the end of the incubation period, the cells were washed 2X with PBS and then lysed with lysis buffer A (1% Triton X-100, 0.1 M KH_2PO_4 pH 7.8, 1 mM DTT). Luciferase assays were performed as described (Ausubel, 1987) with some modifications. Briefly, 20 µL of cellular lysate was added to 30 µL luciferase reaction cocktail (1 M K_2HPO_4, 0.1 M ATP, 0.1 M $MgCl_2$) and injected with 200 µL of a 1 mM D-luciferin solution using a Berthold Lumat LB9501 luminometer. Luciferase activity was expressed as relative luciferase units and then normalized for protein content in the cell lysate to correct for differences in cell numbers among the different treatment groups. The protein concentration of the extract was determined using the Bio-Rad protein assay.

2.9 MAPK Assays

For both the whole cell extract and immunoprecipitation assays, primary chondrocyte cultures were plated at a density of 2 X 10^6 cells/mL in 12 mL of Ham's F-12 medium containing 3% antibiotics and 5% FBS, and were treated for 20 min with IL-1 or C2-ceramide following an overnight deprivation of serum. For the whole cell extract assay, the cells were washed 2X with cold PBS and lysed with 1 mL cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EGTA, 1 µg/mL leupeptin). The lysed cells were scraped into eppendorf tubes and placed on ice for 20 min. Cell debris was pelleted with a 15 min spin at 14000 rpm at 4 °C. The supernatant was collected and protein concentration was measured using the Bio-Rad protein assay. For the phosphorylation reaction, 10 µg protein extract, 4 µg MBP, 0.1 µL ^{32}P-ATP, and 30 µL RIPA buffer were mixed together and incubated at 30 °C for 30 min. The mixtures were boiled for 10 min with the tube caps open to reduce the volume, and then run on a 12% SDS-PAGE at 120V for 1 h. The gel was dried under vacuum and subjected to autoradiography overnight at room temperature. For the immunoprecipitation assays, the cells were washed 2X with cold PBS and harvested with 0.8 mL lysis buffer (50 mM

Hepes pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% NP-40, 10 mM NaF, 0.2 mM Na vanadate, 1 mM PMSF, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 10 mM Na pyrophosphate, 40 mM PNPP). The cell extract was aliquoted into 300 μL samples in eppendorf tubes, and 1-2 μg anti-ERK-1 antibodies was added to each tube. The tubes were rocked for 1 h at 4°C, then 20 μL protein A agarose beads were added, and the tubes rocked for 3 h at 4°C. The beads were spun down with a 3 second spin in a desktop minicentrifuge, and the supernatant discarded. The beads were washed 4X with lysis buffer, and the final wash was aspirated. To each tube was added 20 μL kinase buffer (20 mM Hepes pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM DTT, 25 μM ATP) containing 2 μCi ^{32}P-ATP and 12 μg MBP. The tubes were mixed well and incubated at 30 °C for 30 min, then mixed with 10 μL of Laemmli sample buffer to stop the reaction. The samples were boiled with closed lids for 3 min, and then spun at 14000 rpm for 5 min at 4 °C. Samples of 10 μL were added to a 12% SDS-PAGE, and the gel was run and dried as for the whole cell extract.

2.10 Lipid Studies

Primary chondrocyte cultures were plated at a density of 2 X 10^6 cells/mL in 2 mL Ham's F-12 medium containing 3% antibiotics and 5% FBS in 6-well plates, and treated with 20 μL Bodipy FL-C12-sphingomyelin for 30 min. This lipid contains the fluorescent Bodipy tag on the acyl group such that when it is hydrolyzed, the liberated ceramide is labelled. The cells are then depleted of serum and treated with IL-1 (20 ng/mL) for varying lengths of time. Cells were harvested by washing two times with PBS, and then scraped in 1 mL PBS into a glass test tube. The wells were rescraped with 1 mL PBS and the cells were added to those previously scraped. The cells were pelleted by spinning at 2000 rpm for 10 min. The supernatant was decanted and lipids were extracted by modification of Bligh and Dyer (1959) and Liu and Anderson (1995). First, 750 μL methanol and 500 μL chloroform were added to the pellets and the mixture was vortexed

well. Next, 250 μL chloroform and 250 μL water were added and vortexed well. An additional 100 μL each of chloroform and water were added, vortexed, and then spun at 3000 rpm for 10 min. The aqueous top layer and cellular debris were discarded, and the bottom organic layer, which contains the lipids, was transferred to a clean glass lidded vial. The solvent was evaporated under nitrogen gas, and the residue was redissolved in 40 μL chloroform and spotted on thin layer chromatography (TLC) plates (Merck). The TLC was run for approximately 2 h in chloroform:methanol:H_2O (65:35:4 v/v). After the plates were dry, the areas corresponding to the position of ceramide and sphingomyelin (determined by R_F values of standards) were scraped into glass tubes. The sphingomyelin or ceramide was liberated from the scrapings by the addition of 1 mL chloroform. The scrapings were pelleted by spinning at 1000 rpm for 10 min. The supernatant was transferred to a clean glass tube and the sphingomyelin or ceramide content was estimated by fluorescence intensity of the solvent at an excitation wavelength of 505 nm in an Hitachi F-2000 Fluorescence Spectrophotometer.

CHAPTER 3 RESULTS

3.1 Interleukin-1 stimulates ceramide release in chondrocytes

IL-1 has been shown to induce SM hydrolysis to ceramide in various cell types, including the murine thymoma EL4 cell line (Mathias et al, 1993), cultured granulosa cells (Santana et al, 1996), human umbilical vein endothelial cells (Masamune et al, 1996), and human fibroblasts (Ballou et al, 1992; Liu and Anderson, 1995). It has also been established that IL-1 is found in the synovial fluid of arthritic joints and that this cytokine is involved in promoting cartilage destruction (Goldring et al, 1988; Tyler, 1985; Murphy et al, 1986; Dayer et al, 1986; Frisch et al, 1987). To determine whether IL-1 is involved in ceramide liberation in chondrocytes, a fluorescent-labelled SM was added to primary cell cultures and the cells were treated with IL-1. As shown in Table 1, an approximate three-fold increase in ceramide levels over untreated control cells was observed in chondrocytes treated with IL-1 for 30 or 60 min. These results suggest that IL-1 induces sphingomyelin hydrolysis to ceramide in chondrocytes. Maximal ceramide liberation may occur before 30 min, or may still be occurring after 60 min.

3.2 Ceramide induces an increase in *c-fos* mRNA levels

The effects of this increase in ceramide levels in chondrocytes were determined using cell-permeable ceramide analogues to increase intracellular levels of ceramide. Natural ceramides contain long and very long chain saturated or monosaturated fatty acids and are thus poorly soluble in aqueous solutions. For this reason, synthetic analogues were used, mainly C2-ceramide (where C2 indicates the number of carbon atoms in the acyl group), also called *N*-acetyl-D-sphingosine and abbreviated C2-cer, as well as C6-ceramide (*N*-hexanoyl-sphingosine; C6-cer) (see Figure VII A and B). Since cartilage destruction occurs mainly through the actions of matrix metalloproteinases, genes involved in the induction of these enzymes were first examined. The AP-1 element is

Table 1. **Interleukin-1 stimulates ceramide release in chondrocytes**

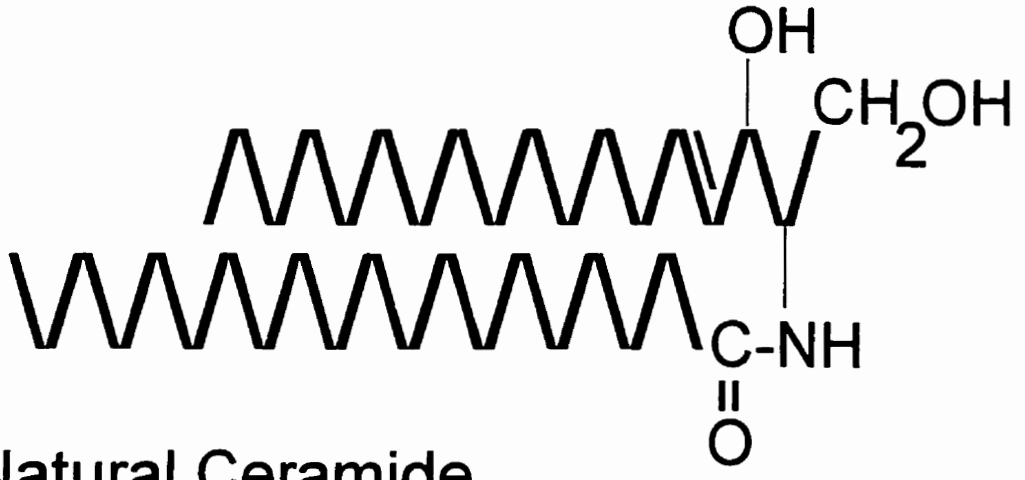
TREATMENT	SPHINGOMYELIN	CERAMIDE
Cells Only	190.1 ± 26.1	208.9 ± 0.6
Control t=2 h	206.9 ± 22.9	205.6 ± 4.5
Cells Only	204.3 ± 14.7	238.1 ± 60.7
Control t=0	160.65 ± 47.6	299.4 ± 44.8
30 min. IL-1	289.75 ± 76.0	661.45 ± 81.2
60 min. IL-1	237.0 ± 42.4	645.75 ± 106.4

Primary chondrocyte cultures were treated with 20 μL of Bodipy FL-C12-sphingomyelin for 30 min. The experimental wells were treated with IL-1 (20 ng/mL) for either 30 or 60 min, and then harvested as described for "Lipid Studies" under "Materials and Methods." Lipids were extracted by the method of Bligh and Dyer (1959) and separated by TLC. Areas corresponding to the R_F values of sphingomyelin and ceramide were scraped and the fluorescence intensity of the isolated lipids was determined. Cells Only refers to the negative control of untreated cells without the addition of fluorescent lipid. Control t = 0 refers to untreated cells with the addition of fluorescent lipid harvested at the time of addition of IL-1 to the experimental cells. Control t = 2 h refers to untreated cells with the addition of fluorescent lipid harvested 2 h after the addition of IL-1 to the experimental cells. Data are expressed as relative fluorescent intensity units of the lipid extract measured at an excitation wavelength of 505 nm ± standard deviation. The first two rows (Cells Only and Control t = 2 h) are a representative experiment independently performed with duplicate samples. The last three rows (Cells Only, Control t = 0, and the experimental cells) are a representative experiment independently performed with duplicate samples.

Figure VII. **The Chemical Structures of Natural and Synthetic Ceramides**

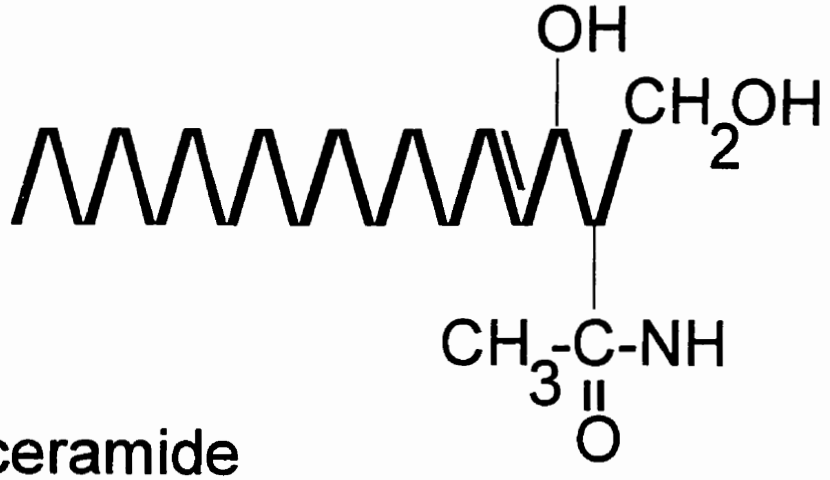
The structures of natural ceramide, the synthetic ceramide analogue C2-ceramide, and the synthetic inactive ceramide analogue dihydro-C2-ceramide are shown

A



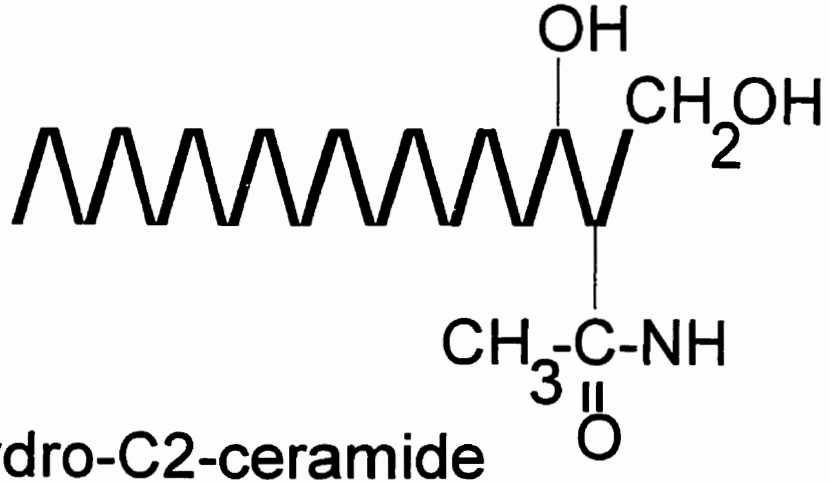
Natural Ceramide

B



C2-ceramide

C



Dihydro-C2-ceramide

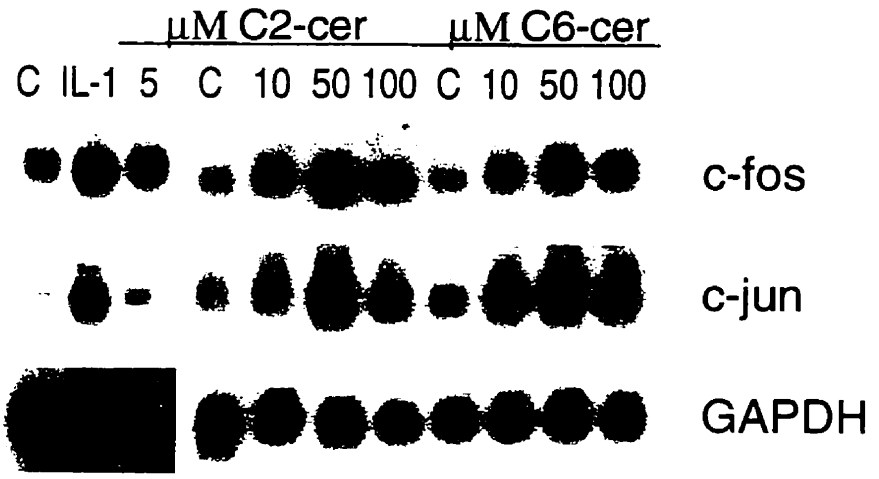
found in the regulatory regions of the *collagenase* and *stromelysin* genes (Angel et al, 1987; Zafarullah et al, 1992), and their protein products are important matrix metalloproteinases; thus the components of AP-1 were first examined to determine their involvement in mediating the biological effects of ceramide. AP-1 is most commonly composed of a heterodimer of c-Fos and c-Jun (Ryseck and Bravo, 1991), and the genes of these proteins were looked at to determine if they could be stimulated by ceramide.

C2-cer at a concentration of 5 μM was able to induce an increase in *c-fos* mRNA levels, but did not induce an increase in *c-jun* mRNA (see Figure 1). Similarly, 10 μM C2-cer also caused an increase in *c-fos* mRNA levels. The slight increase in *c-jun* mRNA levels at 10 μM C2-cer was not commonly observed (see Figures 5A and 9B, for example). At 50 μM, C2-cer induced an elevation in both *c-fos* and *c-jun* mRNA (Figure 1). C6-cer also induced an increase in *c-fos* mRNA at 10 μM and 50 μM, as well as inducing an increase in *c-jun* mRNA. These results indicate that ceramide induces *c-fos* expression in chondrocytes at a wide range of concentrations, as well as inducing *c-jun* at concentrations of 10 μM and higher.

It had previously been shown that C2-cer is able to increase the levels of *c-jun* mRNA, c-Jun protein, and DNA binding activity of AP-1 at concentrations of 1-10 μM in HL-60 cells (Sawai et al, 1995). However, cell death was observed after 2 h of treatment with C2-cer, and no cells were viable after 24 h of treatment. These concentrations of ceramide that induced *c-jun* expression correspond to those at which ceramide induces its biological effects, such as growth inhibition and apoptosis (Sawai et al, 1995). Concentrations of 5 μM C2-cer induced DNA fragmentation of HL-60 cells in 3 h (Obeid et al, 1993; Jarvis et al, 1994), and concentrations of exogenously added ceramide above 5 μM have been shown to induce apoptosis in other cell types also, including PC12 cells by 20 μM C2-cer after 24 h (Hartfield et al, 1997), U937 and HL-60 cells by 10 μM C8-cer for 6 h (Jarvis et al, 1994).

Figure 1. **Effect of C2- and C6-ceramide on *c-fos* and *c-jun* mRNA levels**

Primary chondrocyte cultures were treated with increasing concentrations of C2-ceramide (C2-cer), or C6-ceramide (C6-cer) in serum-free medium for 1 h.
Lanes 1-3 and lanes 4-11 represent two separate experiments.
Total RNA was isolated and Northern blot analyses of *c-fos* or *c-jun* mRNA were performed as described under "Materials and Methods." The blots were subsequently stripped and reprobed with ^{32}P-labelled rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA to confirm roughly equal loading of RNA.


μM C2-cer
μM C6-cer
C IL-1 5 C 10 50 100 C 10 50 100
c-fos
c-jun
GAPDH

It has been suggested that ceramide has different effects depending on the cell type, which may be the reason that *c-fos* is induced at low ceramide concentrations in chondrocytes, while *c-jun* is not; however *c-jun* is induced at these low concentrations in HL-60 cells (Sawai et al, 1995). As well, the chondrocytes used in all experiments in this thesis are primary cultures plated at high density and are non-proliferating; thus, apoptosis does not occur in these cells. Concentrations of ceramide that induce apoptosis in other cell types can be used in chondrocytes to induce *c-fos* and *c-jun* without cell death.

3.3 Ceramide induction of *c-fos* is transient and is regulated at both the transcriptional and post-transcriptional levels

Since *c-fos* appeared to be stimulated more consistently and at lower concentrations than *c-jun*, the interaction of ceramide with this gene was examined further. It has been shown that the IL-1-induced increase in *c-fos* mRNA levels in chondrocytes occurs maximally at 30 - 60 min, and that this induction is transient (Lo, 1995). The increase in *c-fos* mRNA induced by ceramide is also transient, as observed by a time course of stimulation with C2-cer. The elevation in *c-fos* mRNA is maximally induced after 1 h of treatment with C2-cer, with a slight increase still noticeable after 2 h, and levels decreasing to control values by 3 h (Figure 2A).

The regulation of *c-fos* induction by ceramide was examined at the transcriptional and post-transcriptional levels, and was found to occur at both stages, as shown in Figure 2B. Actinomycin D and cycloheximide were used as inhibitors of transcription and translation, respectively. Actinomycin D completely inhibited the increase in *c-fos* mRNA induced by C2-cer. Cycloheximide induced *c-fos* mRNA expression on its own and superinduced this expression when added to chondrocytes together with C2-cer. These results show that C2-cer induction of *c-fos* expression is not observed in the presence of an inhibitor of mRNA transcription. With the addition of ceramide to chondrocytes, *c-fos* mRNA is newly transcribed (when compared with the lack of transcript in untreated

Figure 2. **Regulation of induction of *c-fos* by C2-ceramide**

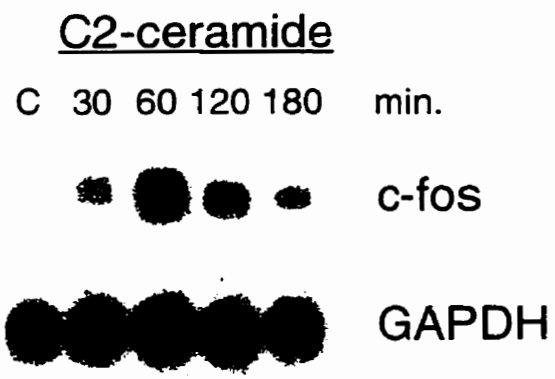
A, time course of induction of *c-fos* by C2-cer.

Primary chondrocyte cultures were treated with 10 μM C2-cer in serum-free medium for the indicated times.

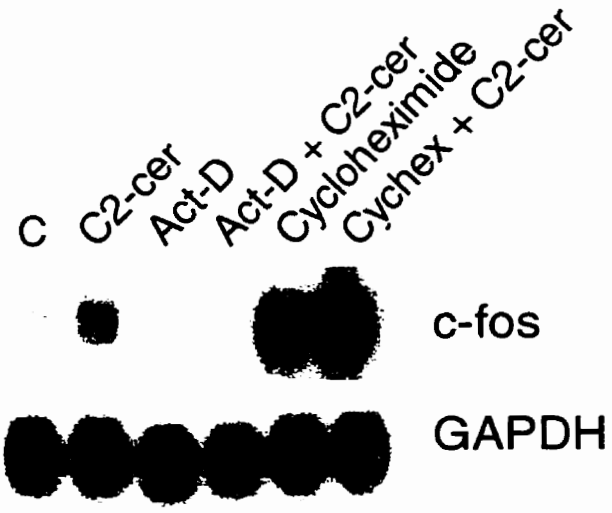
B, effects of actinomycin D or cycloheximide on *c-fos* induction by C2-cer.

Primary chondrocyte cultures were treated with 5 μg/mL actinomycin D or 10 μg/mL cycloheximide in the presence or absence of 50 μM C2-cer in serum-free medium for 1 h. Total RNA was isolated and Northern blot analysis of *c-fos* mRNA was performed as described under "Materials and Methods." The blots were subsequently stripped and reprobed with ^{32}P-labelled rat GAPDH cDNA to confirm roughly equal loading of RNA.

A


C2-ceramide
C 30 60 120 180 min.
c-fos
GAPDH

B


C
C2-cer
Act-D
Act-D + C2-cer
Cycloheximide
Cychex + C2-cer
c-fos
GAPDH

control cells, indicating lack of constitutive transcription); thus, when transcription is inhibited, no *c-fos* mRNA is observed even with ceramide stimulation. If ceramide did not induce an increase in *c-fos* mRNA, then this mRNA would still be observed in the presence of actinomycin-D. The inhibition of transcription inhibits *c-fos* mRNA levels.

c-fos shares similarities with immediate early genes that are rapidly induced by stimulation with serum or purified growth factors in the absence of *de novo* protein synthesis. Their mRNA can be superinduced by protein synthesis inhibitors like cycloheximide (Piechaczyk and Blanchard, 1994). When protein synthesis is inhibited, a build-up of *c-fos* mRNA occurs as it is not being translated to c-Fos. With the addition of C2-cer and cycloheximide, the levels of *c-fos* mRNA increase over those observed with either C2-cer or cycloheximide alone. If no increase in *c-fos* mRNA levels was observed with C2-cer and cycloheximide that was greater than the stimulation observed with cycloheximide, then the regulation of *c-fos* induction by ceramide would not depend on protein synthesis. If the addition of cycloheximide had inhibited the transcription of *c-fos* mRNA, then C2-ceramide-activated transcription of *c-fos* would depend on protein synthesis. As cycloheximide did not inhibit C2-ceramide activation of *c-fos* transcription, the increase in mRNA levels is due to a protein synthesis-independent mechanism. Thus ceramide induction of *c-fos* is regulated at both the transcriptional and post-transcriptional levels.

These results are consistent with the induction of *c-jun* by ceramide reported by Sawai et al. (1995), who found that the elevation in *c-jun* mRNA induced by ceramide was first detected after 30 min, with maximal induction at 1 h, and levels decreasing to baseline after 4 h, as well as regulation of *c-jun* induction by ceramide at both the transcriptional and post-transcriptional levels.

3.4 The induction of *c-fos* by ceramide is specific

Previous reports have shown the specificity of biological activity of ceramide compared with dihydroceramide (Sawai et al, 1995). The structure of dihydroceramide is identical to that of ceramide except that the former lacks the *trans* double bond of ceramide between the fourth and fifth carbons (see Figure VII B and C). Sawai et al. (1995) found that 5 μM dihydro-C2-ceramide (DHC) failed to induce apoptosis in HL-60 cells, whereas apoptosis was observed with the same concentration of C2-ceramide. As well, the increase in *c-jun* mRNA levels observed with 5 μM DHC was approximately one-third that observed with 5 μM C2-cer in HL-60 cells.

To determine the specificity of C2-ceramide in chondrocytes, cells were treated with either 20 ng/mL IL-1, 50 μM C2-cer, or 50 μM DHC for 1 h (Figure 3). Only a slight increase in *c-fos* mRNA levels was observed with DHC, as compared with the induction by IL-1 or C2-cer. These data indicate that C2-ceramide, unlike dihydroceramide, has biological effects in chondrocytes and induces a significant increase in *c-fos* mRNA levels.

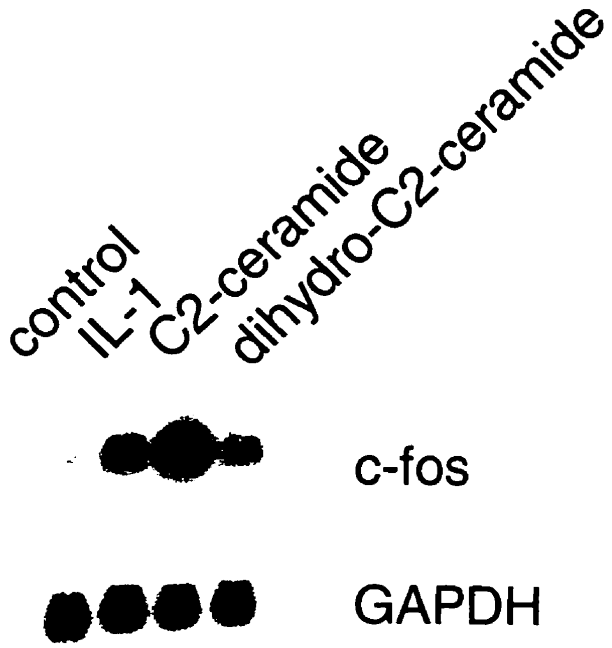
3.5 The induction of *c-fos* by is not affected by Fumonisin B1

The sphingomyelin pathway is composed of several different lipid byproducts, many of which can act as signalling molecules or second messengers. Compounds, such as fumonisins, can be used to determine which part of the pathway is involved in a specific biological effect. Fumonisins are a family of mycotoxins produced by *Fusarium moniliforme* (Sheldon) that contaminate maize and related grains throughout the world (Marasas, 1994). These toxins cause neuronal degeneration, liver and renal toxicity, cancer, and other injury to animals. The structure of Fumonisin B_1 (FB1) resembles the sphingoid base backbone of sphingolipids, making it a competitive inhibitor of ceramide synthase (sphingosine *N*-acyltransferase), the enzyme that catalyzes the acylation of sphinganine in the *de novo* biosynthesis of sphingolipids, and the reutilization of

Figure 3. **Specificity of the effect of ceramide**

Primary chondrocyte cultures were treated with IL-1 (20 ng/mL), C2-ceramide (50 μM), or dihydro-C2-ceramide (50 μM) in serum-free medium for 1 hour.

Total RNA was isolated and Northern blot analysis of *c-fos* mRNA was performed as described under "Materials and Methods." The blots were subsequently stripped and reprobed with ^{32}P-labelled rat GAPDH cDNA to confirm roughly equal loading of RNA.


control
IL-1
C2-ceramide
dihydro-C2-ceramide
c-fos
GAPDH

sphingosine from sphingolipid turnover (reviewed in Merrill Jr. et al, 1996) (see Figure VIII). FB1, as a result of this inhibition, causes an accumulation of sphinganine, and increases both the formation of sphinganine 1-phosphate and cleavage of the sphingoid base backbone to fatty aldehydes and ethanolamine 1-phosphate. FB1 inhibited sphingolipid biosynthesis in every eukaryotic cell type tested, including hippocampal neurons, cultured cerebellar neurons and Purkinje cells, neuroblastoma cells, P388 and U937 cells, rat hepatocytes, MOLT-4 cells, *Xenopus* oocytes, and *Saccharomyces cerevisiae*.

The effects of FB1 (as reviewed in Merrill Jr. et al, 1996) include alteration in cell morphology, cell-cell interactions, behaviour of cell-surface proteins and protein kinases, metabolism of other lipids, and cell growth and viability. However, FB1 is toxic only in cells that divide in culture (Harel and Futerman, 1993), which is not occurring with the chondrocytes used in the above studies. Huang et al. (1995) found that treatment of CV-1 cells with FB1 caused repression of PKC activity and inhibition of transcription that is dependent on the transcription factor AP-1, but stimulated transcription from a promoter containing a CRE. It is known that sphingoid bases are potent inhibitors of PKC (Merrill Jr. et al, 1993a; Merrill Jr. et al, 1989), which may account for the inhibition seen with FB1, which leads to the accumulation of sphinganine. Sphingosine and sphingosine 1-phosphate have also been found to increase cAMP levels and enhance AP-1 activity in Swiss 3T3 cells (Zhang et al, 1990; Goodemote et al, 1995).

To determine whether the induction of *c-fos* by C2-cer was the direct result of ceramide itself, or of the conversion of ceramide to other sphingoid bases, chondrocytes were pre-treated with FB1 (25 μM) for 24 h before the addition of either C2-cer (50 μM) or IL-1 (20 ng/mL). As can be seen in Figure 4, FB1 had no effect on the induction of *c-fos* or *c-jun* by IL-1, nor did it affect gene induction on its own. IL-1 may be able to activate *c-fos* in a manner independent of sphingolipids, whereas ceramide may activate *c-fos* and *c-jun* expression through complex sphingolipid synthesis. If IL-1 induces *c-fos*

Figure VIII. **The Action of FB1 on Sphingolipid Biosynthesis**

The enzymatic reactions inhibited in the sphingolipid biosynthetic pathway by fumonisin B1 (FB1) are shown. FB1 inhibits the action of ceramide synthase.

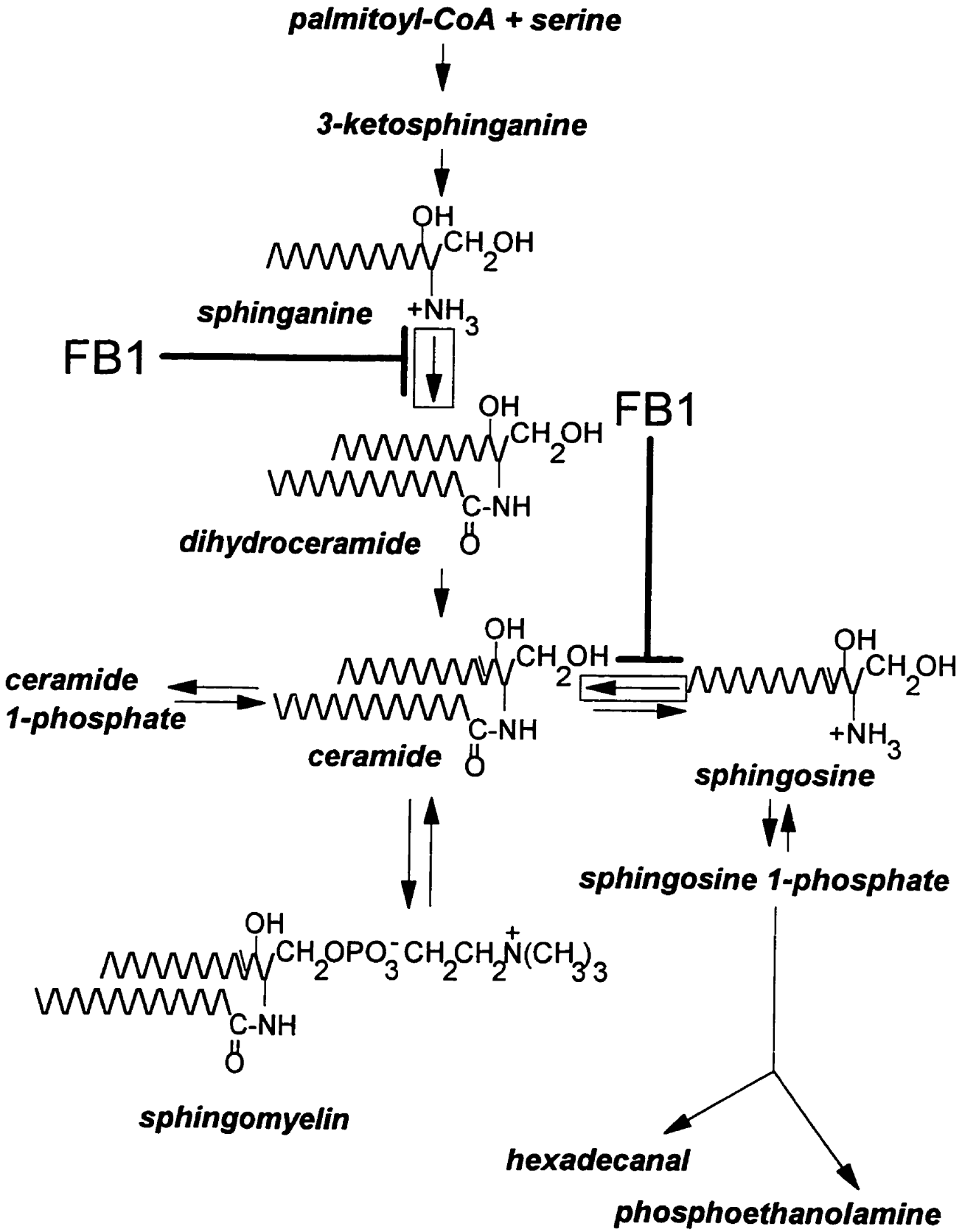
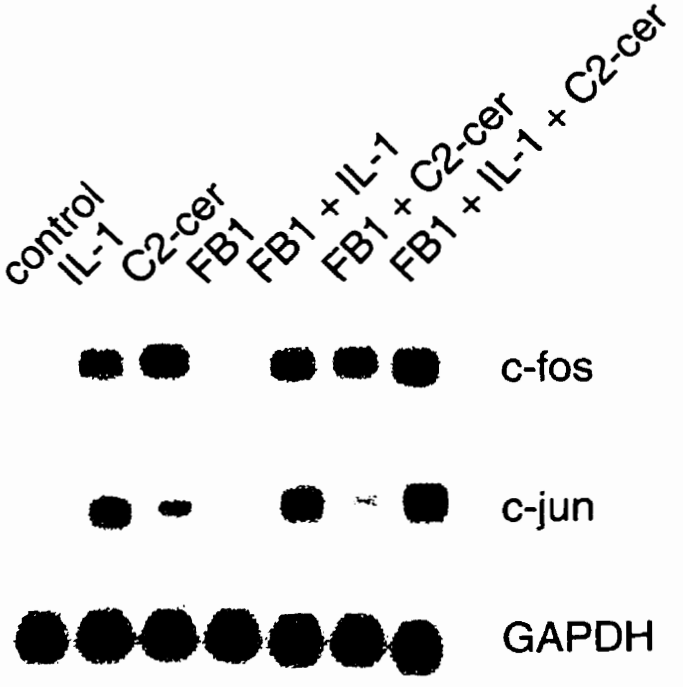

palmitoyl-CoA + serine
3-ketosphinganine
sphinganine
FB1
dihydroceramide
FB1
ceramide
1-phosphate
ceramide
sphingosine
sphingosine 1-phosphate
sphingomyelin
hexadecanal
phosphoethanolamine

Figure 4. **Effect of FB1 on *c-fos* expression**

Primary chondrocyte cultures were treated with 25 μM FB1 for 24 h in serum-free medium and then treated with C2-cer (50 μM) or IL-1 (20 ng/mL) for 1 hour. Total RNA was isolated and Northern blot analysis of *c-fos* mRNA was performed as described under "Materials and Methods." The blots were subsequently stripped and reprobed with ^{32}P-labelled rat GAPDH cDNA to confirm roughly equal loading of RNA.


control
IL-1
C2-cer
FB1
FB1 + IL-1
FB1 + C2-cer
FB1 + IL-1 + C2-cer
c-fos
c-jun
GAPDH

gene expression through the *de novo* biosynthesis of sphingolipids, or through the conversion of the liberated ceramide into sphingosine, then an inhibition in the mRNA levels of *c-fos* would be observed with the addition of IL-1 to cells pretreated with FB1. Since short-chain ceramides are capable of bypassing the inhibition of ceramide synthesis (Merrill Jr. et al, 1996), then the addition of C2-cer to cells pretreated with FB1 should recover any inhibition previously observed. Ceramide thus appears to exert its biological effects directly, although the possibility exists of its conversion to other molecules as IL-1 may induce *c-fos* independent of the sphingomyelin pathway.

3.6 Ceramide potentiates the induction of *c-fos* by interleukin-1

Three criteria have been established for an agent to be designated a second messenger: (1) rapid kinetics of activation of the second messenger pathway by an agonist, (2) ability of cell-permeable analogues to bypass receptor activation and mimic the effects of the agonist, and (3) reconstitution of this cascade in a cell-free system. These criteria have been met for ceramide as a second messenger for IL-1 (Kim et al, 1991; Ballou et al, 1992; Mathias et al, 1993; Obeid et al, 1993; Raines et al, 1993) and TNF (Dressler et al, 1992; Kolesnick and Golde, 1994; Schutze et al, 1994) in other cell systems.

To determine whether ceramide acts solely as a second messenger for IL-1 in chondrocytes, or if it acts through a separate pathway and potentiates the biological effects of IL-1, such as the induction of *c-fos* gene expression, cells were treated with increasing concentrations of IL-1 in the presence or absence of C2-cer for 1 h (Figure 5A), and *c-fos* mRNA levels were determined by Northern blot analysis. If ceramide acts within an IL-1 signalling pathway, then the addition of C2-ceramide to concentrations of IL-1 too low to increase *c-fos* mRNA levels should cause an increase in expression only as large as that seen with C2-ceramide alone. C2-cer, at a concentration of 10 μM, however, when added simultaneously with IL-1, increased the levels of *c-fos* mRNA above those seen with IL-1 alone at equivalent IL-1 concentrations. C2-cer and IL-1 together were

Figure 5. **Effect of C2-ceramide on IL-1 induction of *c-fos* and *c-jun* mRNA.**

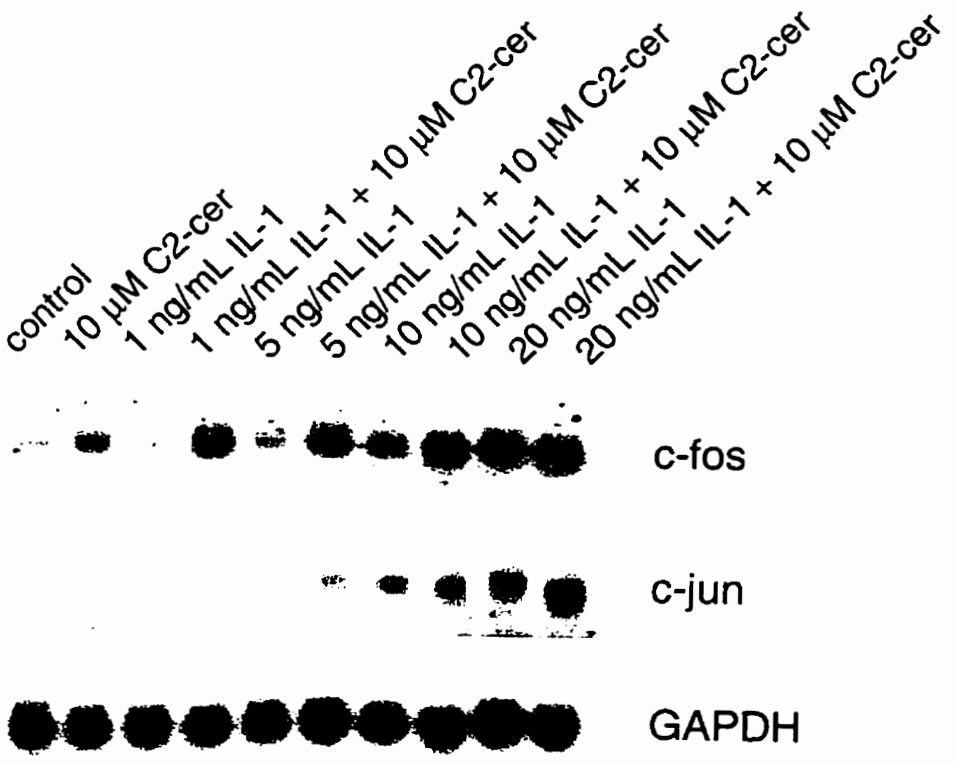
A, addition of 10 μM C2-cer.

B, addition of 50 μM C2-cer.

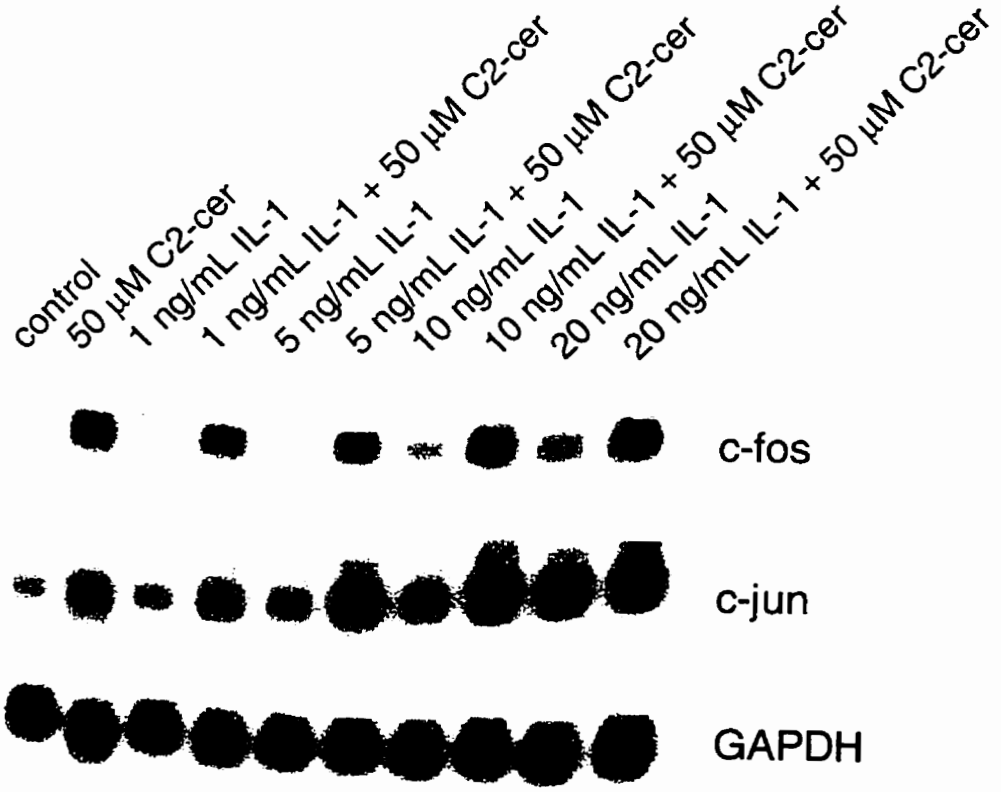
Primary chondrocyte cultures were treated with increasing concentrations of IL-1, with or without the indicated concentration of C2-cer, in serum-free medium for 1 h.

Total RNA was isolated and Northern blot analyses of *c-fos* and *c-jun* mRNA were performed as described under "Materials and Methods." The blot was subsequently stripped and reprobed with ^{32}P-labelled rat GAPDH cDNA to confirm roughly equal loading of RNA.

A


control
10 μM C2-cer
1 ng/mL IL-1
1 ng/mL IL-1 + 10 μM C2-cer
5 ng/mL IL-1
5 ng/mL IL-1 + 10 μM C2-cer
10 ng/mL IL-1
10 ng/mL IL-1 + 10 μM C2-cer
20 ng/mL IL-1
20 ng/mL IL-1 + 10 μM C2-cer
c-fos
c-jun
GAPDH

B


control
50 μM C2-cer
1 ng/mL IL-1
1 ng/mL IL-1 + 50 μM C2-cer
5 ng/mL IL-1
5 ng/mL IL-1 + 50 μM C2-cer
10 ng/mL IL-1
10 ng/mL IL-1 + 50 μM C2-cer
20 ng/mL IL-1
20 ng/mL IL-1 + 50 μM C2-cer
c-fos
c-jun
GAPDH

able to induce *c-fos* expression when the concentration of IL-1 alone was too low to cause induction. Ceramide is thus able to potentiate the induction of *c-fos* seen with IL-1; at IL-1 concentrations too low to induce *c-fos*, the addition of ceramide increased *c-fos* mRNA to levels approximating the induction seen at higher concentrations of IL-1. At low concentrations of IL-1 (≤ 10 ng/mL) ceramide provides an additional signal to induce maximal expression of *c-fos*. It may be that low concentrations of IL-1 do not liberate enough ceramide for *c-fos* induction, and thus exogenously added ceramide is required for induction. Even at high concentrations of IL-1 (> 10 ng/mL), ceramide appears to induce *c-fos* through a separate mechanism from IL-1, such that when IL-1 and ceramide are added together, they are able to increase the levels of *c-fos* mRNA above that seen with either of the agents alone.

At 10 μM C2-cer, induction of *c-jun* mRNA levels was observed only at the higher concentrations of IL-1 (≥ 10 ng/mL), and a slight potentiation of induction was observed at 20 ng/mL IL-1 plus 10 μM C2-cer (Figure 5A). At lower concentrations of IL-1 (< 10 ng/mL), which are unable to induce *c-jun* expression, the addition of C2-cer caused a slight increase in *c-jun* mRNA levels but not to the level of induction observed with 20 ng/mL IL-1. The ability of this concentration of C2-cer to induce *c-jun* expression appears limited to the concentration of IL-1 present.

With the addition of 50 μM C2-cer, the induction of *c-fos* expression was greater than that observed with high concentrations of IL-1 alone (Figure 5B). No potentiation of the induction of *c-fos* by IL-1 was observed; the addition of 50 μM C2-cer to concentrations of IL-1 too low to induce an increase in *c-fos* mRNA levels only increased the induction to the level seen with this concentration of C2-cer alone. With *c-jun* induction, 50 μM C2-cer results in the same potentiation as observed with 10 μM C2-cer in the induction of *c-fos*; this concentration of C2-cer induced an elevation in *c-jun* mRNA levels, and the addition of 50 μM C2-cer to concentrations of IL-1 too low to induce

c-jun expression caused an increase in *c-jun* mRNA levels above those observed with either agent alone.

It thus appears that the second messenger function of ceramide is dose-dependent, and this dose depends on the genes involved. At 10 μM, C2-cer appears to act through an independent mechanism from IL-1 in the induction of *c-fos*; this separate pathway may be the same observed in the induction of *c-jun* expression by 50 μM C2-cer. Once the concentration of C2-cer causes an induction of gene expression that is greater than the induction caused by IL-1 (as seen with 50 μM C2-cer in the induction of *c-fos*), the observed induction is only as great as that found with ceramide alone.

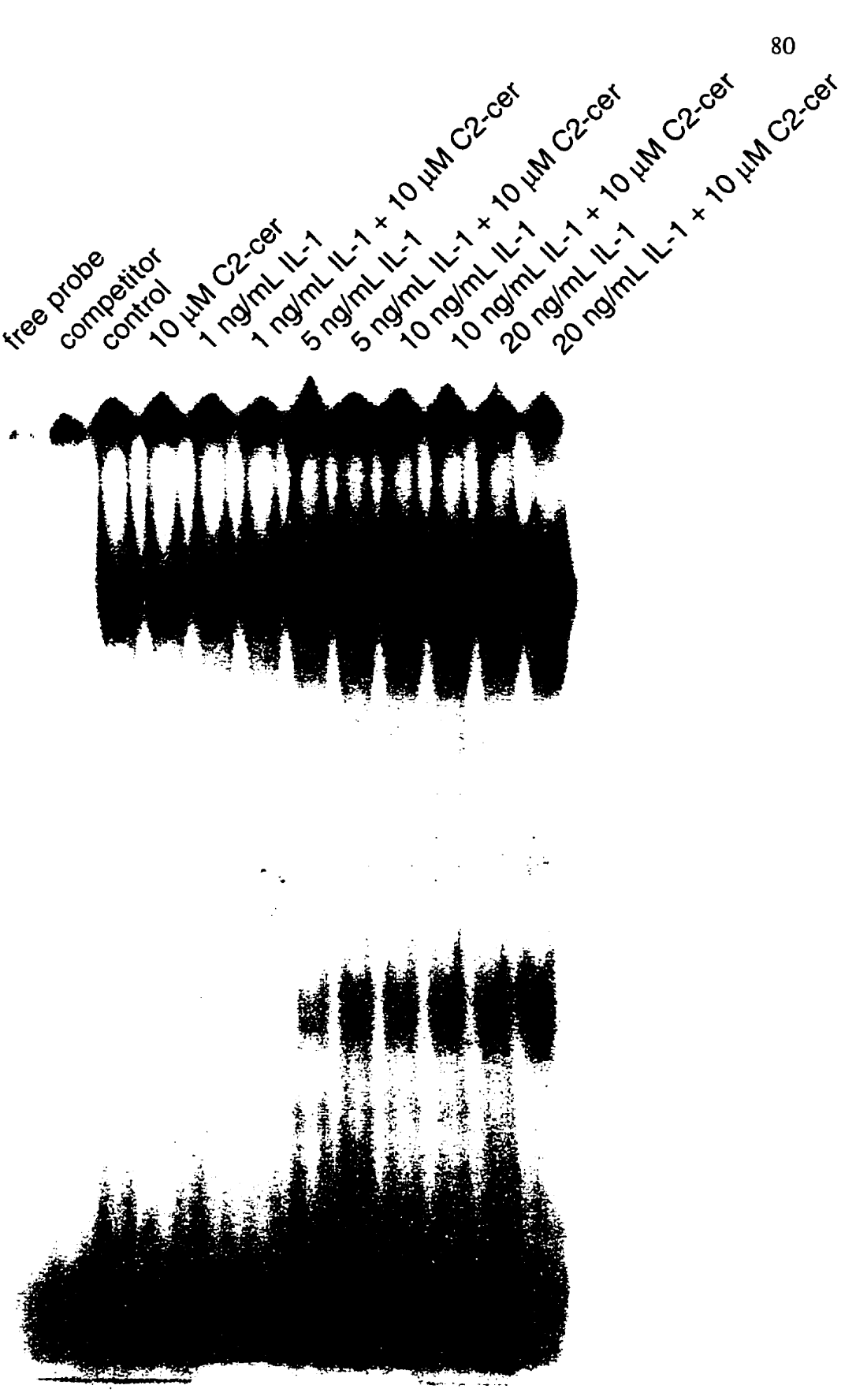
3.7 Ceramide does not induce *c-fos* by an increase in SRE binding

Three proximal elements have been identified on the *c-fos* promoter as major targets for stimulating signals: SIE, SRE, and CRE (Janknecht et al, 1995). IL-1 has been reported to activate MAPK (Guy et al, 1991; Bird et al, 1991), which phosphorylates serine and threonine residues on various subunits including TCFs. Once phosphorylated, TCF has the ability to activate transcription, and, in combination with SRF, activates *c-fos* through the SRE. Thus, IL-1 induces *c-fos* by the stimulation of the SRE through the MAPK pathway. Since it is known that IL-1 can activate the release of ceramide, the ability of ceramide to activate *c-fos* through the SRE was first examined. By the electrophoretic mobility shift assay (Figure 6), it was observed that C2-cer did not increase the binding of nuclear extract to synthetic oligonucleotides containing the SRE consensus sequence after 1 h. IL-1 was able to induce SRE binding starting at a concentration of 5 ng/mL. Interestingly, at 5 ng/mL, IL-1 did not induce an increase in *c-fos* mRNA levels (Figure 5). When ceramide was added to 5 ng/mL IL-1, an increase in *c-fos* mRNA levels was observed. The addition of C2-cer had no effect on IL-1 induction of SRE binding at any concentration of IL-1. With the addition of 100X unlabelled SRE

Figure 6. **Effect of C2-ceramide on SRE DNA binding activity.**

Primary chondrocyte cultures were treated with increasing concentrations of IL-1, with or without 10 μM C2-cer, in serum-free medium for 1 h.

Nuclear extracts were prepared and the electrophoretic mobility shift assay was performed as described under "Materials and Methods." The SRE consensus oligonucleotide used is double stranded with 5' OH blunt ends containing the following sequence 5' - GGA TGT CCA TAT TAG GAC ATC T - 3' (the SRF binding site is underlined). Free probe refers to the addition of free labelled SRE oligonucleotide in the binding mixture without the addition of nuclear extract. Competitor refers to the addition of 100X cold SRE consensus oligonucleotide to the nuclear extract and binding mixture before the addition of labelled SRE consensus oligonucleotide.


free probe
competitor control
10 μM C2-cer
1 ng/mL IL-1
1 ng/mL IL-1 + 10 μM C2-cer
5 ng/mL IL-1
5 ng/mL IL-1 + 10 μM C2-cer
10 ng/mL IL-1
10 ng/mL IL-1 + 10 μM C2-cer
20 ng/mL IL-1
20 ng/mL IL-1 + 10 μM C2-cer

consensus oligonucleotide as a competitor of labelled SRE, the SRE complex induced by IL-1 was not observed. No bands were seen with free probe alone. Basal levels of binding were observed with all cell conditions, even in untreated control cells, as the SRE is occupied by TCF and SRF before, during, and after induction (Janknecht et al, 1995). It appears that an increase in binding to the SRE is not sufficient for an increase in *c-fos* mRNA levels, and, once an increase in binding is induced, there is a limit to the number of complexes that can form at the SRE. Thus, even with increasing concentrations of IL-1, and with the addition of ceramide that was able to potentiate the increase in *c-fos* mRNA levels observed with IL-1, no further increase in binding occurred. The lower band seen at increasing intensity with the increase in IL-1 is possibly another transcription factor capable of binding to the *c-fos* SRE. As shown in Wyke, Lang, and Frame (1996), the pattern of transcription factors binding to the SRE is consistent with the complexity of this region of the promoter. SRE is composed of binding sites for SRF and TCFs, as well as several AP-1/ATF complexes. Their studies revealed five band shifts with the *c-fos* SRE, corresponding (from highest to lowest molecular weights) to SRF, an AP-1/ATF related factor, an AP-1 related factor, another AP-1/ATF related factor, and an unknown complex at the lowest molecular weight band. As only one lower band is observed in Figure 6, this band might result from a shift by the unknown complex. These studies show that ceramide does not induce *c-fos* through an increase in binding to the SRE regulatory promoter site.

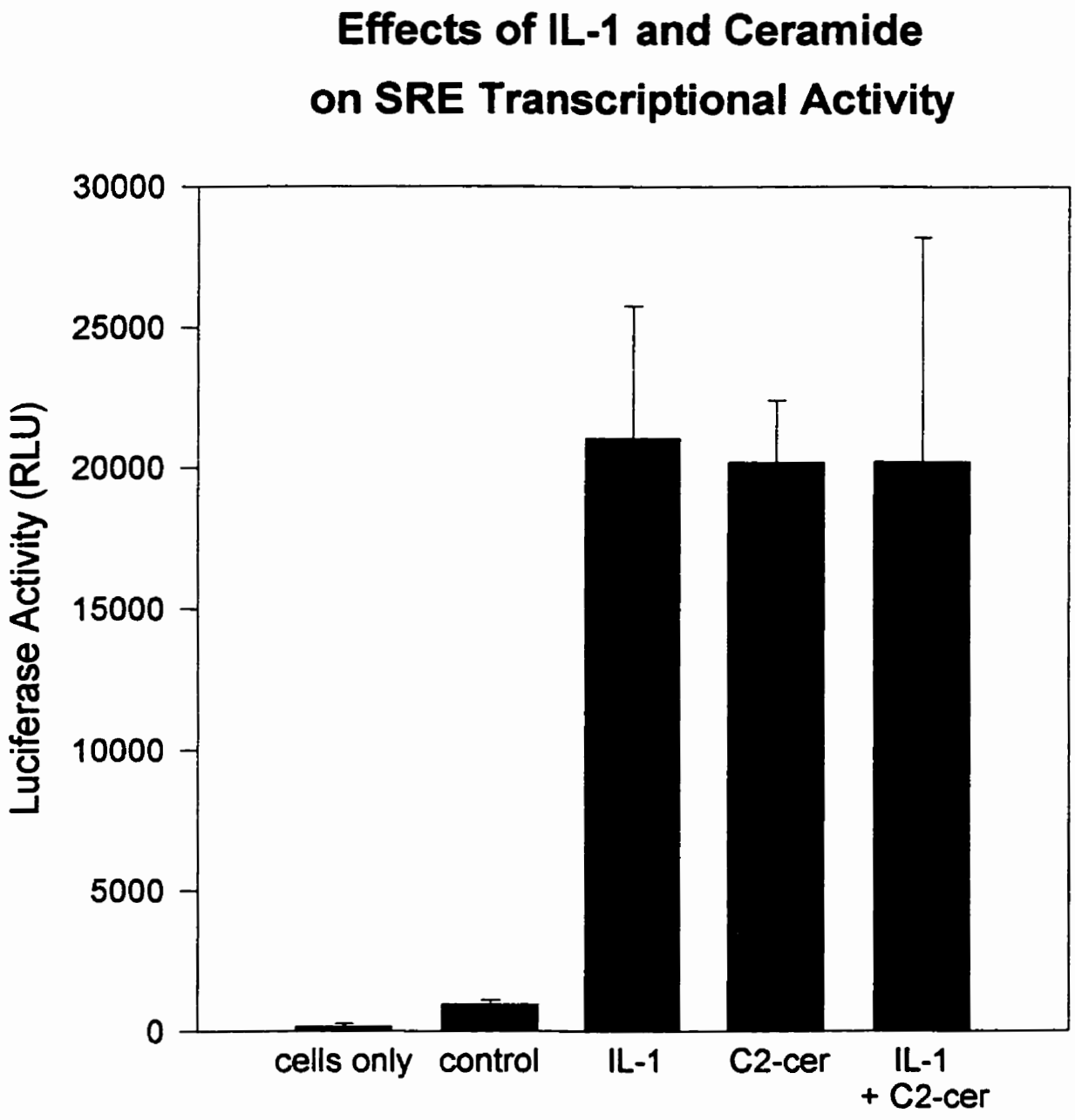
3.8 Ceramide does induce SRE transcriptional activity

Although ceramide is unable to induce an increase in binding to the SRE, it is possible that ceramide activates *c-fos* by inducing transcription through the factors already bound to the SRE. Regulation of *c-fos* transcription by SRE was examined using a plasmid vector containing the SRE of the *c-fos* promoter fused to a luciferase reporter gene containing a thymidine kinase minimal promoter. Transient transfection of chondrocytes with this SRE-Luc reporter construct demonstrated an approximate 21-fold

Figure 7. **Effect of C2-ceramide on SRE transcriptional activity.**

Primary chondrocyte cultures were transfected overnight with the plasmid construct SRE-Luc, which contains a SRE-dependent TK minimal promoter driving the expression of the luciferase reporter gene, and then treated with 20 ng/mL IL-1 or 50 μM C2-ceramide in serum-free medium for 6 h.

Luciferase assays were performed on cell lysates as described in "Materials and Methods." Cells only represents the negative control untransfected, untreated cells, and control represents the background luciferase activity in transfected, but untreated, cells. Data are expressed as relative luciferase units of the cellular lysate. This is a representative experiment independently performed with six identically-treated samples per value. Error bars represent standard deviation of within each set of six samples.


Effects of IL-1 and Ceramide
on SRE Transcriptional Activity
Luciferase Activity (RLU)
30000
25000
20000
15000
10000
5000
0
cells only
control
IL-1
C2-cer
IL-1
+ C2-cer

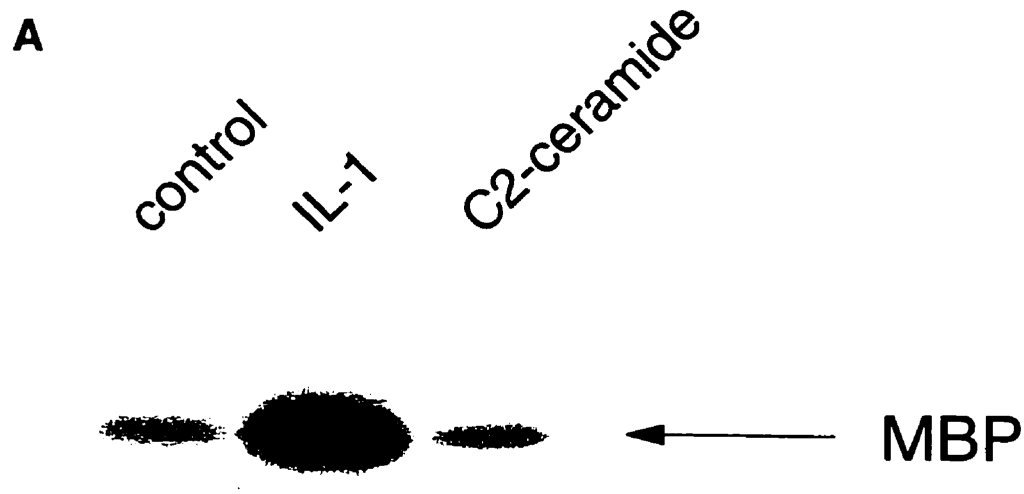
C2-cer stimulation of luciferase activity compared with transfected but untreated control cells (Figure 7). IL-1, as well as IL-1 together with C2-cer, stimulated luciferase activity by approximately the same amount as C2-cer alone. These data suggest that IL-1 can induce *c-fos* by both inducing binding to the SRE and by stimulating transcription from the SRE; ceramide, however, only appears to stimulate the transcriptional activity of the SRE. Ceramide may induce *c-fos* expression by a mechanism independent of the induction of *c-fos* by IL-1.

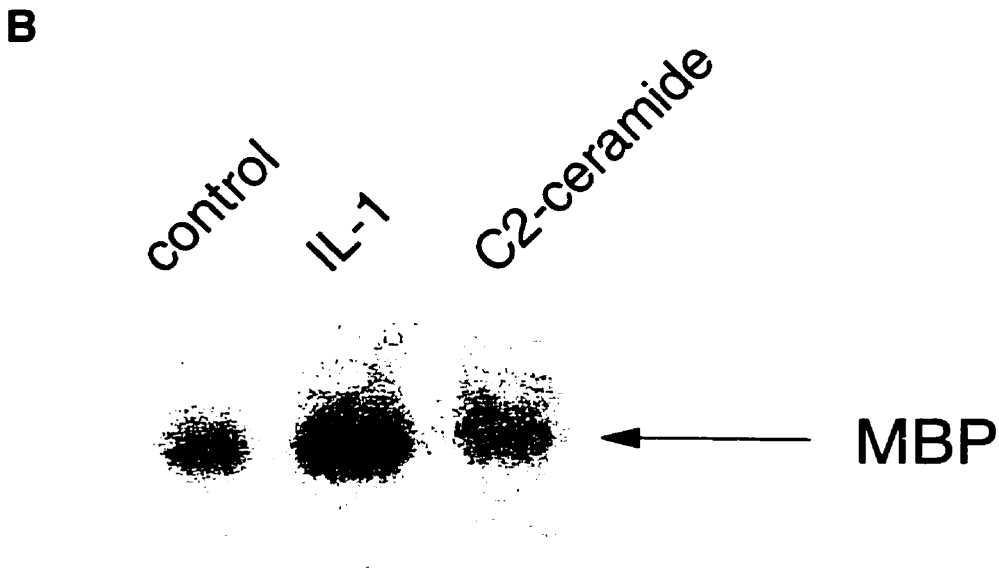
3.9 Ceramide does not activate MAPK in chondrocytes

It is known that IL-1 is able to activate MAPK (ERK1/ERK2) and lead to the phosphorylation of the MAPK substrate myelin basic protein (MBP) (for review see O'Neill, 1992). Ceramide has been shown to stimulate MBP-specific kinase activity in fibroblasts, but to have no effect on kinase activity in other cell types (Bird et al, 1994). To determine whether ceramide-treated chondrocytes were able to induce phosphorylation of MBP, whole cell extracts were mixed with MBP and γ^{32}P-ATP, and proteins were separated by SDS-PAGE. As shown in Figure 8A, IL-1 (20 ng/mL) induced phosphorylation of a protein corresponding to the molecular weight of MBP (18 K), but no induction was observed with C2-ceramide-treated (50 μM) cells. To specifically look at ERK activity, anti-ERK-1 antibodies were used to precipitate the ERKs onto Protein A agarose beads. The beads with their bound immune complex were incubated with MBP and γ^{32}P-ATP. As shown in Figure 8B, IL-1-treated (20 ng/mL) cells induced phosphorylation of MBP, but no induction was observed with C2-ceramide-treated (50 μM) cells. These results indicate that, in chondrocytes, although IL-1 induces activation of ERKs and induces their phosphorylation of MBP, this effect is not seen in ceramide-treated cells. Thus ceramide may use a mechanism independent of the ERK MAPK cascade in its induction of gene expression.

Figure 8. **Role of MAPK in ceramide actions**

A. effect of C2-ceramide on MAPK activity in whole cell extract assays
Primary chondrocyte cultures were treated with IL-1 (20 ng/mL) or C2-ceramide (50 μM) for 20 min in serum-free medium. Whole cell protein extracts were prepared and a whole cell extract MAPK assay was performed as described under "Materials and Methods."
B. effect of C2-ceramide on MAPK activity in immunoprecipitation assays
Primary chondrocyte cultures were treated with IL-1 (20 ng/mL) or C2-ceramide (50 μM) for 20 min in serum-free medium. Protein extracts were prepared and an immunoprecipitation MAPK assay was performed as described under "Materials and Methods."


A
control
IL-1
C2-ceramide
MBP


B
control
IL-1
C2-ceramide
MBP

3.10 Antioxidants do not affect ceramide induction of *c-fos* expression

It has been shown previously that reactive oxygen species (ROS) are necessary for IL-1 signalling through the MAPK pathway and for the induction of *c-fos* expression (Lo and Cruz, 1995). The antioxidants NAC and DPI were used to determine the role of ROS in IL-1 and C2-cer induction of *c-fos* expression (Figure 9). NAC is a free radical scavenger that increases intracellular glutathione levels, which control the concentration of intracellular ROS through glutathione peroxidase (Halliwell and Gutteridge, 1990). DPI is an inhibitor of flavonoid-containing enzymes, such as NADPH oxidase and nitric oxide synthase (NOS), which give rise to various types of ROS (Stuehr et al, 1991).

Using Northern blot analysis, it was shown that pretreatment with 30 mM NAC for 2 h or 2 μM DPI for 30 min before the addition of IL-1 were both able to inhibit the increase in *c-fos* mRNA levels seen with IL-1 (Figure 9A). Pretreatment with 30 mM NAC for 2 h before the addition of C2-cer did not lower the levels of *c-fos* mRNA, while pretreatment with 2 μM DPI for 30 min before the addition of C2-cer increased the levels of *c-fos* mRNA (Figure 9B). The reason for this increase with DPI and C2-ceramide is unknown, since DPI alone is unable to cause induction of *c-fos* (Lo and Cruz, 1995). These studies suggest that *c-fos* induction by ceramide is not signalled through ROS.

Further confirmation of the differing pathways between IL-1 and ceramide is evidenced through the role of ROS as signalling intermediates. Although IL-1 induces *c-fos* expression by signalling through ROS and the MAPK pathway, ceramide does not appear to use ROS as signalling intermediates. While antioxidants inhibit the induction of *c-fos* by IL-1, this inhibition does not bring the levels of *c-fos* mRNA down to control values. This residual stimulation could be the result of ceramide induction of *c-fos* by an alternate pathway from that induced by IL-1.

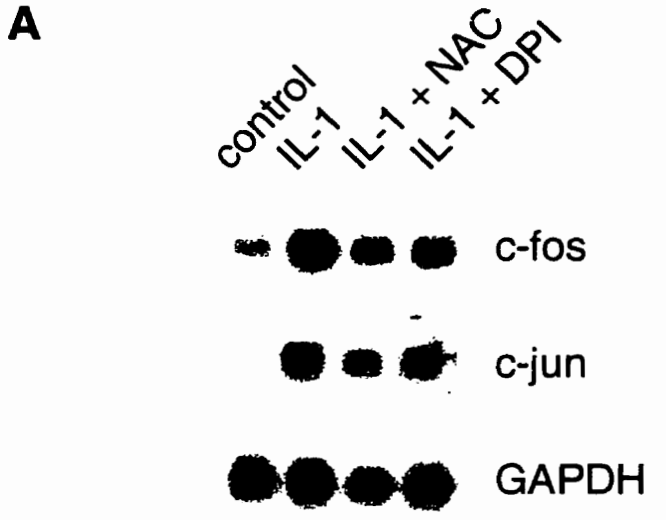
Figure 9. **Effects of antioxidants on *c-fos* expression.**

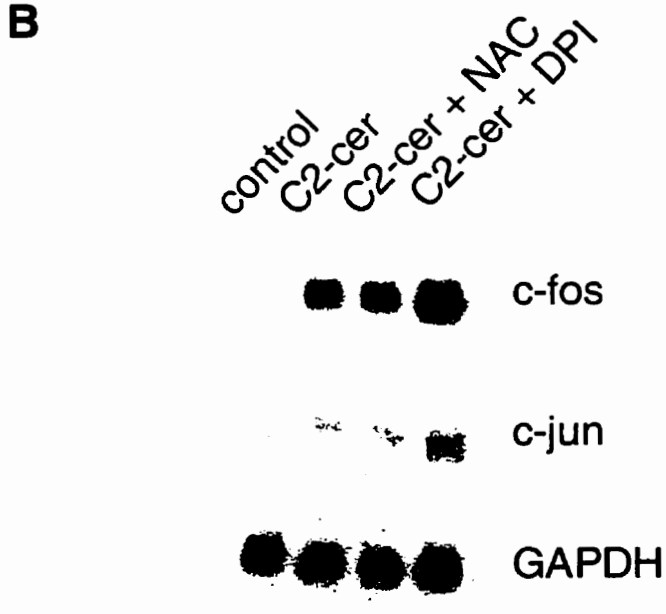
A, effect of antioxidants on IL-1 induction of *c-fos*.

Primary chondrocyte cultures were preincubated in serum-free medium with NAC (30 mM) for 2 h or DPI (2 μM) for 30 min before the addition of IL-1 (20 ng/mL) for 1 h.

B, effect of antioxidants on C2-ceramide induction of *c-fos*.

Primary chondrocyte cultures were preincubated in serum-free medium with NAC (30 mM) for 2 h or DPI (2 μM) for 30 min before the addition of C2-cer (10 μM) for 1 h. Total RNA was isolated and Northern blot analyses of *c-fos* and *c-jun* mRNA were performed as described under "Materials and Methods." The blots were subsequently stripped and reprobed with ^{32}P-labelled rat GAPDH cDNA to confirm roughly equal loading of RNA.


A
control
IL-1
IL-1 + NAC
IL-1 + DPI
c-fos
c-jun
GAPDH


B
control
C2-cer
C2-cer + NAC
C2-cer + DPI
c-fos
c-jun
GAPDH

3.11 Ceramide does not induce MMP expression

Since ceramide was shown to induce an increase in *c-fos* and *c-jun* mRNA levels (see Figure 1), and the protein products of these genes comprise AP-1, which is necessary for IL-1 induction of the MMP collagenase, levels of *collagenase* mRNA were examined in ceramide-treated cells (Figure 10A). No increase in *collagenase* mRNA was observed with either 5 μM or 10 μM C2-ceramide. Higher concentrations of C2-ceramide (50 μM and 100 μM) appear to inhibit the expression of *collagenase* mRNA. This inhibition appears to be specific for *collagenase*, as the housekeeping gene GAPDH is not affected. Perhaps this inhibition of *collagenase* mRNA levels at high ceramide concentrations occurs due to the length of exposure time to ceramide. Cells are treated for 16 h to induce *collagenase* expression, while treatment is only for 1 h to induce *c-fos* and *c-jun*. The effects of ceramide appear to be more complex than simple gene induction. In the presence of ceramide, there appears to be interactions occurring that prevent *collagenase* induction. At low concentrations of ceramide, *collagenase* mRNA is still present, although not increased above control values; at higher concentrations of ceramide, no *collagenase* mRNA is observed.

It is not surprising that an increase in *c-fos* and *c-jun* mRNA do not lead to an increase in *collagenase* mRNA, as activation of the AP-1 site is necessary but not sufficient for *collagenase* induction. Since the AP-1 site is necessary, however, the effect of ceramide on AP-1 transcriptional activity was examined (Figure 10B). Cells transfected with an AP-1-Luc plasmid construct (4 AP-1 consensus sequences driving the expression of a luciferase reporter gene containing an SV40 minimal promoter) showed increased transcriptional activity from the TRE with both IL-1-treated and C2-ceramide-treated cells. The increase in AP-1 transcriptional activity over untreated control cells appears to be greater for chondrocytes treated with 10 μM C2-ceramide than with 50 μM C2-ceramide, but the error bars do overlap. Based on the average relative luciferase activity for 10 μM C2-cer and 50 μM C20cer, this decrease in AP-1 transcriptional

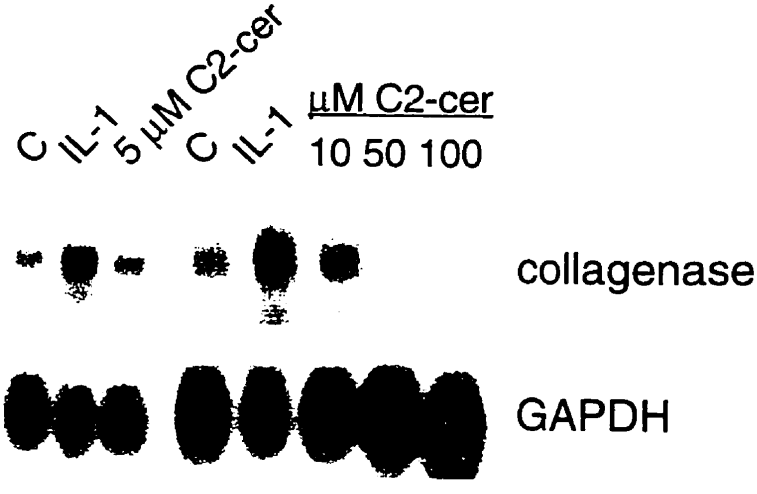
activity appears to correspond to the decrease in *collagenase* mRNA levels observed with 50 μM C2-ceramide (Figure 10A). As the presence of higher concentrations is normally associated with apoptosis in proliferating cell types, the activation of inhibitory factors may be induced, such as the TGFβ-inhibitory element, or JunB, which inhibits c-Jun.

Figure 10. **Role of ceramide in the induction of MMPs**

A. Effect on *collagenase* mRNA levels by C2-ceramide

Primary chondrocyte cultures were treated with increasing concentrations of C2-cer in serum-free medium for 16 h.

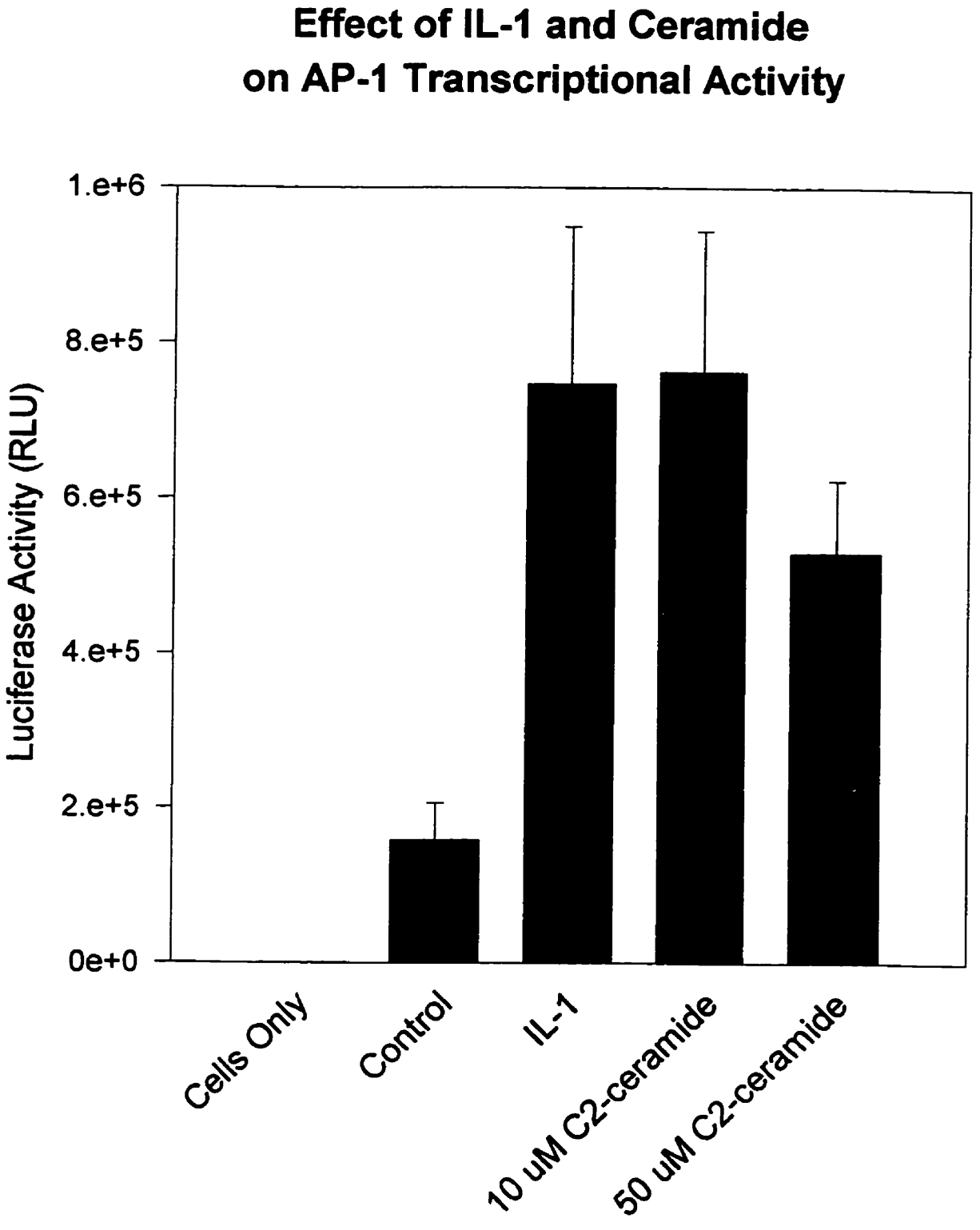
Total RNA was isolated and Northern blot analysis of *collagenase* mRNA was performed as described under "Materials and Methods." The blot was subsequently stripped and reprobed with ^{32}P-labelled rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA to confirm roughly equal loading of RNA.


C
IL-1
5 μM C2-cer
C
IL-1
μM C2-cer
10 50 100
collagenase
GAPDH

B. Effect of C2-ceramide on AP-1 transcriptional activity

Primary chondrocyte cultures were transfected overnight with the plasmid AP-1-Luc, which contains an AP-1 dependent SV40 minimal promoter driving the expression of the luciferase reporter gene, and then treated with 20 ng/mL IL-1 or 10 μM or 50 μM C2-ceramide in serum-free medium for 6 h.

Luciferase assays were performed on cell lysates as described in "Materials and Methods." Cells only represents the negative control untransfected, untreated cells, and control represents the background luciferase activity in transfected, but untreated, cells. Data are expressed as relative luciferase units of the cellular lysate. This is a representative experiment independently performed with four identically-treated samples per value. Error bars represent standard deviation within each set of four samples.


Effect of IL-1 and Ceramide
on AP-1 Transcriptional Activity
Luciferase Activity (RLU)
1.e+6
8.e+5
6.e+5
4.e+5
2.e+5
0e+0
Cells Only
Control
IL-1
10 uM C2-ceramide
50 uM C2-ceramide

CHAPTER 4 DISCUSSION

In Canada alone, more than four million people are afflicted with arthritis, a chronic condition that causes pain, inflammation, and joint stiffness. The two most common types of arthritis are osteoarthritis, in which joints (usually in the hands, hip, and feet) deteriorate, causing cartilage break-down and rubbing together of the underlying bones, and rheumatoid arthritis, which occurs when the body's immune system attacks healthy tissue, usually in the smaller joints. Present research is attempting to decipher the etiology and pathogenesis of this debilitating disease to allow for increased understanding and establishment of better treatments.

The focus of this research has been on the role of IL-1 signalling pathways in the development and progression of arthritis. Specifically, the role of the sphingomyelin pathway and its second messenger ceramide was examined. Since it is known that AP-1 is necessary for IL-1 induction of matrix metalloproteinases, such as collagenase, the expression of *c-fos* and *c-jun* was investigated, as the protein products of these genes comprise AP-1.

Previous research demonstrated that ceramide played contradictory roles as both an agent of cell growth and differentiation, and as an agent of cell death. As reviewed in Hannun (1994), exogenous ceramides were found to mimic the actions of TNFα, Vitamin D3, and IFN-γ by inducing differentiation of HL-60 cells. C2-ceramide also mimicked TNFα-induced apoptosis in myeloid and lymphoid cells. Studies in human fibroblasts revealed that ceramide plays a role in the induction of PGE_2 secretion in response to IL-1, and also enhances IL-2 secretion in lymphocytes. Since both IL-1 and TNF are involved in inflammation, and are found at elevated levels in the synovial fluid of arthritic joints, the role of ceramide in mediating the effects of these agonists in arthritis was examined. Arguments for the role of ceramide in TNF and IL-1 signalling (as outlined in Andrieu et al, 1995) are based on observations that these three agents all stimulate a

membrane-bound serine/threonine protein kinase, a cytosolic protein phosphatase, a MAPK, and promote down-regulation of *c-myc* proto-oncogene, enhancement of cyclooxygenase gene expression, and activation of transcription factors, such as NF-κB and AP-1.

In this study, it was found that synthetic ceramide analogues induced *c-fos* and *c-jun* expression in chondrocytes, but caused no induction of *collagenase* gene expression. No studies to date have focused on the induction of *c-fos* by ceramide, and a paper focused on the requirement of AP-1 for ceramide-induced apoptosis examined only the induction of *c-jun* by ceramide (Sawai et al, 1995). This thesis, then, provides novel findings on the role of ceramide in the induction of *c-fos* gene expression in chondrocytes.

4.1 INDUCTION OF *c-fos* BY CERAMIDE

This study showed that the synthetic ceramide analogue C2-ceramide induced an increase in *c-fos* mRNA levels within a concentration range of 5-100 μM (Figure 1). C2-ceramide showed a rapid and transient increase in *c-fos* mRNA levels (Figure 2A), similar to that observed for *c-jun* mRNA levels (Sawai et al, 1995), and for IL-1 and TNF stimulation of *c-jun* (Lo, 1995). Maximal induction of *c-fos* was observed at 1 h, the same time for maximal induction of *c-jun* by ceramide found by Sawai et al. (1995). Similar to the regulation of *c-jun* by ceramide (Sawai et al, 1995), the induction of *c-fos* by C2-ceramide is regulated both at the transcriptional and post-transcriptional levels (Figure 2B). These results are consistent with the evidence that the transcriptional induction of *c-fos* is independent of *de novo* synthesized proteins and inhibition of protein synthesis leads to a stronger and prolonged induction of *c-fos* (Greenberg et al, 1986).

The *c-fos* gene shares common regulation with other early response genes. Their accumulation is normally transient, due to the brief period of gene activation and the instability of the mRNA transcripts (Barcellini-Couget et al, 1993). During the transient accumulation of *c-fos* mRNA, the apparent size of the mRNA decreases as stimulation

proceeds (Piechaczyk and Blanchard, 1994). The poly(A) tail of the *c-fos* mRNA accounts for most of the newly synthesized transcript, and deadenylation of this tail is slowed by the presence of cycloheximide or the deletion of the AU-rich element in the *c-fos* transcript. Shortening of the poly(A) tail of *c-fos* begins within minutes of serum addition to cells, and the entire tail is removed in about 10 min. Degradation of the mRNA body occurs abruptly after this deadenylation is complete. Either a 400 nucleotide fraction of the coding region of *c-fos*, or the AU-rich element in the untranslated region is sufficient to direct removal of the poly(A) tail (Piechaczyk and Blanchard, 1994).

Protein synthesis inhibitors, such as cycloheximide, could either stabilize a transcriptional activator or inhibit the production or activity of a repressor, thereby causing an increase in mRNA levels. Labile proteins could act in *trans* to alter the half-life of *c-fos* mRNA, or protein synthesis inhibitors could trap mRNAs within polysomes, protecting them from cytoplasmic ribonucleases (Veyrune et al, 1995). It is also possible that degradation is linked to continuous translation.

In the absence of inhibitors of protein synthesis, *c-fos* mRNA is preferentially degraded within minutes after it is synthesized. Many transiently expressed eukaryotic mRNAs contain several types of instability determinants located within their 3' untranslated regions (UTRs) or within their coding regions (for review see Belasco and Brawerman, 1993). The 3' untranslated regions (UTRs) of unstable mRNAs contain an $A(U)_nA$-rich segment (ARE), which leads to their short half-lives. The 3' UTR of *c-fos* has the following sequence: GUUUUUAAUUUAUUUAUUAAGAUGGAUUCU CAGAUAUUUAUAUUUUUAUUUU (the AU-rich regions are underlined). The inactivation or decay, resulting from inhibition of protein synthesis, of the cytoplasmic RNase that specifically recognizes this mRNA could account for the superinduction of *c-fos* mRNA synthesis seen with cycloheximide. The ARE in the 3' UTR of *c-fos* is mainly responsible for *c-fos* degradation in exponentially growing cells, while another ARE also

responsible for *c-fos* mRNA instability exists within the coding region (Piechaczyk and Blanchard, 1994).

Also within the coding region of *c-fos* is an intragenic regulatory element (FIRE) located at the end of exon 1 (Lamb et al, 1990). This sequence has been shown to block transcriptional elongation. Under conditions where the *c-fos* gene is actively transcribed this intragenic element is inoperative. This effect could result from either the synthesis of a factor that binds to the FIRE, relieving its inhibitory effect, or the posttranslational modification of the FIRE. This element represents another mechanism of transcriptional regulation of *c-fos*.

Studies by Subramaniam et al. (1989) showed that a synthetic copy of the *c-fos* SRE was sufficient to confer cycloheximide-dependent inducibility on a heterologous promoter, and that several mutations in the SRE that impaired serum-inducibility also impaired cycloheximide-inducibility. They found that serum-responsive enhancer elements were negatively regulated by one or more labile proteins, and that both positive and negative regulators of enhancer activity required a functional CArG box.

The CArG box is an AT-rich region within the SRE region of the *c-fos* promoter (AATTCCAGGATGTCCATATTAGGACATCTGCA). Mutations in the CArG box eliminate the binding of the SRF to the SRE. The putative repressor of SRE-dependent transcription of *c-fos* may exert its effect not by binding directly with the DNA, but by protein-protein contact, perhaps with the SRF itself (Subramaniam et al, 1989).

The *c-fos* promoter also contains a MyoD consensus binding site that overlaps the SRE. MyoD acts as a negative regulator of *c-fos* transcription by blocking serum responsiveness through the SRE. Mutation of the CArG site did not have any effect on MyoD binding, indicating that the MyoD binding site does not contain the CArG box (Subramaniam et al, 1989). The SRE appears to play a role in both the transcriptional and post-transcriptional regulation of *c-fos*.

The induction of *c-fos* by ceramide is specific for the biological action of ceramide. In the biosynthesis of sphingolipids, ceramide is formed by the addition of a *trans*-4,5 double bond to dihydroceramide; the chemical structures of these two molecules are identical except for this bond (Spiegel et al, 1996). The addition of the double bond creates a biologically active molecule, as ceramide, but not dihydroceramide, induces a significant increase in *c-fos* mRNA levels (Figure 3).

The possibility exists for the conversion of ceramide to other sphingolipids, as break-down and reuse of components of sphingolipid biosynthesis occurs regularly in cells (Spiegel et al, 1996). Cleavage of the amide-linked fatty acid from ceramide by a ceramidase forms sphingosine, another lipid second messenger, which, like ceramide also has a phosphorylated form (sphingosine 1-phosphate). Sphingosine is also both mitogenic and growth inhibitory in various cell types (for reviews see Merrill Jr. et al, 1996; Spiegel et al, 1996). Downstream signalling for sphingosine 1-phosphate involves activation of the MAPK cascade (Wu et al, 1995), and of AP-1 (Su et al, 1994). PDGF has been shown to induce a rapid, transient elevation in sphingosine and sphingosine 1-phosphate levels in several cell types (Olivera and Spiegel, 1993; Coroneos et al, 1995); this growth factor is also a simulator of the SIE on the *c-fos* promoter, perhaps indicating a role for sphingosine and sphingosine 1-phosphate in the regulation of *c-fos*. Both ceramide, sphingosine, and sphingosine 1-phosphate could be present in the same cell simultaneously, and the levels of these lipids could determine cell fate. Sphingosine 1-phosphate has been found to inhibit intranucleosomal DNA fragmentation and morphological changes that result from increased concentrations of ceramide (Cuvillier et al, 1996). Fumonisin B_1 (FB1) is a fungal toxin that has been shown to inhibit sphingolipid synthesis by affecting ceramide synthase (for review see Merrill Jr. et al, 1996). FB1 bears structural similarity to the sphingoid base backbone of sphingolipids, and inhibits the metabolic reactions of conversion of sphinganine to dihydroceramide and of ceramide to sphingosine. It therefore causes accumulation of sphinganine and, sometimes, sphingosine

due to inhibition of the reacylation of sphingosine produced by sphingolipid turnover. As well, there is an elevation of sphingoid base 1-phosphates, increased sphingoid base turnover to hexadecanal and ethanolamine phosphate, and inhibition of *de novo* biosynthesis of more complex sphingolipids (Merrill Jr. et al, 1996). In chondrocytes (as shown in Figure 4), no effect was observed with the addition of FB1 to cell cultures. FB1 did not induce *c-fos* expression, nor did it inhibit the induction of *c-fos* observed with IL-1. These results would indicate that the elevation in *c-fos* mRNA levels observed with C2-ceramide does not occur by the conversion of ceramide to other sphingolipids, such as sphingosine. Ceramide, then, appears to be the bioeffector involved in the induction of *c-fos* in chondrocytes when the level of ceramide is increased in culture by the addition of short-chain ceramide analogues.

The increase in *c-fos* mRNA observed with C2-ceramide was independent of an increase in SRE binding (Figure 6), suggesting the activation of another site on the *c-fos* promoter, or the requirement of an additional signal for induction of binding. Although no increase in SRE binding was observed with ceramide treatment, an increase in SRE-dependent transcription occurred (Figure 7). It is believed that signalling to the *c-fos* promoter nearly always involves the SRE, identifying this element as the main regulatory sequence of *c-fos* (Janknecht et al, 1995). Most signals targeting the SIE also stimulate the SRE. However, there is evidence that the other elements of the *c-fos* promoter may be activated independently of a simultaneous activation of the SRE. Calcium signalling in hippocampal neurons can activate preferentially either the SRE or the CRE, with the other element being activated only slightly, depending on the mode of Ca^{2+} entry into the cells (Bading et al, 1993). It is unknown, though, if the slight activation of the SRE is necessary for the resulting induction of *c-fos*. In other cell lines, the CRE and the SRE may jointly upregulate *c-fos* transcription. The induction of PKA by cAMP has been shown to block the Ras-dependent activation of Raf-1 and thus also block the MAPK pathway (Cook and McCormick, 1993; Wu et al, 1993; Hafner et al, 1994). This scenario would lead to

PKA-induced activation of *c-fos* solely by the CRE because any potential MAPK-induced stimulation of the SRE would be inhibited. In PC12 cells, however, cAMP can activate MAPK independently of Raf-1 (Frodin et al, 1994); thus, it is possible for cAMP to cause stimulation of both the CRE and the SRE to induce *c-fos*.

In exponentially growing Rat-1*ts*LA29 cells, AP-1/ATF-related factor binding was induced by v-Src stimulation at the expense of SRF, suggesting that the TRE may play a role in downregulating the *c-fos* transcriptional response, and that binding does not occur simultaneously at the SRE and the TRE (Wyke et al, 1996). Binding of transcription factors at the TRE may require an intact SRE, because in quiescent NIH 3T3 cells the SRE and the AP-1/ATF-like sequence appear to function cooperatively in the repression of *c-fos*. It was found that no ternary complex formation was observed with the activation of MAPK by v-Src, but that a complex was induced to form at the SIE under conditions in which *c-fos* transcription is stimulated. In growing Rat-1*ts*LA29 fibroblasts, *c-fos* transcription was found to be uncoupled from the MAPK pathway (Wyke et al, 1996). Perhaps in the chondrocytes used in the studies in this thesis, *c-fos* transcription induced by ceramide is also uncoupled from the MAPK pathway, such that SRE transcriptional activity (Figure 6), but not binding (Figure 7) is induced, and no phosphorylation of the ERK1/ERK2 MAPK substrate MBP is observed (Figure 8).

The *c-fos* SRE is occupied by TCF and SRE before, during, and after induction (Herrera et al, 1989; Konig, 1991), and thus phosphorylation of TCF could occur *in situ* at the *c-fos* promoter, but may also involve rapid exchange of proteins bound to the promoter. TCFs interact with the SRE only in conjunction with the SRF, and once phosphorylated are able to stimulate transcription (Gille et al, 1992; Hill et al, 1993; Janknecht et al, 1993; Marais et al, 1993). Dimerized SRF, however, can autonomously interact with the SRE (Norman et al, 1988). The down-regulation of *c-fos* after induction involves the repression of SRE-dependent transcription through dephosphorylation of TCF. Both TCF dephosphorylation and *c-fos* down-regulation are blocked by inhibitors of

protein phosphatases (Zinck et al, 1993). The protein phosphatases 3CH134/CL100 and PAC1, which dephosphorylate MAPKs, are inducible by stimuli known to induce *c-fos* (Charles et al, 1992; Keyse and Emslie, 1992; Rohan et al, 1993). These phosphatases could be responsible for the transient nature of MAPK activation and TCF phosphorylation, and the transient transcriptional activation of *c-fos* (Janknecht et al, 1995).

As reviewed in Hill and Treisman (1995), combinatorial interactions of multiple transcription factors increase specificity of the transcriptional response. Transcription from a single promoter containing multiple signal-regulated elements will be activated more efficiently by stimuli that can simultaneously activate all of these elements rather than stimuli that only activate some of the elements. Transcription from the *c-fos* promoter seems to occur through the action of stimuli that activate more than one proximal regulatory element (Janknecht et al, 1995). Under cellular conditions where ceramide is released from the hydrolysis of SM, other stimuli are likely to be present causing biological effects on their own. Agents that activate SMases also activate kinases and transcription factors independent of the action of ceramide (for example, as discussed for TNF in Johns et al, 1994, and for IL-1 in Bird et al, 1994). It could be that simultaneous signals mediated by IL-1 and ceramide converge on the *c-fos* promoter, activating transcription by the action of both together. The addition of exogenous ceramide at levels above those found under normal physiologic conditions may be sufficient to activate multiple elements on the *c-fos* promoter, thus activating transcription (as seen in Figure 7).

Ceramide may be able to activate transcriptional activity of *c-fos* from the SRE by inducing phosphorylation of TCF through the action of CAPK. Phosphorylation of $p42^{MAPK}$ (ERK2) in HL-60 cells was found to be induced by C2-ceramide within seconds (Raines et al, 1993). Also in HL-60 cells, studies indicate that CAPK phosphorylates Raf1 on Thr-269, increasing its activity towards MEK and leading to activation of MAPK (Yao et al, 1995). In rat mesengial cells, IL-1 induces the expression of a specific type IV

collagenase, a group II phospholipase A_2, eicosanoids, an inducible nitric oxide synthase, a variety of chemokines, and ceramide production (Huwiler et al, 1996). The ceramide liberated was found to specifically bind to and activate protein kinase c-Raf, leading to activation of the MAPK cascade (Huwiler et al, 1996). Ceramide was not found to bind to any other member of the MAPK cascade nor to PKCζ. In human aortic smooth muscle cells, however, incubation of cells with ceramide did not significantly stimulate $p44^{MAPK}$ (ERK1) activity (Bhunia et al, 1996). Studies by Bird et al (1994) also indicate a difference in MAPK stimulation between IL-1 and ceramide. In gingival fibroblasts and HepG2 hepatoma cells, IL-1 activated a kinase that phosphorylated Thr-669 peptide, but not MBP [both MBP and a peptide containing Thr-669 of the EGFR are substrates of ERK1/ERK2, whereas p54 MAPK phosphorylates EGFR-based peptides and c-Jun, but MBP only poorly], and failed to cause tyrosine phosphorylation of ERK1/ERK2. In contrast, C2-ceramide stimulated predominantly MBP-specific kinase activity in fibroblasts, and had no effect in HepG2 cells. In HL-60 cells, C2-ceramide treatment inhibited ERK1/ERK2 activity, perhaps through the action of CAPP (Westwick et al, 1995). The response of ceramide in the activation of the MAPK pathway could be cell-type dependent in the same manner that cellular responses to ceramide differ in various cell types. Ceramide could be indirectly involved in the phosphorylation of previously bound TCF and SRF on the SRE, but not in the recruitment of new molecules to the *c-fos* promoter. Increased transcriptional activity of *c-fos* by ceramide could also occur through FRK, a proline-directed kinase regulated independently of the ERKs and JNK/SAPKs that phosphorylates the Fos transcriptional activation domain at Thr-232 (Deng and Karin, 1994).

In this study, phosphorylation of MBP by ceramide-treated cells was examined (Figure 8), but no phosphorylation of this protein was observed. As well, induction of an increase in binding to both the *c-fos* SIE and CRE were investigated (data not shown), but no increase was observed with either C2-ceramide or IL-1. It appears that, in

chondrocytes, ceramide induces *c-fos* expression through the activation of molecules already bound to the SRE. Perhaps, activation of other regulatory sites on the *c-fos* promoter may also occur without an increase in binding.

4.2 INDUCTION OF *c-jun* BY CERAMIDE

C2-ceramide was generally shown to induce an increase in *c-jun* mRNA starting at a concentration of 50 μM (Figure 1). This induction did not involve ROS (Figure 9B), or the conversion of ceramide to sphingosine (Figure 4). Previous research has demonstrated that C2-ceramide increased the levels of *c-jun* mRNA, c-Jun protein, and AP-1 DNA binding in HL-60 cells (Sawai et al, 1995). This induction of *c-jun* was dose-dependent (1-10 μM), and maximal expression was achieved after 1 h of treatment. Ceramide, thus, shows differential effects in various cell types. As well, although the effects of C2-ceramide and C6-ceramide on *c-fos* were similar, C6-ceramide was able to induce an increase in *c-jun* mRNA levels at a lower concentration than the shorter chain analogue C2-ceramide (Figure 1). The effects of ceramide on *c-jun* expression appears to depend on both the cell type and the length of the acyl chain on the analogue.

The regulation of *c-jun* occurs through MAPKs similar to those that regulate *c-fos*. JNK/SAPKs are proline-directed like ERK1/2, and require Tyr and Ser/Thr phosphorylation for activity, but differ in their substrate specificity (Kyriakis et al, 1994). C2-ceramide was found to mimic the action of TNF (Westwick, 1995) and IL-1 (Welsh, 1996) in the activation of JNK/SAPK, and ceramide-initiated JNK/SAPK signalling was found to be required for stress-induced apoptosis (Verheij et al, 1996). Ceramide appears to signal both of the major effects of TNF--inflammation and apoptosis--through two opposing pathways (Kolesnick and Golde, 1994). Ceramide signals apoptosis in U937 and BAE cells through the SAPK cascade (Verheij et al, 1996), but in HL-60 cells, links the 55KDa TNFR to the inflammatory response through the MAPK cascade (Yao et al, 1995). It is unknown, however, whether ceramide signalling through the MAPK and

SAPK cascades is cell-type specific, or whether one cell can shift between the two pathways (Verheij et al, 1996).

4.3 ROLE OF THE SPHINGOMYELIN PATHWAY IN IL-1 SIGNALLING

Several studies exist on the role of ceramide as a second messenger in various signalling pathways, such as IL-1 and TNF (Hannun, 1994; Kolesnick and Golde, 1994; reviewed in Divecha and Irvine, 1995 and in Spiegel et al, 1996). In some cell types, criteria for establishing a pathway as a signalling system and a molecule as a second messenger were met for the SM pathway and ceramide (Dressler et al, 1992; Yanaga and Watson, 1992; Ballou et al, 1992; Mathias et al, 1993). These criteria include rapid kinetics of activation of a potential signalling pathway, the ability of analogues of a purported second messenger to bypass receptor activation and mimic agonist action, and reconstitution of events in a cell-free system (Kolesnick and Golde, 1994). It has also been shown, however, that IL-1 and ceramide cause different effects in the same cell type. The IL-1-mediated activation of Thr-669-specific MAPK was not mimicked by ceramide in gingival fibroblasts and HepG2 cells (Bird et al, 1994). In fact, ceramide exerted an opposite effect from IL-1 in kinase activation. IL-1 also was found not to signal to MAPK through Raf-1, although other studies found that ceramide not only activates Raf-1 (Yao et al, 1995), but also binds specifically to it (Huwiler et al, 1996). Experiments have shown that the addition of ceramide to cells induces the translocation of NF-κB to the nucleus (Schutze et al, 1992; Johns et al, 1994), mimicking the effect of TNF; however, inhibition of the ceramide pathway by phorbol 12-myristate (PMA) in SW480 cells resulted in the loss of the ceramide-induced NF-κB response, but had no effect on the TNF-induced NF-κB activation (Johns et al, 1994). In Jurkat cells, however, C2-ceramide was unable to induce NF-κB under multiple conditions, but did enhance the activation of NF-κB by TNF in a dose-dependent manner (Dbaibo et al, 1993). In HL-60 cells, as well, C2-ceramide did not mimic the effect of TNF in stimulating NF-κB DNA binding activity

(Westwick et al, 1995). In human vascular endothelial cells, ceramide was not found to be an important second messenger for TNF signalling, as the addition of exogenous SMase or cell-permeable ceramide analogues did not completely activate NF-kB, and only activated JNK at late times; as well, gene transcription and surface expression of endothelial leukocyte adhesion molecules that are readily induced by TNF were not induced by ceramide nor SMase (Slowik et al, 1996). Ceramide appears to mimic only some of the effects of TNF and IL-1 in a cell-dependent manner.

Since ceramide is believed to be a second messenger involved in mediating some of the effects of IL-1, it could be involved in positive or negative feedback regulation of IL-1 effects. The ability of ceramide to modulate the induction of *c-fos* and *c-jun* by IL-1 was examined in Figure 5. C2-ceramide, like IL-1, is able to induce an elevation in mRNA levels of these two genes. When C2-ceramide is added to chondrocytes together with IL-1, the observed increase in *c-fos* (Figure 5A) or *c-jun* (Figure 5B) mRNA levels is greater than that observed with C2-ceramide alone. Note that in Figure 5A, the concentration of C2-ceramide was too low to induce *c-jun* expression alone, and in Figure 5B, the concentration of C2-ceramide caused an increase in *c-fos* mRNA levels on its own that is greater than the induction observed with IL-1 alone. The results for *c-fos* at 10 μM C2-ceramide and for *c-jun* at 50 μM C2-ceramide are similar, and thus potentiation for each gene will be considered at these concentrations of C2-ceramide. At concentrations of IL-1 too low to induce *c-fos* or *c-jun* expression, the addition of C2-ceramide together with IL-1 caused an increase in mRNA levels approximating the induction seen at high concentrations of IL-1 (≥ 20 ng/mL). The induction of *c-fos* or *c-jun* by IL-1 is concentration-dependent, but at concentrations of IL-1 too low to cause maximal induction, C2-ceramide is able to potentiate the effects of IL-1. At 5 ng/mL IL-1, no increase in *c-fos* mRNA is observed; however, this concentration is enough to induce an increase in SRE binding (as shown in Figure 6). Ceramide appears to induce *c-fos* and *c-jun* through a separate mechanism from IL-1, such that when IL-1 and ceramide are

added together, they are able to increase mRNA levels above that seen with either agent alone.

Further confirmation of the differing pathways between IL-1 and ceramide is evidenced through the role of ROS as signalling intermediates. Although IL-1 has been shown to induce *c-fos* and *c-jun* expression by signalling through ROS (Lo, 1995), ceramide does not appear to use ROS as signalling intermediates (Figure 9B). While antioxidants inhibit the induction of *c-fos* by IL-1, this inhibition does not bring the levels of *c-fos* mRNA down to control values. This residual stimulation could be the result of ceramide induction of *c-fos* by an alternate pathway from that activated by IL-1.

These results suggest that ceramide provides an additional signal that adds to the one provided by IL-1 to induce *c-fos*. It may be that the induction of SRE binding seen with IL-1 is not sufficient to induce *c-fos* expression without the activation of another site on the *c-fos* promoter. Higher concentrations of IL-1 may be able to activate additional sites from the SRE, thus inducing *c-fos* expression. However, it may be that IL-1 induces ceramide liberation in a concentration-dependent manner, such that the activation of SMases and release of ceramide occurs only at concentrations higher than 10 ng/mL, which provide the additional signal necessary to induce *c-fos*.

4.4 ROLE OF CERAMIDE IN INDUCTION OF COLLAGENASE

Most of the studies on the second messenger properties of ceramide have examined the activation of immediate early genes, such as *c-fos*, *c-jun*, and *c-myc*, or have focused on stimulation of the MAPK pathway or NF-κB. As reviewed in Ballou et al. (1996), ceramide transduces downstream signals through its ability to affect the activities of target enzymes and transcriptional activators, such as CAPK, CAPP, PKCζ, Vav, NF-κB, MAPKs, Ras, PLA_2, Cox, IL-6, IL-2, c-Myc, and Rb. Since it is established that AP-1 activity is a general requirement for MMP expression, the expression patterns of *fos* and *jun* may provide clues to MMP expression. In chondrocytes, the induction of *c-fos*

and *c-jun* gene expression by ceramide does not correlate with an increase in *collagenase* mRNA levels. As well as AP-1 complexes, NF-κB and PEA3 sites also function as MMP transcriptional activators. It has been found that there may be selective cooperativity of specific Ets family members with Fos or Jun family members, or the influence of specific cofactors required for productive Ets/AP-1 interactions (Crawford and Matrisian, 1996). The expression of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) also appears to be controlled by AP-1 and Ets binding (Logan et al, 1996). The TIMP-1 promoter contains two AP-1 binding sites and multiple Ets binding sites. It is possible that ceramide activates TIMP-1, (perhaps the arrangement of positive regulatory sites on its promoter is activated more readily by ceramide's targets than MMP promoters), which leads to the inhibition of MMP expression. Considerable cross-talk exists between lipid signalling pathways, such as sphingolipids and glycerolipids (Brindley et al, 1996). DAG is an activator of PKC, while phosphatidate and lysophosphatidate stimulate tyrosine kinases and activation of the Ras-Raf-MAPK pathway. Ceramide inhibits activation of PLD by decreasing its interaction with G proteins. As well, ceramide stimulates the degradation of phosphatidate, lysophosphatidate, ceramide 1-phosphate, and sphingosine 1-phosphate through the multifunctional phosphohydrolase phosphatidate phosphohydrolase, whereas sphingosine inhibits this enzyme. It is possible, after 16 h treatment with C2-ceramide to examine *collagenase* induction, that ceramide is converted to other sphingolipids or activates other lipid signalling pathways, leading to the inhibition of *collagenase* expression.

SUMMARY AND CONCLUSIONS

In this study, the effect of ceramide on gene expression in chondrocytes was examined. Ceramide, a proposed lipid second messenger, has been shown to mediate the effects of mitogens, such as cytokines and growth factors, in various cell types. The ability of ceramide to mimic the biological effects of IL-1, specifically the induction of *c-fos*, *c-jun*, and *collagenase* gene expression, and to act as a second messenger of IL-1 were studied.

This study showed that the synthetic ceramide analogue C2-ceramide induced *c-fos* expression in chondrocytes at a concentration 10-fold less than that required for it to induce *c-jun* expression. Ceramide caused a potentiation of the effects of IL-1, inducing an increase in *c-fos* and *c-jun* mRNA levels greater than that seen with either IL-1 or ceramide alone. Ceramide, unlike IL-1, did not induce an increase in binding to the SRE of the *c-fos* promoter; but, like IL-1, did induce an increase in SRE transcriptional activity. As well, ceramide did not appear to use ROS as signalling intermediates in its induction of *c-fos* and *c-jun* gene expression, unlike IL-1, which has been shown to induce *c-fos* expression through ROS, the MAP kinase cascade, and activation of the SRE on the *c-fos* promoter. Ceramide did not appear to affect the induction of MMPs. It did, at low concentration, increase transcriptional activity from the AP-1 site, which is found on MMP gene promoters, but did not induce an increase in *collagenase* (a major MMP) mRNA. In fact, ceramide at high concentration appeared to inhibit the activation of MMPs. The role of ceramide appears to be both concentration- and cell type-dependent, and can exert complex effects even within the same cell type.

Taken together, these data seem to suggest that ceramide is not solely a second messenger of IL-1, although it might function as such in various cell types; rather, ceramide appears to function through a signalling pathway separate from that used by IL-1 in the induction of genes such as *c-fos*.

Thus, although not being directly involved in the induction of *collagenase* gene expression, ceramide may still be involved in the induction of AP-1-regulated genes by its ability to induce *c-fos* and *c-jun* gene expression.

FUTURE DIRECTIONS

The role of ceramide in a signalling pathway inducing early gene expression, such as that of *c-fos* and *c-jun* is still not clearly elucidated. It remains to be identified the exact mechanism of induction of *c-fos* and *c-jun* gene expression in chondrocytes. A nuclear run-off assay could be performed to determine specific gene transcription as a function of cell state. Doing this type of experiment on ceramide- and IL-1-treated cells would determine whether changes in mRNA levels of *c-fos* and *c-jun* occur as a result of changes in synthesis or changes in mRNA degradation or transport from the nucleus to the cytoplasm. Analysis of regulatory sites on the promoters of *c-fos* and *c-jun*, including the SIE and CRE of *c-fos*, as well as further analysis of the SRE, and the Jun-AP-1 sites of *c-jun*, using EMSAs, are required to determine if ceramide induces an increase in binding of transcription factors regulating these genes. Immunoprecipitation kinase assays involving JNK/SAPK and p38 kinase would assess the pathway of phosphorylation and activation to determine how ceramide is able to induce gene expression.

The importance of ceramide in chondrocytes should be further analysed. Although present methods cannot distinguish between the structural and signalling pools of sphingolipids nor measure stimulated elevations over the entire cell, it would be helpful to determine how large a role ceramide plays in the progression of arthritic diseases. Are matrix metalloproteinases, such as collagenase and stromelysin, upregulated by IL-1 signalling through the sphingomyelin pathway? Are separate signals from IL-1 and ceramide required for gene regulation? An examination of other regulatory sites on MMP promoters, such as the PEA3 site, using EMSAs and transfections, would be necessary to

determine how ceramide is able to induce its inhibitory effect on *collagenase* mRNA levels and AP-1 transcriptional activity.

As the role of the sphingomyelin pathway and its putative second messenger ceramide are complex, widespread, and inconsistent (based on cell-type, concentration, and stimulus), further analysis of this pathway is necessary to understand the effects of ceramide. It is also necessary, however, to examine the roles of other lipid signalling molecules in this pathway. The effects of sphingosine and its phosphate, as well as ceramide phosphate, need to be studied in chondrocytes (using the same methods as above in the study of ceramide) to determine if they play any role in the progression of arthritic diseases. If ceramide does not solely act as a second messenger for IL-1, then it remains to be identified what determines when and how ceramide acts and how much overlap in effects is present between IL-1 and ceramide.

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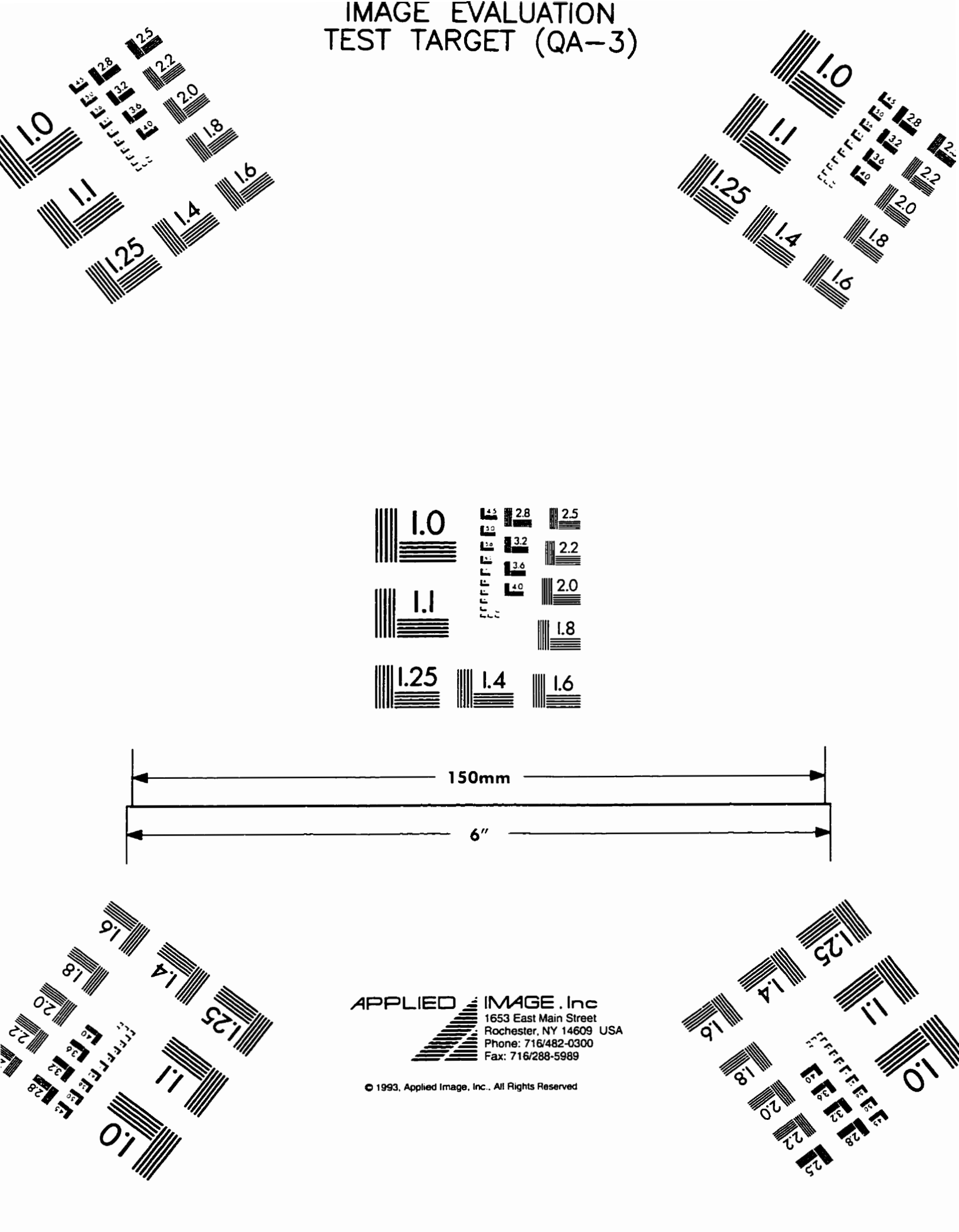
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4.0
150mm
6"
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