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REGULATION OF MITOGENIC SIGNAL TRANSDUCTION BY GANGLIOSIDES IN U-1242 MG HUMAN GLIOMA CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of the Ohio State University

By

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The Ohio State University
1996

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ABSTRACT

Several lines of evidence have implicated gangliosides in the regulation of cellular proliferation. Specific effects of gangliosides on signal transduction molecules have been demonstrated in vitro, but little evidence exists that gangliosides have these effects in whole cells. Exogenously added gangliosides inhibit DNA synthesis in Swiss 3T3 cells stimulated with platelet-derived growth factor (PDGF). Antiphosphotyrosine western blots showed that this correlates with the inhibitory effects of several gangliosides (except GM3) on tyrosine phosphorylation of the PDGF receptor. Experiments using a protein crosslinker demonstrated that the same gangliosides prevented dimerization of PDGF receptor monomers in both Swiss 3T3 and human glioma U-1242 MG cells. These results demonstrate that all gangliosides tested, except GM3, probably inhibit PDGF-mediated growth by preventing dimerization and tyrosine phosphorylation of PDGF receptor monomers.

Exogenously added gangliosides have a bimodal effect on proliferation in U-1242 MG glioma cells, inhibiting DNA synthesis in growing cells and stimulating it in quiescent cells. Immune complex kinase assays and
[\[^{3}\text{H}\]thymidine incorporation experiments were used to identify the signal transduction pathways responsible for GM1-stimulated DNA synthesis. GM1 caused activation of two kinases known to be important for mitogenic signaling, the MAP kinase isoform Erk2 and p70 S6 kinase (p70\(^{66\text{k}}\)). Erk2 activation and GM1-stimulated DNA synthesis could be inhibited by pretreatment with the inhibitor of MAP kinase kinase activation, PD98059. p70\(^{66\text{k}}\) activation was prevented by pretreatment with rapamycin. Rapamycin partially inhibited GM1-stimulated DNA synthesis. Platelet-derived growth factor also activated both kinases, but did not cause DNA synthesis, suggesting that GM1 may stimulate other cascades which are involved in GM1-mediated DNA synthesis.

Investigation of the mechanism of Erk2 activation by GM1 revealed that tyrosine phosphorylation, Protein Kinase C and changes in cAMP levels were not involved. c-Raf-1 was activated by GM1 but B-Raf was not. In addition, activation of both Erk2 and p70\(^{66\text{k}}\) was prevented by the phosphatidylinositol 3'-kinase inhibitor wortmannin, but GM1-stimulated DNA synthesis was not. These results demonstrate that gangliosides can modulate specific signal transduction pathways in whole, living cells and that these effects can be correlated with a biological outcome such as cell growth.
Dedicated to my parents, 
whose unwavering support 
made achieving this goal possible.
ACKNOWLEDGMENTS

I am grateful for the advice and support of the members of my dissertation committee, especially my adviser, Dr. Allan Yates and Dr. Arfaan Rampersaud. The many hours of discussions I've had with both of them have taught me much more about being a scientist than can be summarized in a dissertation.

I would also like to thank the people that I've worked with in the lab for their help and friendship, especially Dr. Hany Saqr, Dr. Ching-Ching Sung, DiAnna Hynds, and Ted Comas.

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**ABSTRACTS**


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Major Field: Pathology
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<tr>
<td>BCIP/NBT</td>
<td>5-bromo-4-chloro-3-indolyliophosphate/p-nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>BGG</td>
<td>bovine gamma globulin</td>
</tr>
<tr>
<td>BS3</td>
<td>bis (sulfosuccinimidyl) suberate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CDTA</td>
<td>cyclohexanediaminetetraacetic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(b-aminoethyl ether) N,N,N',N'-taetaacetic acid</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>HEPES</td>
<td>4(-2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Jnk</td>
<td>jun N-terminal kinase</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKAP kinase 2</td>
<td>MAP kinase-activated protein kinase 2</td>
</tr>
<tr>
<td>MAPKK</td>
<td>MAP kinase kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MEK</td>
<td>Map kinase/Erk1 activating kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>p70S6k</td>
<td>p70 S6 kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>cPKA</td>
<td>cAMP-dependent protein kinase</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>PI 3'-kinase</td>
<td>phosphatidylinositol 3'-kinase</td>
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<td>PLC-γ</td>
<td>Phospholipase C-γ</td>
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<tr>
<td>PMSF</td>
<td>phenyl-methyl-sulfonyl-fluoride</td>
</tr>
<tr>
<td>RSK</td>
<td>p90 ribosomal S6 kinase</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SH2</td>
<td>src-homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>src-homology 3</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-Tetradecanoylphorbol 13-Acetate</td>
</tr>
<tr>
<td>TTBS</td>
<td>0.5% Tween Tris Buffered Saline</td>
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CHAPTER 1

INTRODUCTION

GANGLIOSIDES

Structure

The term ganglioside was coined by Klenk (Klenk, 1942), who isolated them from bovine brain tissue. Gangliosides are defined as sialic acid containing glycosphingolipids. The structure of a ganglioside which is used in many experiments in this dissertation (GM1a, which I will refer to in subsequent chapters as GM1) is shown in Figure 1.1. The lipid portion of glycosphingolipids is called ceramide, which consists of a long chain base, most commonly sphingosine (4-sphingenine, d18:1trans) (Karlsson, 1970), to which a fatty acid is attached by an amide linkage (Figure 1.1). The hydrophilic portion consists of carbohydrate residues which are attached to the 1-OH position of the ceramide. Although a wide variety of structures exist for the neutral carbohydrate chain, the major brain gangliosides, termed the ganglio-series contain the structure: galactosyl-β-(1-3)N-acetylgalactosaminyl-β-(1-4)-galactosyl-β-(1-4)glucosyl-β-(1-1)-ceramide, or
a shortened derivative thereof (Wiegandt, 1985). The core structures of the other series are as follows: lacto - galactosyl-β-(1-3)N-acetylgalactosaminyl-β-(1-3)-galactosyl-β-(1-4)-glucosyl-β-(1-1)-ceramide; neolacto - galactosyl-β-(1-4)N-acetylgalactosaminyl-β-(1-3)-galactosyl-β-(1-4)-glucosyl-β-(1-1)-ceramide; globo - N-acetylgalactosaminyl-β-(1-3)-galactosyl-α-(1-4)-galactosyl-β-(1-4)-glucosyl-β-(1-1)-ceramide. Sialic acid residues are linked to either galactose at the 3 position or another sialic acid at the 8 position (Svennerholm, 1970).

Nomenclature

Specific gangliosides are usually named according to the system of Svennerholm (Svennerholm, 1963). In this system, for the ganglio-series, the upper case letter G stands for ganglioside. This is followed by a second upper case letter which signifies the number of sialic acid residues: M - mono; D - di; T - tri; Q - quadra; P - penta, etc. This is then followed by a number which designates the length of the core neutral carbohydrate chain by the formula 5-n. Thus, the number is inversely proportional to the length of the carbohydrate chain. This is sometimes followed by a lower case letter which designates the positions of the sialic acid residues. Figure 1.2 shows that these letters are derived from the pattern of synthesis such that they designate the number of sialic acid residues attached to the inner galactose as follows: o-series, no sialic acid; a-series, one sialic acid; b-series - two sialic acids; c-series, three sialic acids (Sandhoff and van Echten, 1993).
Expression

Klenk originally thought that gangliosides were expressed only in neurons, hence the name (Klenk, 1942). They are most concentrated in the central nervous system, however it has become clear that gangliosides are ubiquitously expressed in mammalian cells (Ledeen, 1989; Puro et al., 1969; Stults et al., 1989). It is generally thought that gangliosides are located in the outer leaflet of the plasma membrane, however there is evidence that some gangliosides exist in other subcellular locations. While a portion of the intracellular gangliosides may be due to sorting and recycling of cell surface components (Hoekstra and Kok, 1992; Sandhoff and van Echten, 1993), some may have biological roles in intracellular organelles (Gillard et al., 1993; Ledeen et al., 1988; Wu et al., 1995).

Ganglioside metabolism

Ganglioside biosynthesis begins at the cytosolic face of the endoplasmic reticulum (Mandon et al., 1992) with the condensation of serine and palmitoylCoA to form 3-dehydrosphinganine (Mandon et al., 1991; Stoffel and Bister, 1974), which is then reduced to sphinganine (Stoffel and Bister, 1974). After the amide linked fatty acid is added, the 4-trans double bond is introduced forming ceramide (Rother et al., 1992). The ceramide is transferred to the Golgi by an unknown mechanism where glycosyltransferases form first GlcCer and then LacCer on the cytosolic face of the golgi (Sandhoff and van Echten, 1993). Later additions
to form the more complex glycosphingolipids occur on the luminal surface. All the various sugars are provided by nucleotide sugar donors. The exact step at which the transfer from cytosolic to luminal topology occurs and the mechanism by which it takes place are unknown. Figure 1.2 shows a scheme for the additions of sugars to form more complex gangliosides. The glycosyltransferases responsible for these steps are located in more distal layers of the Golgi (Sandhoff and van Echten, 1993).

These glycosyltransferases appear to be rather nonspecific in regard to the ganglioside used as substrate. For example, the same GalNAc transferase can catalyze the formation of GA2 from LacCer, GM2 from GM3, GD2 from GD3 or GT2 from GT3 (Iber et al., 1992). This promiscuity appears to be the rule rather than the exception and is similar at subsequent steps in ganglioside biosynthesis (Figure 1.2) (Sandhoff and van Echten, 1993).

Gangliosides residing in the plasma membrane are slowly internalized by endocytosis. Most endocytosed gangliosides are catabolized in lysosomes, and the components are reused for synthesis of new glycosphingolipids (Schwartzman and Sandhoff, 1990). However, a small portion of the endocytosed gangliosides make their way back to the Golgi where they are glycosylated to form more complex gangliosides and are thus recycled (Sandhoff and van Echten, 1993).
Biological Functions of Gangliosides

The biological functions of gangliosides are not clearly understood. However, many studies have demonstrated effects of gangliosides that hint at a variety of possible roles. For example, gangliosides are thought to enhance neuritogenesis (Schengrund, 1990; Yates, 1986). A variety of cultured neural cells, including both primary cultured cells and established neuroblastoma and pheochromocytoma cell lines, have shown enhanced neurite outgrowth when grown in the presence of gangliosides (Cannella et al., 1988; Roisen et al., 1981; Spoerri and Roisen, 1988). Other evidence for roles in neuritogenesis, include changes in ganglioside composition during development of the nervous system (Yates, 1986), the appearance of meganeurites in neurons from individuals with gangliosidosis (Purpura and Baker, 1978; Purpura and Suzuki, 1976), and enhanced recovery from neural traumas (Schengrund, 1990; Yates, 1986).

Gangliosides have also been proposed to play important roles in cell adhesion (Hakomori and Igarashi, 1995). Possible roles include binding to sugar binding proteins (Hattori et al., 1995; Tiemeyer and Scnaar, 1989), or to other glycosphingolipids on apposing cells (Brewer and Thomas, 1984; Kojima and Hakomori, 1989), and modulation of adhesion strength by direct interaction with integrins (Zheng et al., 1993).

There is also considerable evidence that gangliosides are involved in the regulation of cellular proliferation. Changes in ganglioside composition have been noted in several conditions associated with alterations in cell
growth, such as malignant transformation (Hakomori, 1985). For example, human melanoma cells express higher levels of GM3 and GD3 than do normal melanocytes (Pukel et al., 1982; Thampoe et al., 1989). GD3 expression is also enhanced in hepatocellular carcinoma cells (Ye et al., 1990). The overall pattern of changes in ganglioside composition is usually a simplification, that is a loss of gangliosides containing more complex oligosaccharides (Hakomori, 1985; Smid et al., 1989; Yates et al., 1979). In some cases, this has been shown to be due to a decrease in enzymatic activity of a glycosyltransferase involved in ganglioside biosynthesis (Cumar et al., 1970; Keenan and Doak, 1973; Ye et al., 1990).

Several studies have examined ganglioside composition of glioma cells. Kostic and Buchheit (1970) noted that gliomas contained lower amounts of gangliosides in comparison to normal human brain tissue, and that the gliomas contained a higher proportion of the less polar gangliosides. An increase in GD3, as well as an overall simplification, was noted in glioblastomas (Traylor and Hogan, 1980). Simplification of ganglioside patterns also correlates with the histological grade of gliomas. Yates et al. (1979) showed that the simple gangliosides GM2 and GM3 made up the highest proportion of gangliosides in the most malignant gliomas. The more complex polysialogangliosides, except GD2, were consequently lower with increasing malignancy. Berra et al. (1985) demonstrated a correlation between decreased polysialylated gangliosides and an increased GD3, and increasing malignancy of astrocytomas. Loss of gangliosides of the 1b
pathway and the presence of a neolacto-series ganglioside, 6'-LM1, were found to correlate with increasing histological grade of malignancy of gliomas (Sung et al., 1994) and with patient survival (Sung et al., 1995).

Changes in ganglioside composition have also been seen with increases in cell density and with contact inhibition (Hakomori, 1970; Hakomori, 1981; Liepkalns et al., 1981). These changes usually take the form of an increase in glycolipid complexity. This response has been shown to be absent in some transformed cells (Hakomori, 1970; Kijimoto and Hakomori, 1971).

In addition to studies on ganglioside composition, much of the evidence for the effects of gangliosides on cell growth comes from studies in which exogenous gangliosides have been added to cells growing in culture. Gangliosides added in this way are slowly incorporated into the plasma membrane (Saqr et al., 1993) thus this technique has been used to artificially increase the concentration of gangliosides in the cells. Most of the studies done in this way have shown that gangliosides inhibit the growth of cultured cells. Such effects have been demonstrated for: GM3 on fibroblast growth factor (FGF)-, platelet-derived growth factor (PDGF)-, or epidermal growth factor (EGF)-stimulated proliferation of fibroblasts (Bremer and Hakomori, 1982; Bremer et al., 1984); GM1 on PDGF- but not EGF-stimulated growth of fibroblasts (Bremer et al., 1984); and sialosylparagloboside on insulin-stimulated growth (Nojiri et al., 1991).
The mechanism for inhibition of growth factor-stimulated proliferation by gangliosides may be related to effects of gangliosides on the growth factor receptors. Hakomori and colleagues showed that the same gangliosides which inhibited proliferation decreased tyrosine phosphorylation of receptors in membrane preparations (Bremer et al., 1984; Bremer et al., 1986). Such effects have only been seen in intact cells for insulin (Nojiri et al., 1991) and for EGF (Song et al., 1991). However, the effects of GM3 on EGF receptor autophosphorylation in vitro were dependent on the concentration of detergent used in the assay, such that GM3 inhibited at low detergent concentration but stimulated autophosphorylation at high detergent concentrations (Hanai et al., 1988).

In addition to inhibition of growth factor receptor tyrosine phosphorylation, gangliosides have been shown to have effects on several other protein phosphorylation systems. GT1b was shown to stimulate the activity of Ca\textsuperscript{2+}/calmodulin-dependent kinase II (Fukunaga et al., 1990), and GM1 stimulated a Ca\textsuperscript{2+}-dependent kinase in NGF treated PC12 cells (Hilbush and Levine, 1991). GT1b also activated phosphorylase \textit{b} kinase in skeletal muscle (Chan, 1989). Protein kinase C may also be modulated by gangliosides, although some reports have shown inhibition by gangliosides (Kim et al., 1986; Kreutter et al., 1987), while another reports stimulation (Momoi, 1986). GD1b affected the phosphorylation of several proteins in rat brain membrane preparations, increasing phosphorylation of several proteins and decreasing phosphorylation of several others (Bassi et al.,
Cyclic-AMP-dependent protein kinase is inhibited by gangliosides at micromolar concentrations, although cyclic nucleotide phosphodiesterase is stimulated at nanomolar concentrations (Yates et al., 1989). Although these studies hint at potential roles for gangliosides in modulation of protein phosphorylation, they have been performed on purified or partially purified kinases rather than on whole cells. Whether gangliosides have access to these kinases at the required concentrations in living cells is unknown. Thus, the biological relevance of these effects must be interpreted with caution.

Although many studies have shown gangliosides to be inhibitors of cellular proliferation, a few have shown that under the proper conditions gangliosides (Pettman et al., 1988; Saqr et al., 1995a) or the cholera toxin B subunit, used as a probe for endogenous GM1 (Spiegel and Fishman, 1987), can also stimulate growth. Swiss 3T3 cells have a bimodal response to treatment with the B subunit of cholera toxin, which binds specifically to the monosialoganglioside GM1. Growth of ras-transformed or rapidly dividing normal Swiss 3T3 cells is inhibited by the B subunit. However, DNA synthesis is stimulated by the B subunit in quiescent Swiss 3T3 cells (Spiegel and Fishman, 1987). A similar effect was seen in the human glioma cell line U-1242 MG using exogenously added gangliosides (Saqr et al., 1995a). Addition of gangliosides caused an increase of DNA synthesis in U-1242 MG glioma cells, provided that they were rendered relatively
quiescent by serum starvation, whereas serum-stimulated proliferation of U-1242 MG cells was inhibited by gangliosides. GM1 also increased proliferation of rat astrocytes in serum-free medium (Pettman et al., 1988). The mechanisms by which gangliosides stimulate cellular proliferation are entirely unknown.

PLATELET-DERIVED GROWTH FACTOR

PDGF and PDGF receptors

Platelet-derived growth factor (PDGF) is a 30 kDa dimer of two polypeptide chains (Westermark and Heldin, 1993), which is a potent mitogen for fibroblasts (Kohler and Lipton, 1974), smooth muscle cells (Ross et al., 1974), and glial cells (Westermark and Wasteson, 1976). Two types of chains exist, A and B, and the PDGF molecule can consist of either a homodimer or a heterodimer (Hammacher et al., 1988b; Hart et al., 1990) although the predominant form in platelets is PDGF-AB (Hammacher et al., 1988a). Two types of PDGF receptor, termed PDGF receptor α and β, bind PDGF on the surface of cells (Hart et al., 1988). Both receptors belong to the family of growth factor receptor tyrosine kinases (Claesson-Welsh et al., 1989; Yarden et al., 1986). As PDGF is a bivalent molecule, binding causes the dimerization of two receptor monomers (Bishayee et al., 1989; Seifert et al., 1989). Whereas the α receptor can bind either PDGF chain, the β
receptor binds only the B chain. Thus PDGF-AA will bind only to α homodimers, PDGF-AB will bind α homodimers or αβ heterodimers, and PDGF-BB will bind all three possible receptor dimers (Heldin et al., 1989; Herren et al., 1993). Dimerization of the receptor monomers correlates with, but is not dependent upon, activation of the tyrosine kinase activity of the receptor (Heldin et al., 1989). The two receptors in the dimer phosphorylate each other in trans (Kelly et al., 1991), and this is thought to be essential for activation of the receptor and subsequent transmission of its mitogenic signal (Kanakaraj et al., 1991; Ueno et al., 1991).

**PDGF and its receptors in gliomas**

There is considerable evidence that PDGF may be involved in the proliferation of gliomas (Westermark et al., 1995). The oncogene v-sis of the simian sarcoma virus encodes a homologue of the PDGF B chain (Chiu et al., 1984; Doolittle et al., 1983; Waterfield et al., 1983). Intracerebral injection of simian sarcoma virus causes the formation of gliomas in marmosets (Deinhardt, 1980). Several glioma cells have been shown to express both PDGF and its receptors (Nistér et al., 1991; Nistér et al., 1988). Although various glioma cell lines express PDGF receptor α and β, glioma tissue expressed only the PDGF receptor α in the tumor cells (Fleming et al., 1992; Hermanson et al., 1992). The PDGF receptor β is expressed in proliferating vasculature associated with the tumor. The tumor cells expressed both PDGF A and B chains but only at Grade III or IV, suggesting that an
autocrine loop may exist for the tumor cells utilizing PDGF-AA, AB or BB and PDGF receptor α and a paracrine loop could drive neovascularization by stimulation of PDGF receptor β on endothelial cells (Hermanson et al., 1992; Plate et al., 1992). Kumabe et al. (1992) found an amplified mutant PDGF receptor α in a human glioma, which lacked a portion of the extracellular domain. The adjacent normal tissue contained an amplified PDGF receptor α gene but not the deletion.

There is also evidence that PDGF may drive proliferation of glioma cells. Several glioma cell lines in culture can be driven to proliferate with PDGF (Pollack et al., 1990a; Pollack et al., 1990b). The transformed phenotype of the human glioblastoma cell line A172 could be reversed by treatment with antibodies to PDGF (Vassbotn et al., 1994). Expression of a truncated form of the PDGF receptor β in rat C6 glioma cells reduced their ability to grow in culture, form colonies in soft agar and grow as xenografts in nude mice (Strawn et al., 1994).

Signal transduction by PDGF receptors

PDGF receptors are large transmembrane proteins consisting of ligand binding and cytoplasmic signaling domains separated by a single transmembrane domain (Heldin et al., 1993; Westermark and Heldin, 1993). Both PDGF receptors contain a tyrosine kinase domain located in the intracellular portion of the molecule which is interrupted by a kinase insert domain (Claesson-Welsh et al., 1989; Yarden et al., 1986). Activation of the
receptors, upon PDGF binding and dimerization, is accompanied by phosphorylation of the receptors on several tyrosine residues which are located in the juxtamembrane, kinase insert, and the C-terminal tail regions of the cytoplasmic domain as well as in the kinase domain (Claesson-Welsh, 1994). The tyrosine phosphorylation site in the kinase domain, Tyr 857, is thought to play a role in positive regulation of kinase activity (Claesson-Welsh, 1994). Once phosphorylated, the tyrosine residues in the juxtamembrane, kinase insert and C-terminal tail region serve as binding sites for signal transduction molecules containing src-homology 2 (SH2) domains (Claesson-Welsh, 1994; Malarkey et al., 1995). SH2 domains are modular domains contained in a variety of proteins which specifically bind to phosphotyrosine residues in the context of the neighboring amino acids (Pawson, 1995). In this way the PDGF receptor serves as the nucleus for the assemblage of a large complex of signal transduction proteins. Several of the phosphorylated tyrosines on the PDGF receptor β and the proteins that bind to them have been identified (Claesson-Welsh, 1994; Malarkey et al., 1995). Figure 1.3 depicts the intracellular domain of the PDGF receptor β. Some of the known tyrosine phosphorylation sites and the proteins that bind to them are shown. These proteins either have enzymatic activity or are adapter proteins, consisting only of modular domains such as SH2 and SH3 domains, which bind to polyproline sequences (Cohen et al., 1995; Pawson, 1995). The adapter proteins link the receptor to signaling enzymes. Association in this signaling complex is thought to activate these enzymes.
which then propagate the signal through several pathways described below. Figure 1.4 diagrams these signal transduction pathways activated by the PDGF receptor. Although other pathways are also likely to be involved in PDGF signaling, these are the best understood and have been shown to be important for mediating the biological effects of PDGF.

**Phosphatidylinositol 3'-kinase**

One protein that associates with the PDGF receptor is the p85 subunit of phosphatidylinositol 3'-kinase (PI 3'-kinase). PI 3'-kinase consists of a regulatory subunit, p85, and a catalytic subunit p110, which catalyzes the phosphorylation of phosphoinositides on the 3 position (Varticovski et al., 1994). The p85 subunit becomes tyrosine phosphorylated following activation of PDGF receptors, however the role of phospho-p85 for activation of PI 3'-kinase is unclear. The details of the signaling pathway downstream of PI 3'-kinase are also unclear, however several potential effectors have been identified. These include p70 S6 kinase (p70S6k) which phosphorylates the S6 protein of 40S ribosomal subunits on serine and threonine residues. Activation of p70S6k is blocked by the PI 3'-kinase inhibitor wortmannin (Han et al., 1995; Wilson et al., 1996). The small GTP-binding protein rac (Parker, 1995), and the serine/threonine kinase Protein Kinase B/Akt (Burgering and Coffer, 1995; Franke et al., 1995) are also potential downstream effectors of PI 3'-kinase.
The role of PI 3'-kinase, and other signaling molecules, in PDGF-stimulated mitogenesis has been examined by mutating tyrosines 740, 751, 771, 1009 and 1021 of the PDGF receptor β to phenylalanines and adding testing tyrosine add-back mutations one at a time for restoration of the mitogenic response (Valius and Kazlauskas, 1993). These experiments demonstrated that restoration of the PI 3'-kinase binding sites at tyrosines 740 and 751 is sufficient to restore a mitogenic response to PDGF, suggesting that binding and/or activation of PI 3'-kinase plays an important role in PDGF-stimulated mitogenesis.

**Phospholipase C-γ**

Phospholipase C-γ (PLC-γ) also associates with activated PDGF receptors (Kashishian and Cooper, 1993). Association leads to tyrosine phosphorylation of PLC-γ and an increase in its catalytic activity (Kim et al., 1991). PLC-γ hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce inositol trisphosphate and diacylglycerol (Berridge and Irvine, 1989). These second messengers cause release of calcium from intracellular stores and the activation of protein kinase C. Tyrosine add back experiments like the one described above show that restoration of the PLC-γ binding site at Tyr 1021 to mutant PDGF receptors is also sufficient to restore PDGF-stimulated mitogenesis (Valius and Kazlauskas, 1993).
**The ras/MAP kinase pathway**

Association of the adapter protein Grb2 with receptor tyrosine kinases has been shown to lead to activation of the small GTP binding protein ras (Malarkey et al., 1995). Although Grb2 can bind directly to the PDGF receptor β it may also bind indirectly through association with Shc (Rozakis-Adcock et al., 1992) or with the tyrosine phosphatase Syp ( Bennet et al., 1994; Li et al., 1994). The relative contribution of these various methods of linking Grb2 to the PDGF receptor is not clear.

Grb2 is constitutively bound to the guanine nucleotide exchange factor sos (Rozakis-Adcock et al., 1993). By associating with signaling complexes, Grb2 brings Sos to the membrane proximal to ras (Buday and Downward, 1993; Li et al., 1993). Sos causes the conversion of ras from its inactive GDP-bound form to its active GTP-bound form (Egan et al., 1993; Gale et al., 1993). Activated ras undergoes a conformational change which allows it to associate with downstream effector molecules (Milburn et al., 1990).

Although ras may have several effectors (Khosravi-Far and Der, 1994) the best understood is a cascade of kinases leading to the activation of the mitogen-activated protein (MAP) kinases known as Erks (extracellular signal-regulated kinases) (Cobb and Goldsmith, 1995; Seger and Krebs, 1995). The serine/threonine kinase and proto-oncogene c-Raf-1, which functions as a MAP kinase kinase kinase, interacts directly with activated ras (Moodie et al., 1993; Warne et al., 1993; Zhang et al., 1993). Although this association does not directly activate c-Raf-1, it is thought to lead to its
activation by bringing Raf to the inner surface of the membrane where it is activated by a poorly understood mechanism. Targeting c-Raf-1 to the membrane by engineering a CAAX box, which is required for farnesylation and a polybasic domain, leads to Raf activation without the need for active ras, suggesting that membrane localization is sufficient to activate Raf (Leevers et al., 1994). c-Raf-1 then phosphorylates and activates MAP kinase kinase (Dent et al., 1992; Kyriakis et al., 1992). MAP kinase kinase is a dual-specificity kinase which activates the MAP kinases Erk1 and Erk2 by phosphorylation on threonine and tyrosine residues (Crews and Erikson, 1992; Rossomando et al., 1992a).

Activated MAP kinases can phosphorylate a wide variety of substrates, some of which are located in the cytosol and some in the nucleus (Robbins et al., 1994). Cytosolic substrates include phospholipase A2, p90 ribosomal S6 kinase (RSK), and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase 2) (Davis, 1993). They can also phosphorylate cytoskeletal proteins such as microtubule-associated proteins (Ray and Sturgill, 1987) and this can affect microtubule polymerization/depolymerization (Gotoh et al., 1991). In addition Erks translocate to the nucleus when activated (Gonzalez et al., 1993; Lenormand et al., 1993) where they phosphorylate the transcription factor p62TCF/Elk-1 and thus lead to increased transcription from the serum response element (Marais et al., 1993).
Although many potential Erk substrates have been identified it is not understood how phosphorylation of these substrates leads to the ultimate biological effects. However, several lines of evidence suggest that Erks are essential for mitogenic stimulation. Preventing Erk activation using either antisense RNA or transfection of a kinase-inactive dominant negative mutant inhibits fibroblast proliferation (Pagès et al., 1993). PDGF-stimulated DNA synthesis in Swiss 3T3 cells can be blocked by treatment with the pharmacological inhibitor of MAP kinase kinase, PD98059 (Dudley et al., 1995). In addition, transfection of NIH 3T3 cells with a constitutively active mutant of MAP kinase kinase is sufficient to lead to transformation (Cowley et al., 1994; Mansour et al., 1994).

STATEMENT OF THE PROBLEM

There is considerable evidence that gangliosides are involved in the regulation of cell growth, such as the changes in ganglioside composition of cells in conditions associated with cell growth and the effects of gangliosides on cells growing in culture. Specifically, gangliosides may have effects on the proliferation of glioma cells that are important for the maintenance of their transformed phenotype as suggested by several lines of evidence: 1) Gangliosides are highly expressed in the central nervous system. 2) Changes in ganglioside composition of glioma cells correlate with
malignancy and patient survival. 3) Gangliosides inhibit the PDGF-stimulated proliferation of cells in culture. They also can inhibit tyrosine phosphorylation of PDGF receptors \textit{in vitro}. Since there is considerable evidence that PDGF is involved in the proliferation of glioma cells it is reasonable to suggest that gangliosides could modulate the proliferation of glioma cells by affecting PDGF-stimulated signal transduction. 4) Gangliosides inhibit serum-stimulated proliferation of the glioma cell line U-1242 MG. 5) Gangliosides can stimulate the proliferation of U-1242 MG cells when the cells are serum starved.

These lines of evidence, together with the various other effects that gangliosides have been shown to cause on protein phosphorylation, indicate that gangliosides may affect cellular proliferation through a variety of mechanisms. However, caution must be exercised in interpreting these data. The details of how gangliosides affect proliferation are entirely unknown. The majority of the studies of the modulation of signal transduction systems by gangliosides discussed above were performed entirely \textit{in vitro}. It is unclear whether gangliosides would have access to these molecules in whole, living cells. As a result, the effects seen on specific enzymes can not be correlated with any biological phenomena.

We formed the general \textbf{hypothesis} that gangliosides affect cellular proliferation by modulating signal transduction pathways involving protein phosphorylation. Specifically, we hypothesized that gangliosides inhibited PDGF-stimulated proliferation by inhibiting PDGF receptor tyrosine kinase
activation. In addition, we hypothesized that the stimulation of DNA synthesis in U-1242 MG cells by gangliosides, in the absence of added growth factors or serum, was mediated by activation of mitogenic protein phosphorylation cascades. To test this hypothesis it was therefore necessary to address the following questions:

1) Do gangliosides inhibit PDGF-stimulated PDGF receptor activation in whole cells?

2) If so by what mechanism?

3) What is the role of protein phosphorylation cascades on the proliferation of U-1242 MG glioma cells?

4) Do gangliosides stimulate mitogenic phosphorylation cascades in U-1242 MG glioma cells?

To address these questions the following strategies were used:

1) PDGF receptor tyrosine phosphorylation was examined in Swiss 3T3 and U-1242 MG cells which had been incubated with exogenous gangliosides. Swiss 3T3 cells were used because they have many PDGF receptors, respond to PDGF with a strong mitogenic response and their proliferation can be inhibited by exogenous gangliosides.

2) PDGF receptor dimerization was examined under similar conditions as above by use of a chemical crosslinker.

3) Stimulation of the MAP kinase pathway by known mitogens and its involvement in mitogenesis was studied in U-1242 MG cells. This pathway was chosen because it has been extensively studied and is thought to be
crucial for mitogenic stimulation, however little work has been done on MAP kinases in glioma cells.

4) The stimulation of the MAP kinase pathway by gangliosides was examined in U-1242 MG cells under conditions in which gangliosides stimulated DNA synthesis. Several other enzymes which have been shown to be involved in mitogenic signal transduction were also examined.

Figure 1.5 lists several signal transduction molecules which will be discussed in this dissertation. Comments are included to provide a general concept of the enzymes' functions. Those whose roles in ganglioside modulation of signal transduction are more extensively examined are discussed in some detail above. Others, such as JNK, B-Raf, and FRAP, which are not mentioned above, are discussed in subsequent chapters.

![Figure 1.1. Structure of the monosialoganglioside GM1a.](image)
Figure 1.2. Scheme for ganglioside biosynthesis. The carbohydrates are donated by sugar-nucleotides which are omitted from the diagram for clarity (Sandhoff and van Echten, 1993).
Figure 1.3. Diagram of the intracellular domain of the PDGF receptor β showing phosphorylated tyrosines and several signal transduction proteins which bind to them (Claesson-Welsh, 1994; Malarkey et al., 1995).

Figure 1.4. Signal transduction pathways activated by the PDGF receptor.
<table>
<thead>
<tr>
<th>enzyme</th>
<th>full name</th>
<th>other names</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
<td>Erk</td>
<td>phosphorylates nuclear and cytosolic substrates may be critical for cell growth</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP kinase or ERK kinase</td>
<td>MAP kinase kinase</td>
<td>phosphorylates and activates MAP kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
<td>Stress-Activated Protein (SAP) Kinase</td>
<td>phosphorylates and activates c-Jun protein activated by many stresses</td>
</tr>
<tr>
<td>c-Raf-1</td>
<td>MAP kinase kinase kinase</td>
<td></td>
<td>proto-oncogene / interacts with ras phosphorylates and activates MEK</td>
</tr>
<tr>
<td>B-Raf</td>
<td>MAP kinase kinase kinase</td>
<td></td>
<td>Raf isoform expressed in neural tissue phosphorylates and activates MEK</td>
</tr>
<tr>
<td>PI 3'-kinase</td>
<td>Phosphatidylinositol 3'-kinase</td>
<td></td>
<td>phosphorylates phosphoinositides on 3' position may be critical for cell growth</td>
</tr>
<tr>
<td>p70^{66k}</td>
<td>p70 S6 kinase</td>
<td></td>
<td>phosphorylates ribosomal protein S6 increases translation of certain mRNAs</td>
</tr>
<tr>
<td>FRAP</td>
<td>FKBP-rapamycin-associated protein</td>
<td>TOR</td>
<td>inhibited by rapamycin</td>
</tr>
</tbody>
</table>

Figure 1.5. Summary of signal transduction molecules discussed.
Comments are not meant to be complete descriptions of biological roles, but to provide a general concept of the enzymes' functions.
CHAPTER 2

INHIBITION OF PLATELET-DERIVED GROWTH FACTOR RECEPTOR ACTIVATION BY GANGLIOSIDES

ABSTRACT

Exogenously added gangliosides inhibit DNA synthesis in Swiss 3T3 cells stimulated with platelet-derived growth factor (PDGF). Antiphosphotyrosine Western blots showed that this correlates with the inhibitory effects of several gangliosides (except GM3) on tyrosine phosphorylation of the PDGF receptor. \(^{35}\text{S}\)methionine-labeled Swiss 3T3 cells were incubated either with or without gangliosides, stimulated with PDGF, and proteins crosslinked with bis (sulfosuccinimidyl) suberate (BS3). SDS-PAGE revealed that two protein bands (170 kDa and 350 kDa) were specifically immunoprecipitated with an anti-PDGF receptor antibody. Using both Swiss 3T3 and human glioma U-1242 MG cells, Western blots with anti-PDGF receptor and anti-phosphotyrosine antibodies confirmed
that these bands were the PDGF receptor monomer and dimer respectively, and that phosphotyrosine was present in these bands only after cells were stimulated with PDGF. Of the gangliosides tested GM1, GM2, GD1a, GD1b, GD3 and GT1b, but not GM3, inhibited the formation of the 350 kDa band. These results demonstrate that all gangliosides tested, except GM3, probably inhibit PDGF mediated growth by preventing dimerization and tyrosine phosphorylation of PDGF receptor monomers. Loss of more complex gangliosides in human gliomas would permit unregulated activation of PDGF receptors contributing to uncontrolled growth stimulation. We propose that ganglioside inhibition of receptor dimerization is a novel mechanism for regulating and coordinating several trophic factor mediated cell functions.

INTRODUCTION

Gangliosides are sialic acid containing glycosphingolipids which are found in most vertebrate cells, but are particularly enriched in the central nervous system. Changes in ganglioside composition occur with growth, development and regeneration, and exogenous gangliosides have many biological effects including enhancement of neuritogenesis, improved recovery from some forms of neural trauma (Schengrund, 1990; Yates, 1986),
and growth inhibition of cultured cells (Hakomori, 1984; Igarashi et al., 1989).

The mechanisms through which gangliosides exert these varied effects are not clear, however elucidation of these is important for understanding their biological roles. Recent studies have focused on the effects of sphingolipids and their metabolites following internalization (Bielawska et al., 1992; Igarashi et al., 1989; Kolesnick and Hemer, 1990) on protein phosphorylation systems including protein kinase C (Kim et al., 1986; Kreutter et al., 1987; Miyagi et al., 1988; Momoi, 1986), calcium-calmodulin-dependent protein kinase (Fukunaga et al., 1990), cAMP-dependent protein kinase (Miyagi et al., 1988; Yates et al., 1989), cyclic nucleotide phosphodiesterase (Yates et al., 1989), and calcineurin (Bassi et al., 1991; Yates et al., 1988). There is also evidence that gangliosides exert effects on receptors for growth factors, i.e. epidermal growth factor (Bremer et al., 1986), fibroblast growth factor (Bremer and Hakomori, 1982), platelet-derived growth factor (PDGF) (Bremer et al., 1984), and insulin (Nojiri et al., 1991). Previous evidence has shown that gangliosides can inhibit tyrosine phosphorylation of the PDGF receptor in response to PDGF stimulation (Bremer et al., 1984), but the mechanism for this has been unknown. It has been suggested that a similar effect of gangliosides on the EGF receptor might involve inhibition of receptor dimerization (Hakomori, 1990).

While the above mentioned studies provide important clues to how gangliosides may be regulating signal transduction enzymes, their
interpretation is limited since they were performed in vitro using purified enzymes or membrane preparations. It is therefore important to demonstrate that gangliosides have similar effects in whole cells to support the hypothesis that these modulatory events are biologically relevant. In the studies described in this chapter the effects of gangliosides on PDGF-stimulated tyrosine phosphorylation and dimerization of PDGF receptor monomers in whole Swiss 3T3 and U-1242 MG glioma cells were investigated. These processes are thought to be important for activation of the PDGF receptor tyrosine kinase and transduction of the PDGF signal (Heldin et al., 1989; Ueno et al., 1991).

MATERIALS AND METHODS

Materials

Gangliosides were obtained from the following sources: GM1-Fidia Pharmaceuticals (Abano Terme, Italy); GM2 and GD1a-Matreya (Pleasant Gap, PA); GM3 and GT1b-Sigma Chemical Co. (St. Louis, MO). Recombinant human PDGF-BB (PDGF) was from R&D Systems (Minneapolis, MN). Monoclonal anti-phosphotyrosine antibody 4G10 and anti-PDGF receptor β antiserum were from Upstate Biotechnology Inc. (Lake Placid, NY). Bis (sulfosuccinimidyl) suberate (BS3), normal rabbit serum and protein A agarose beads were from Pierce (Rockford, IL). [35S]methionine was from American Radiolabeled Chemicals (St. Louis,
MO). Entensify autoradiography enhancer was from (New England Nuclear, Boston, MA). Goat anti-mouse or goat anti-rabbit IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolylphosphate/p-nitroblue tetrazolium chloride (BCIP/NBT), and nitrocellulose membranes were from BioRad (Hercules, CA).

**Ganglioside Preparation**

Gangliosides were dissolved in chloroform/methanol (2/1) and quantitated by the resorcinol method (Svennerholm, 1957). Gangliosides were prepared for experiments by evaporating the chloroform/methanol under nitrogen. Gangliosides were dissolved in serum-free culture medium by incubation at 37°C for one hour, during which the gangliosides were vortexed and sonicated for 15 seconds at 20 minute intervals.

**Cell Culture**

Swiss 3T3 cells were maintained in culture in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum (Gibco, Grand Island, NY). They were routinely passed every five days by removal with trypsin and dilution into fresh flasks at a ratio of 1:20. U-1242 MG cells were cultured as above except that cells were grown in Minimum Essential Medium (MEM) containing 10% calf serum, essential and non-essential amino acids, and vitamins (GIBCO). All cells were cultured at 37°C in a humidified environment in 5% CO₂.
Western Blot Analysis

Swiss 3T3 or U-1242 MG cells were seeded at 13,000 cells/cm² or 10,000 cells/cm² respectively. Cells were incubated 48 hours and the medium was changed to fresh medium. Swiss 3T3 cells were incubated 5 additional days until confluent and quiescent. U-1242 MG cells were incubated 3 additional days and then washed twice with phosphate-buffered saline (PBS) and incubated one day more in serum-free MEM containing vitamins and amino acids. For both cell lines the medium was then replaced with serum-free medium containing gangliosides and the cells were incubated for the indicated time. Cells were then stimulated by the addition of PDGF-BB for 10 minutes at 37°C or one hour at 4°C. The plates were washed once with cold PBS and the cells lysed in 1% SDS, containing 5 mM EDTA, 5 mM EGTA and 1 mM Na₃VO₄. The lysates were boiled 5 minutes.

Cell lysates were separated by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) on 7-15% gradient gels (for PDGF receptor monomers) or 3-10% gradient gels (for PDGF receptor dimers) and transferred to nitrocellulose (BioRad, Richmond, CA). Membranes were blocked with 3% gelatin in Tris Buffered Saline (TBS) for 30 minutes. They were then washed with 0.5% Tween Tris Buffered Saline (TTBS) and probed with anti-PDGF receptor β anti-serum at 1:100 dilution or a monoclonal anti-phosphotyrosine antibody at 1 µg/ml in TTBS with 1% gelatin overnight. Membranes were washed twice with TTBS and incubated 2 hours
with goat anti-mouse or goat anti-rabbit IgG alkaline phosphatase conjugate (BioRad) at 1:3000 in TTBS with 1% gelatin. They were then washed twice with TTBS and once with TBS and developed with 5-bromo-4-chloro-3-indolylyphosphate/p-nitroblue tetrazolium chloride (BCIP/NBT).

**Immunoprecipitation**

Cells were lysed in 1% NP-40 and lysates were incubated overnight at 4°C with 50 µl protein A-conjugated beads preloaded with either anti-PDGF receptor antibody or normal rabbit serum (Pierce, Rockford, IL). The beads were pelleted, washed three times with lysis buffer, and boiled 5 min in treatment buffer. The supernatant was then used for Western blot analysis.

**PDGF Receptor Dimer Crosslinking**

Swiss 3T3 cells were washed twice with DMEM without methionine. Methionine-free DMEM with 100 µCi/ml [³⁵S]methionine was added and the cells were incubated overnight. The medium was removed, DMEM either with or without gangliosides was added and the cells incubated 2 hours at 37°C. The cells were stimulated with 50 ng/ml PDGF-BB for 1 hour at 4°C to prevent internalization of the receptors. The medium was then removed and proteins were crosslinked using 8.7 mM bis (sulfo succinimidyl) suberate (BS3) in 25 mM Hepes (pH 8.5), 120 mM NaCl, 6 mM KCl, 1 mM MgCl₂, and 10 mM EGTA. The reaction was terminated by the addition of ethanolamine to a final concentration of 1%. Cells were then lysed by the
addition of 1% NP-40 in 25 mM Tris (pH 7.4), 150 mM NaCl, 1.5 mM EGTA and 1 mM Na$_3$VO$_4$ (lysis buffer). Cell lysates were then centrifuged at 20,000 $g$ for five minutes at 4°C. Lysates were precleared twice (Harlow and Lane, 1988) using Immunoprecipitin (Gibco) and once with normal rabbit serum and Immunoprecipitin for 1 hour at 4°C, then immunoprecipitated overnight at 4°C with anti-PDGF receptor antiserum bound to protein A agarose beads. Beads were washed three times with lysis buffer and then boiled 5 minutes in reducing sample buffer. Proteins were separated by SDS-PAGE on 3-10% gradient gels. The gels were prepared for fluorography using Entensify according to the manufacturers instructions and exposed to Kodak X-OMAT film for 24 hours.

Alternatively, the crosslinking experiment was performed on unlabeled cells. In these experiments whole cell lysates were analyzed by western blot analysis using antibodies to the PDGF receptor $\beta$ or phosphotyrosine as described above.

RESULTS

PDGF-Stimulated PDGF Receptor Tyrosine Phosphorylation

To determine the optimal time of PDGF treatment confluent U-1242 MG cells were treated with 100 ng/ml PDGF for various times ranging from 1 min to 2 hours. The cells were then lysed and equal amounts of protein
separated by SDS-PAGE. The proteins were then transferred to nitrocellulose membranes which were probed with antibodies to phosphotyrosine. As shown in Figure 2.1A, one minute after addition of PDGF tyrosine phosphorylation of a protein migrating at 170 kDa was evident. The intensity of this band peaked at 2.5 minutes and declined thereafter. By 40 minutes tyrosine phosphorylation of the 170 kDa protein was no longer detectable.

To determine the dose of PDGF required for maximal tyrosine phosphorylation of the 170 kDa protein, confluent U-1242 MG cells were treated with PDGF for 2.5 min at various concentrations ranging from 10-100 ng/ml. Tyrosine phosphorylation of the 170 kDa protein was then determined as above. Figure 2.1B shows that tyrosine phosphorylation of a 170 kDa band was seen when cells were treated with as little as 20 ng/ml PDGF. Maximal tyrosine phosphorylation of this protein was seen when cells were treated with 50 ng/ml PDGF. Therefore, for subsequent experiments U-1242 MG cells were stimulated with 50 ng/ml PDGF for 2.5 minutes.

Swiss 3T3 fibroblasts are known to express a high level of PDGF receptors (approximately 300,000/cell) (Bowen-Pope and Ross, 1982). A 170 kDa protein was also tyrosine phosphorylated when Swiss 3T3 cells were treated with 50 ng/ml PDGF for 2.5 min. The PDGF receptor β is known to migrate at approximately 170 kDa on SDS-PAGE. To determine whether the 170 kDa protein was the PDGF receptor β immunoprecipitation was
performed using an antiserum specific for PDGF receptor β on lysates from unstimulated and PDGF-stimulated Swiss 3T3 cells. The immunoprecipitates as well as whole cell lysates were separated by SDS-PAGE and Western blotting was performed with anti-phosphotyrosine antibodies as above. Figure 2.2A shows that a 170 kDa protein was tyrosine phosphorylated in lysates from PDGF-stimulated but not unstimulated cells (lanes 4 & 1, respectively). The 170 kDa protein was also present in anti-PDGF receptor immunoprecipitates (lane 6) but not in control normal rabbit serum immunoprecipitates (lane 5) from PDGF-stimulated cells confirming that the 170 kDa tyrosine phosphorylated protein is the PDGF receptor β.

Effect of Gangliosides on PDGF Receptor Tyrosine Phosphorylation

Prolonged incubation of cells with gangliosides added to cell culture medium leads to the gradual incorporation of the gangliosides into the plasma membranes of the cells (Saqr et al., 1993). Addition of gangliosides in this way has been shown to decrease the proliferation of Swiss 3T3 cells (Bremer et al., 1986; Yates et al., 1993). To determine if exogenously added gangliosides could inhibit PDGF-stimulated PDGF receptor tyrosine phosphorylation in cultured cells, Swiss 3T3 cells were incubated with the 100 μM GM1, GM2, GM3, GD1a or GT1b for 2 hours prior to stimulation with PDGF. Tyrosine phosphorylation of PDGF receptors was assessed as above and the intensity of the 170 kDa PDGF receptor band was quantitated by scanning densitometry. Figure 2.2B shows that pretreatment with all of
the gangliosides tested except GM3 decreased PDGF-stimulated tyrosine phosphorylation of PDGF receptors. The results were quantitated by scanning densitometry and are shown in Figure 2.2B. The order of potency of the gangliosides tested was: GT1b>GD1a>GM1>GM2; GM3 - no effect. Although in some experiments PDGF receptor tyrosine phosphorylation was slightly lower in GM3-treated cells, in others it appeared to be slightly greater (Figure 2.2B). Over three separate experiments no significant change was seen in GM3-treated cells compared to control (Figure 1.2C).

The effects of gangliosides on PDGF receptor tyrosine phosphorylation in U-1242 MG glioma cells was also examined. Confluent U-1242 MG cells were treated with 100 μM GM1, GM2, GM3, GD1a, GD1b, GD3 or GT1b for 2 hours. Figure 2.3A shows that the inhibition of PDGF-stimulated PDGF receptor tyrosine phosphorylation by gangliosides was similar to that seen in Swiss 3T3 cells. GD1b and GD3 were also effective.

This inhibition was dose-responsive as shown in Figure 2.3B, for the gangliosides GM1 and GM2. U-1242 MG cells were pretreated as above with 25, 50 or 100 μM GM1, GM2 or GM3, and then stimulated with PDGF. GM1 and GM2 caused a similar reduction in tyrosine phosphorylation of the 170 kDa protein at 100 μM, but GM1 was more effective at lower concentrations. GM3 caused only a slight reduction in PDGF-stimulated tyrosine phosphorylation even at 100 μM.
PDGF-Stimulated Receptor Dimerization

To demonstrate the dimerization of PDGF receptors in PDGF-stimulated cells, $[^{35}S]$methionine labeled Swiss 3T3 cells were stimulated with PDGF-BB and proteins were subsequently crosslinked with BS3. Autoradiography of SDS-PAGE gels revealed bands at 170 kDa and 350 kDa (Figure 2.4, lane 4) which were specifically immunoprecipitated using anti-PDGFβ receptor antiserum (anti-PDGF receptor). Immunoprecipitates from cells which were treated with PDGF-BB or BS3 alone contained only the 170 kDa band, indicating that these bands were the PDGFβ receptor monomer and dimer, respectively (Figure 2.4, lanes 1-3).

To confirm this we performed immunoblots of whole cell lysates using either the anti-PDGF receptor or monoclonal anti-phosphotyrosine antibody. The blot probed with anti-PDGF receptor revealed bands at 170 kDa and 350 kDa identical to those seen on autoradiography of PDGF receptor immunoprecipitates (Figure 2.5A, lanes 1-4). Furthermore, the blot stained with anti-phosphotyrosine had a band at 170 kDa which contained phosphotyrosine when the cells were stimulated with PDGF-BB and a band at 350 kDa when proteins from PDGF-stimulated cells were crosslinked with BS3 (Figure 2.5B, lanes 1-4). The same results were seen when this experiment was performed using U-1242 MG glioma cells (Figure 2.6).
Effect of Gangliosides on PDGF Receptor Dimerization

Preincubation of Swiss 3T3 cells with 100 μM GM1, GM2, GD1a, GD1b, GD3 or GT1b for two hours at 37°C abolished the 350 kDa band seen on autoradiography in immunoprecipitates from cells treated with PDGF and BS3. GM3 was the only ganglioside tested which was without effect (Figure 2.4). Western blots of whole cell lysates also showed that GM1 abolished the 350 kDa band in a dose-responsive fashion (Figure 2.5A) and that PDGF receptor monomers from the cells treated with 50 or 100 μM GM1 did not contain phosphotyrosine (Figure 2.5B). GM3 had no effect on either formation of the 350 kDa band or tyrosine phosphorylation of receptor monomers. GM1 alone, or in combination with either PDGF or BS3, did not cause formation of receptor dimers or decreased intensity of receptor monomer bands visualized with anti-PDGF receptor (not shown).

The same effects were observed in U-1242 MG glioma cells (Figure 2.6). Preincubation with 100 μM GM1, GM2, GD1a or GT1b abolished the 350 kDa band seen on Western blots probed with anti-PDGF receptor. Western blots probed with anti-phosphotyrosine demonstrated that GM1, GM2, GD1a and GT1b prevented tyrosine phosphorylation of PDGF receptor monomers in U-1242 MG cells. GM3, at 100 μM, had only a partial inhibitory effect. These results demonstrate that the same gangliosides prevent both tyrosine phosphorylation of PDGF receptors, and dimerization of receptor monomers, and that GM3 has only a slight effect on either process in U-1242 MG cells.
DISCUSSION

Bremer et al. (Bremer et al., 1984) showed that GM1 inhibited the tyrosine phosphorylation of partially purified PDGF receptors and that GM3 inhibited tyrosine phosphorylation of the EGF receptor in similar membrane preparations (Bremer et al., 1986). However, these effects were dependent on the detergent concentration present in the assay (Hanai et al., 1988). The purpose of the experiments described in this chapter was to determine whether gangliosides had a similar effect on PDGF receptors in whole cells, thus supporting the hypothesis that inhibition of growth factor receptor activation is the mechanism by which gangliosides inhibit the proliferation of cells in culture.

The results clearly show that PDGF treatment of both Swiss 3T3 and U-1242 MG cells leads to tyrosine phosphorylation of a 170 kDa protein identified as the PDGF receptor β. This phosphorylation was inhibited by incubation with all the gangliosides tested except GM3. Inhibition of PDGF-stimulated PDGF receptor phosphorylation was not due to a decrease in PDGF binding to its receptor as determined by [125I]PDGF competitive binding experiments (Yates et al., 1993).

All five of the gangliosides tested, including GM3, were able to prevent PDGF-stimulated DNA synthesis in Swiss 3T3 cells (Yates et al., 1993), whereas GM3 did not prevent PDGF receptor tyrosine
phosphorylation. Therefore, although GM1, GM2, GD1a and GT1b may be decreasing PDGF-stimulated Swiss 3T3 cell growth at least partially through inhibition of PDGF receptor tyrosine phosphorylation, a different mechanism may account for inhibition of DNA synthesis by GM3. Interestingly, GM3 does decrease both the percentage of cells responding to PDGF with increased \([\text{Ca}^{2+}]_i\) and the level of \([\text{Ca}^{2+}]_i\) in those cells which do respond, although to a lesser degree than GM1, GM2 or GT1b (Yates et al., 1993).

Results presented here also show that stimulation of Swiss 3T3 cells with PDGF causes the formation of a 350 kDa protein that becomes tyrosine phosphorylated. The 350 kDa protein is specifically immunoprecipitated by antibodies to PDGF receptor \(\beta\). A similar size band is also immunoblotted with this antiserum on Western blots of whole cell lysates from PDGF-stimulated, crosslinked cells. Therefore, it is interpreted as being a dimer of the PDGF receptor. Preincubation with several gangliosides, but not GM3, abolished the 350 kDa band and prevented phosphorylation of the monomer at 170 kDa. Furthermore, GM1 inhibited both the dimerization and tyrosine phosphorylation in a dose-responsive fashion over the same concentration range (10-100 \(\mu\)M) as was previously found to inhibit Swiss 3T3 cell growth, PDGF receptor phosphorylation (Bremer et al., 1984; Yates et al., 1993) and increases in intracellular calcium (Guan et al., 1992; Yates et al., 1993). These combined results demonstrate that gangliosides inhibit PDGF-stimulated tyrosine phosphorylation of PDGF receptors by preventing
dimerization of receptor monomers. The same gangliosides that inhibit both PDGF-stimulated phosphorylation of the PDGF receptor and DNA synthesis in Swiss 3T3 cells prevent receptor dimerization. However, the moderate inhibitory effect of GM3 on PDGF-stimulated DNA synthesis does not appear to involve inhibition of either PDGF receptor dimerization or phosphorylation of the receptor on tyrosine.

Nistér et al. (Nistér et al., 1991) found that several different glioma cell lines, including U-1242 MG, expressed both PDGF and its receptors. On the basis of this, they suggested that autocrine stimulation by PDGF may be involved in the uncontrolled proliferation of gliomas. The ganglioside composition of human gliomas is different than normal neural tissues suggesting that gangliosides may play a role in the pathogenesis of gliomas (Yates et al., 1979). Our hypothesis was that the loss of growth inhibitory gangliosides contributes to uncontrolled stimulation of glioma cell division by mitogenic factors such as PDGF. It was therefore important to verify that the same inhibitory effects of gangliosides on PDGF receptor phosphorylation and dimerization seen in Swiss 3T3 cells occurred in glioma cells. This was tested by studying the effects of specific gangliosides on dimerization of the PDGF receptor in U-1242 MG cells. As with Swiss 3T3 cells, GM1 and GT1b prevented dimerization and phosphorylation of PDGF receptor monomers. GM3 had a slight effect on both tyrosine phosphorylation and dimerization in U-1242 MG cells. The reason that a slight inhibitory effect of GM3 is seen in U-1242 MG cells but not in Swiss
3T3 cells is not clear, but it may be related amount of PDGF receptor expressed, as Swiss 3T3 cells are known to express large numbers of PDGF receptors (Bowen-Pope and Ross, 1982).

There is a correlation between the loss of complex gangliosides and histological grade of malignancy in gliomas (Berra et al., 1985; Sung et al., 1994; Sung et al., 1995), and those gangliosides lost are the same ones that inhibit dimerization and activation of the PDGF receptor. GM3 is one of the most abundant ganglioside in high grade gliomas and it has the least effect on PDGF receptor dimerization. This raises the interesting possibility that the shift in ganglioside composition during malignant progression of gliomas may relate to both PDGF receptor signal transduction and uncontrolled growth.

We previously suggested that, as with the PDGF receptor, different gangliosides could affect a variety of molecular processes mediated by different trophic factors to variable degrees (Yates et al., 1993). As an extension of this, we developed the hypothesis that alterations in cellular ganglioside composition could be a method of regulating and coordinating diverse cellular functions involving trophic factor-receptor interactions. Changes in the activities of a few select enzymes responsible for metabolizing gangliosides could thus lead to specific effects on ganglioside-sensitive signal transduction pathways, as demonstrated here for the PDGF receptor. Receptor dimerization is a common mechanism of activation for many trophic factor receptors. Therefore, we propose: first, that inhibition of
receptor dimerization by gangliosides may be a major, novel mechanism through which a variety of cellular functions are regulated and coordinated; and second, that alterations in ganglioside composition will lead to aberrant signal transduction through ganglioside regulated receptors as demonstrated here for a human glioma cell line.
Figure 2.1. Time and concentration-dependence of PDGF-stimulated tyrosine phosphorylation. Confluent U-1242 MG cells were stimulated with (A) 100 ng/ml PDGF-BB for the indicated time or (B) the indicated concentration of PDGF-BB for 2.5 min. Western analysis was performed on cell lysates using a monoclonal antibody to phosphotyrosine (4G10). Arrows indicate position of 170 kDa band.
Figure 2.2. Effects of gangliosides on PDGF-stimulated tyrosine phosphorylation of 170 kDa protein in Swiss 3T3 cells. Panels A and B are antiphosphotyrosine western blots from Swiss 3T3 cells treated with or without 50 ng/ml PDGF-BB for 2.5 min. (A) Lanes 4, 5 and 6 were stimulated with PDGF but Lanes 1, 2 and 3 were not. Lanes 1 and 4 are whole cell lysates, 3 and 6 were immunoprecipitated with anti-PDGF receptor β, and 2 and 5 with normal rabbit serum. The arrow indicates position of the 170 kDa band. (B) All lanes except the first were stimulated with PDGF. The second lane was not pretreated with gangliosides. The remaining lanes were pretreated with the indicated ganglioside at 100 μM for 2 hours. The arrow indicates position of the 170 kDa band. The - indicates no gangliosides added. (C) The graph shows the means plus and minus standard deviations as determined by scanning densitometry for three separate experiments.
Figure 2.3. Effects of gangliosides on PDGF-stimulated tyrosine phosphorylation of 170 kDa protein in U-1242 MG cells. Antiphosphotyrosine western blots from U-1242 MG cells treated with or without 50 ng/ml PDGF-BB for 2.5 min. Cells were pretreated with (A) the indicated ganglioside at 100 μM or (B) the indicated concentration of GM1, GM2 or GM3 for 2 hours at 37°C. The - indicates no gangliosides added.
Figure 2.4. Inhibition of PDGF receptor
dimerization by gangliosides in Swiss 3T3 cells.

Lysates from $^{[35S]}$methionine labeled Swiss 3T3 cells were immunoprecipitated with anti-PDGF receptor and autoradiography was performed. All lanes except 1 and 3 were stimulated with PDGF. All lanes except 1 and 2 were crosslinked with BS3. Lanes 5 to 11 were pretreated with 100 μM of various gangliosides as follows: 5-GM1, 6-GM2, 7-GM3, 8-GD1a, 9-GD1b, 10-GD3, 11-GT1b. The sample in Lane 12 was immunoprecipitated with normal rabbit serum.
Figure 2.5. Dose-response effect of GM1 on PDGF receptor dimerization in Swiss 3T3 cells. Both panels are western blots of whole cell lysates probed with (A) anti-PDGF receptor and (B) anti-phosphotyrosine. All lanes except 1 and 3 were stimulated with PDGF. All lanes except 1 and 2 were crosslinked with BS3. Lanes 5-10 were pretreated with gangliosides as follows: 5-1 µM GM1, 6-10 µM GM1, 7-25 µM GM1, 8-50 µM GM1, 9-100 µM GM1, 10-100 µM GM3.
Figure 2.6. Effect of gangliosides on PDGF receptor dimerization in U-1242 MG glioma cells

Both panels are western blots of whole cell lysates probed with (A) anti-PDGF receptor and (B) anti-phosphotyrosine. All lanes except 1 and 3 were stimulated with PDGF. All lanes except 1 and 2 were crosslinked with BS3. Lanes 5-9 were pretreated with gangliosides as follows: 5-100 μM GM1, 6-100 μM GM2, 7-100 μM GM3, 8-100μM GD1a, 9-100μM GT1b.
CHAPTER 3

THE ROLE OF THE MAP KINASE ERK2 IN BASAL AND MITOGEN-STIMULATED PROLIFERATION OF U-1242 MG CELLS

ABSTRACT

MAP kinases are essential components of a signal transduction cascade used by growth factor receptors to trigger proliferative responses. Although glioma progression correlates with overexpression of growth factor receptors and the presence of autocrine growth factor loops, MAP kinases have not been studied in human glioma cells for their potential role in proliferation. Using gel shift, in-gel and immune complex kinase assays we have identified the MAP kinase isoforms Erk1 and Erk2 in U-1242 MG glioma cells. Erk2 was activated no more than three-fold by mitogen-treatment, which was considerably less than that found in Swiss 3T3 cells when stimulated with EGF, PDGF, TPA or serum. In a comparison of confluent and subconfluent U-1242 MG cells stimulated with EGF, PDGF,
TPA or serum, there was more effective activation of MAP kinase in subconfluent than in confluent cells. Thus activation of MAP kinase in U-1242 MG cells is affected by growth conditions. In subconfluent U-1242 MG cells, only serum caused a substantial increase in $[^3]$H]thymidine incorporation, although significant Erk2 activation was observed with all mitogens tested. These results indicate that Erk2 activation is insufficient to stimulate DNA synthesis in these cells. However, an inhibitor of MAP kinase activation lowered $[^3]$H]thymidine incorporation suggesting that Erk2 may be important for U-1242 MG proliferation.

INTRODUCTION

Intracellular signal transduction pathways invariably involve protein phosphorylation cascades which link receptor-associated events to downstream effector molecules (Karin, 1992; Nigg, 1990; Pawson, 1993; Wang, 1992). Such cascades are essential for cell proliferation with many converging on a class of closely related serine/threonine protein kinases termed mitogen-activated protein kinases (MAP kinases); also called Extracellular signal-regulated protein kinases (Erks) (Blenis, 1993; Cobb et al., 1991; Davis, 1993; Mordret, 1994; Robbins et al., 1994). Two well characterized MAP kinase isoforms are Erk1 and Erk2 (Cobb et al., 1991; Mordret, 1994; Robbins et al., 1994). Both proteins (Erks 1 and 2) are
proline-directed protein kinases having overlapping substrate specificities (Cobb et al., 1991; Mordret, 1994; Robbins et al., 1994) and they phosphorylate a wide range of cytosolic and nuclear effector proteins (Cobb et al., 1991; Davis, 1993; Karin, 1995; Robbins et al., 1994). Erks require dual phosphorylation at threonine and tyrosine residues for full catalytic activity (Payne et al., 1991; Rossomando et al., 1992b; Sturgill et al., 1988).

Erks coordinate signaling cascades originating from activated cell surface receptors containing tyrosine kinase domains or from G protein-linked receptors (Cobb et al., 1991). While the sequence of events for most of these has not been completely determined, a basic mechanism has been put forward for the platelet-derived growth factor (PDGF) receptor and the epidermal growth factor receptor (EGFR) (Avruch et al., 1994; Claesson-Welsh, 1994; Schlessinger and Ulrich, 1992). Briefly, autophosphorylation of the receptors at tyrosine residues creates binding sites for proteins having Src homology 2 (SH2) domains such as the Grb2/Sem5 protein (Claesson-Welsh, 1994) and the phosphotyrosine phosphatase, Syp (Li et al., 1994). Grb2 recruits the SOS protein (Egan et al., 1993; Rozakis-Adcock et al., 1993; Rozakis-Adcock et al., 1992) a nucleotide exchange factor which stimulates the conversion of Ras from its inactive GDP-bound form to its active GTP-bound state (Khosravi-Far and Der, 1994; McCormick, 1993). Activated Ras interacts with the Raf-1 kinase (Avruch et al., 1994) and stimulates its protein kinase activity towards the dual specificity kinase MEK, (Map kinase/Erk1 activating kinase) (Avruch et al., 1994; Kyriakis et
al., 1992). MEKs are activated by phosphorylation at serine residues and subsequently phosphorylate Erks at threonine and tyrosine residues (Dent et al., 1994; Guan, 1994; Robbins et al., 1993; Rossomando et al., 1992a).

Glioma cells have not been characterized for the role of MAP kinases in cellular proliferation. However, genetic studies have shown amplification and mutation of genes encoding proteins that are components of the MAP kinase signaling pathway, including PDGF receptors (Fleming et al., 1992; Hermanson et al., 1992; Kumabe et al., 1992), and EGF receptors (Bigner et al., 1990; Ekstrand et al., 1991; Ekstrand et al., 1992; Wong et al., 1992). Additionally, several cell lines have been characterized for growth factor and growth factor receptor expression (Nistér et al., 1991; Nistér et al., 1988) as well as protein kinase C (PKC) activity (Couldwell et al., 1992; Couldwell et al., 1994; Pollack et al., 1990b), all of which would be expected to activate MAP kinase.

In order to understand the modulation of a mitogenic signal transduction pathway by gangliosides in glioma cells, it is important to first characterize the relevance of that pathway for the proliferation of the cells. Therefore, in this chapter the role of the MAP kinase Erk2 in U-1242 MG cell proliferation has been examined.
MATERIALS AND METHODS

Materials

Materials were obtained from the following sources. U-1242 MG cells were obtained from the laboratory of Dr. Bengt Westermark, Uppsala, Sweden. Swiss 3T3 cells were from ATCC (Rockville, MD). C16 anti-Erk1 and C14 anti-Erk2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Erk2 antiserum 98 was prepared from rabbits immunized with a peptide corresponding to the 12 C-terminal amino acids of mouse Erk2. Alkaline phosphatase-conjugated goat anti-rabbit antibody was from BioRad (Hercules, CA). [3H]thymidine was from American Radiolabeled Chemicals (St. Louis, MO). [γ-32P]ATP was from ICN Biomedicals (Costa Mesa, CA). Myelin Basic Protein, 12-O-Tetradecanoylphorbol 13-Acetate (TPA) and epidermal growth factor (EGF) were from Sigma (St. Louis, MO). Platelet-derived growth factor (PDGF) was from R&D Systems (Minneapolis, MN). Cell culture media and serum were from Gibco (Grand Island, NY).

Cell Culture

U-1242 MG cells were grown in minimum essential medium (MEM) supplemented with 10% calf serum, essential and non-essential amino acids and vitamins (Gibco) in a humidified atmosphere at 37°C with 5% CO2. Cells were passaged at a ratio of 1:20 once per week and the medium was
changed three days after passaging. For experiments, cells were plated at a density of 5000 cells/cm² and grown for either 2 days for subconfluent density or a week for confluent. They were then washed twice with phosphate buffered saline (PBS) and serum-starved 24-48 hours in MEM with vitamins and amino acids without serum. Swiss 3T3 cells were grown as above except in Dulbecco's modified Eagle's medium with 10% calf serum. For experiments these cells were grown to confluence and incubated an additional 2 days without changing the medium to render them quiescent.

[^H]Thymidine Incorporation

Cells were grown to either confluent of subconfluent density in 24 well tissue culture plates (Corning), serum starved for 24 hours and then stimulated by the addition of EGF (200 ng/ml), PDGF (50 ng/ml), TPA (100 ng/ml) or 10% calf serum. [Methyl-[^H]thymidine (American Radiolabeled Chemicals) was then added to the wells to a final concentration of 0.5 μCi/ml. The cells were then incubated for 24 hours after which they were washed twice with cold PBS, and 0.5 ml cold 5% TCA was added to each well. After 5 minutes, the TCA was removed and 250 mM NaOH was added for 30 minutes to dissolve TCA precipitated material. Dissolved precipitate (50 μl) was then added to 5 ml Aquasol scintillation cocktail (NEN) and incorporated radioactivity was determined by scintillation counting.
Western Blot Analysis

Cells were grown and serum-starved as detailed above in 6 well tissue culture plates. They were then stimulated with various mitogens as in thymidine incorporation experiments for 5 minutes. Cells were then washed in PBS and lysed by the addition of 1% sodium dodecyl sulfate (SDS) containing 1 mM sodium orthovanadate, 5 mM EDTA. Cell lysates were separated by SDS-PAGE on 10% gels (acrylamide:bis 37.5:1) and transferred to nitrocellulose (Micron Separations) using an Idea electroblotter at 400 mAmperes for 1 hour. Membranes were blocked with 3% gelatin in Tris Buffered Saline (TBS) for 30 minutes. They were then washed with 0.5% Tween Tris Buffered Saline (TTBS) and probed with C16 anti-ERK1 (Santa Cruz Biotechnology) at 1:1000 dilution in TTBS with 1% gelatin overnight. Membranes were washed twice with TTBS and incubated 2 hours with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) at 1:3000 in TTBS with 1% gelatin. They were then washed twice with TTBS, once with TBS and developed with 5-bromo-4-chloro-3-indolylphosphate/p-nitroblue tetrazolium chloride (BCIP/NBT).

Immune Complex Kinase Assay

Cells were grown to confluence in 100 mm plates and stimulated as above. They were then lysed by the addition of 500 µl 1% Triton X-100 containing 20 mM 4(-2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 1 mM dithiothreitol (DTT), 30 mM sodium pyrophosphate,
50 mM NaF, 1 mM ethylene glycol-bis(b-aminoethyl ether)N,N,N',N'-tertaacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na3VO4 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 0.2 units/ml aprotinin for 10 minutes on ice. Lysates were centrifuged 15 min at 4°C. Anti-MAP kinase anti-serum (10 µl, antiserum 98 provided by Dr. Jackie Vandenbeede, Katholieke Universiteit Leuven, Belgium) was then added and lysates were incubated 2 hours at 4°C with rocking. Protein A Sepharose beads (Pierce) (50 µl of a 50% suspension) were then added and the incubation continued for an additional 30 minutes. The beads were then pelleted and washed three times in lysis buffer and twice in 20 mM HEPES pH 7.4, 1 mM DTT. The kinase assay was begun by resuspending the beads in 50 µl of 20 mM HEPES pH 7.4, 10 mM MgCl2, 50 µM ATP, 0.5 mg/ml MBP and 5.7 µCi [γ-32P]ATP (sp. act. 5,000 dpm/pmol), and incubating 30 minutes at 30°C. The beads were then spun down and 25 µl of the supernatant was spotted on P81 phosphocellulose papers. The papers were then washed 4 times for 15 minutes each in 75 mM phosphoric acid at 4°C. They were then allowed to dry, placed in scintillation cocktail and the radioactivity was counted.

In-gel Kinase Assay

Cells were grown to confluence and stimulated, lysed and MAP kinase was immunoprecipitated as detailed for the immune complex kinase assay. Immunoprecipitates were run on a 10% SDS polyacrylamide gel containing
0.5 mg/ml myelin basic protein. The gel was incubated one hour in 50 mM Tris-HCl pH 8.0, 20% isopropanol, and then one hour in 50 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol at room temperature to remove SDS. The proteins were then denatured by incubation in 6 M guanidine HCl for 1 hour at room temperature. Proteins were gradually renatured by washing the gel with successive solutions of 3 M, 1.5 M, 0.75 M and 3 changes of guanidine-free 50 mM Tris HCl pH 8.0, 5 mM β-mercaptoethanol, and 0.04% Tween 40 overnight at 4°C with shaking. The gel was then preincubated 30 min in 50 mM HEPES pH 7.4, 2 mM dithiothreitol, 10 mM MgCl₂ at room temperature. The kinase assay was performed by incubation in preincubation buffer plus 50 μM ATP and 10 μCi/ml [γ³²P]ATP for 1 hour at room temperature. The gel was washed repeatedly with 1% sodium pyrophosphate, 5% trichloroacetic acid until no more radioactivity eluted. The gel was then dried and exposed to film for autoradiography.

RESULTS

MAP kinase catalytic assays

Kinase assays were performed on anti-MAP kinase immunoprecipitates from U-1242 MG using myelin basic protein (MBP) as substrate. Autoradiographs showed a set of phosphorylated low molecular weight bands which are consistent with the expected size of MBP (results
not shown). Prolonged exposure of autoradiographs failed to detect additional bands. The amount of radioactivity in the MBP band excised directly from the nitrocellulose filter was at least 95% of that obtained from P81 filters. Thus, phosphorylated MBP was the major phosphoprotein formed in these immune complex kinase assays.

Figure 3.1 shows MBP phosphotransferase activity for mitogen-treated U-1242 MG cells. The results were compared with identical experiments on Swiss 3T3 cells which have been well characterized for MAP kinase activity following mitogen treatment (Ahn and Krebs, 1990; Ahn et al., 1991; Chung et al., 1991). These experiments were performed on plates of U-1242 MG and Swiss 3T3 cells containing a similar number of cells. In these experiments basal phosphotransferase activity in unstimulated Swiss 3T3 cells and U-1242 MG cells was typically 1.7 and 1.2 pmol/µg MBP, respectively. However, in some experiments basal MBP phosphotransferase activity in U-1242 MG cells ranged from 1.2 pmol phosphate/µg MBP, to as much as 5.3 pmol phosphate/µg MBP.

Relative to untreated cells MBP phosphotransferase activity was approximately seven-fold higher in Swiss 3T3 cells treated with EGF, PDGF, TPA and calf serum but was only slightly higher in cells treated with aFGF. In U-1242 MG cells all of the mitogens tested caused an increase in MBP phosphotransferase activity, although to a lesser degree than in Swiss 3T3 cells. EGF, PDGF and serum were found to be the best mitogens for
activation of MBP phosphotransferase activity with results from three independent experiments yielding 3 fold increased activity.

in gel MAP kinase assay

Since U-1242 MG cells produce both Erk1 and 2 (Fig 3.3, see also Northern analysis, Appendix) it was important to determine whether one or both isoforms were contributing to the MBP phosphotransferase activity measured above. To do this, kinase assays of immunoprecipitates were performed within a polyacrylamide gel containing MBP. Figure 3.2 shows the results from one such experiment using the same antibody employed for the immune complex kinase assay described above. Only one band, representing MBP kinase activity, was detected in the immunoprecipitates from either U-1242 MG or Swiss 3T3 cells. This band had an estimated size of approximately 42 kDa, and indicates that the antiserum used for our assay immunoprecipitates only one, active Erk isoform.

The relative intensity of the phosphorylated band seen by in-gel kinase assays reflects the overall extent of MBP phosphotransferase activity (Hutchcroft et al., 1991; Kameshita and Fujisawa, 1989). Relative to the unstimulated cells, there was a clear increase in MBP phosphotransferase activity in immunoprecipitates prepared from PDGF-stimulated 3T3 cells (compare lanes 1 and 2, Fig 3.2) as well as in U-1242 MG cells (compare lanes 3 and 4, Fig 3.2). However, the intensity of the phosphorylated band for PDGF-stimulated U-1242 MG cells was clearly weaker than that in
PDGF-stimulated 3T3 cells (compare lanes 2 and 4, Fig. 3.2). This is consistent with the results of the immune complex kinase assay (Figure 3.1). Overexposure of the dried gel to film did not detect any additional phosphorylated bands (result not shown).

To unequivocally identify the kinase detected by the in-gel kinase assay as either Erk1 or Erk2, Western blotting of anti-MAP kinase immunoprecipitates was performed using the antibody C16 (Santa Cruz), which recognizes both Erk isoforms (Figure 3.3). Two well separated MAP kinase bands were observed in SDS lysates prepared from unstimulated U-1242 MG cells (Panel A, lane 3). The upper band correlated with the expected size of the Erk1 isoform, 44 kDa, while the lower one represented the Erk2 molecule, 42 kDa. This interpretation is based on the mobilities of Erk1 and Erk2 in unstimulated 3T3 cells which were easily identified on the Western blot (Panel A, lane 1). Corresponding immunoprecipitates contained only the lower band which co-migrated with Erk2 from the whole cell lysates (Panel B, lanes 1 and 3), indicating that Erk2 is present in these immunoprecipitates. Thus, Erk2 is responsible for the MBP phosphotransferase activity seen with the immune-complex kinase assay and the in-gel kinase assay.

Figure 3.3 also shows band-shifted Erks from PDGF-stimulated cells, which migrated more slowly in comparison with Erks from unstimulated cells (lanes 2 and 4, Fig 3.3 panel A). Previous reports have shown that the retarded electrophoretic mobility is due to phosphorylation of Erks and
correlates with their kinase activity (Posada and Cooper, 1992). While PDGF-treated Swiss 3T3 cells exhibited a complete shift of both Erk1 and Erk2 bands to a slightly slower mobility, only approximately 50% of the Erk2 protein present in PDGF-stimulated U-1242 MG cells was shifted to the slower mobility. These results were also observed in the corresponding immunoprecipitates of PDGF-treated cells (lanes 2 and 4 panel B).

MAP kinase mobility shift assays

The electrophoretic shift of Erks in U-1242 MG cells indicated that band shifts could be used to examine activation of MAP kinase in U-1242 MG cells following mitogen treatment. Western blot analysis of SDS lysates from unstimulated as well as mitogen-stimulated U-1242 MG cells probed with anti-MAP kinase antibody C16 are shown in Figure 3.4. Unstimulated U-1242 MG cell lysates contained a band that corresponded to the more slowly moving Erk2, suggesting that some of the MAP kinase in these cells was activated in the absence of mitogenic stimulation. For the Western blot shown, approximately 30% of the Erk2 protein present in U-1242 MG lysates, as determined by scanning densitometry, was shifted to the more slowly moving band, while in other experiments up to 50% of the Erk2 protein present in the cells was shifted.

Treatment of U-1242 MG cells with EGF, PDGF, TPA or serum caused a partial increase in the amount of mobility shifted Erk2. None of the mitogens caused a complete band-shift of the Erk2 protein in these cells.
Of the mitogens tested, EGF and serum were most potent, whereas PDGF and TPA only slightly increased the amount of shifted Erk2.

The Erk1 bands seen on Western blots of U-1242 MG cell lysates were not as clearly resolved as the Erk2 bands and did not show a clear mobility shift. However, the Erk1 band from Swiss 3T3 was clearly shifted in response to mitogenic stimulation.

**MAP kinase activation in confluent vs. subconfluent U-1242 MG cells**

The above experiments were performed on confluent monolayers of cells. To determine whether the incomplete activation of Erk2 in U-1242 MG cells was an inherent characteristic of this cell line or was related to the growth conditions of the culture, mitogen-stimulated confluent and subconfluent U-1242 MG cells were compared for their extent of shifted Erk2 protein.

U-1242 MG cells were treated with several mitogens at confluent and subconfluent densities, and MAP kinase mobilities analyzed on Western blots probed with C16 antibody. Figure 3.5 shows that in subconfluent U-1242 MG cells all the mitogens tested were capable of almost completely converting Erk2 to the more slowly migrating form. The mitogen-stimulated Erk2 band shift was less complete in confluent U-1242 MG cells; only EGF led to a nearly complete shift of Erk2. Thus, mitogen treatment leads to a band shift of MAP kinase more effectively in subconfluent than in confluent U-1242 MG cells.
Stimulation of DNA synthesis in confluent and subconfluent U-1242 MG cells

Since activation of the MAP kinase pathway is thought to be important for mitogenic signal transduction (Mansour et al., 1994; Pagès et al., 1993; Sun et al., 1994; Troppmair et al., 1994), we next tested whether activation of MAP kinase in U-1242 MG cells correlated with stimulation of DNA synthesis as measured by incorporation of \[^{3}H\]thymidine. Figure 3.6 shows that \[^{3}H\]thymidine incorporation in confluent, EGF-, PDGF-, TPA- and serum-stimulated U-1242 MG cells was no different than that seen in control, unstimulated cells. Thus none of the mitogens tested was able to stimulate DNA synthesis in confluent U-1242 MG cells, whereas they all led to partial activation of MAP kinase as determined by immune complex kinase assay (Figure 3.1).

To determine whether a more complete activation of MAP kinase was necessary to stimulate U-1242 MG cells to synthesize DNA, we also tested \[^{3}H\]thymidine incorporation in subconfluent cells, a condition in which all the mitogens tested caused a band shift of MAP kinase. However, only serum was capable of stimulating DNA synthesis in subconfluent U-1242 MG cells. EGF, PDGF, and TPA caused no increase in \[^{3}H\]thymidine incorporation above that seen in the unstimulated control. These results suggest that MAP kinase activation by mitogenic stimulation is not in itself sufficient to stimulate DNA synthesis in U-1242 MG cells.
PD98059 decreases DNA synthesis in U-1242 MG cells

U-1242 MG cells proliferate in culture even in the absence of added mitogens. In addition, the presence of a mobility-shifted Erk2 band in unstimulated U-1242 MG cells suggests that a portion of the Erk2 is active under these conditions. Therefore, it is possible that, although Erk2 activation is not sufficient to stimulate further DNA synthesis, it may be involved in maintaining this basal proliferation. To test this possibility subconfluent, serum starved U-1242 MG cells were treated with a specific, pharmacological inhibitor of the MAP kinase pathway known as PD98059 (Alessi et al., 1995), and [3H]thymidine incorporation was measured. Figure 3.7 shows that PD98059 treatment caused a decrease in DNA synthesis in unstimulated U-1242 MG cells. This result suggests that MAP kinase may be involved in continual proliferation of U-1242 MG cells in the absence of mitogenic stimulation.

DISCUSSION

Three different techniques were used to examine the activation of MAP kinase in U-1242 MG glioma cells. First, the immune-complex kinase assay showed that the Erk2 specific antiserum 98 immunoprecipitated an MBP phosphotransferase activity from U-1242 MG cell lysates which was activated in response to mitogen treatment. Second, an in-gel kinase assay of immunoprecipitates using the same antibody showed that MBP
phosphotransferase activity correlated with a protein migrating at approximately 42 kDa. This activity was increased in immunoprecipitates from cells treated with PDGF. Third, Western blots of cell lysates and antiserum 98 immunoprecipitates identified bands at 42 and 44 kDa. These correspond to the molecular weights of Erk2 and Erk1 respectively. Approximately 30% of the protein migrating at 42 kDa was shifted to one with a slower mobility in lysates from PDGF-treated cells. Previous reports have demonstrated that mitogenic activation of MAP kinases causes slight retardation of their electrophoretic mobility by SDS-PAGE (Posada and Cooper, 1992). This appears to be due to their phosphorylation on regulatory threonine and tyrosine residues which are required for activation of MAP kinase phosphotransferase activity (Posada and Cooper, 1992). The antiserum 98 immunoprecipitates contained the 42 kDa band and its mobility shifted form, but not the 44 kDa band. Taken together these results indicate that, Erk1 and Erk2 are expressed in U-1242 MG cells, but only Erk2 is immunoprecipitated by antiserum 98.

Both band shift as well as catalytic activity assays show that Erk2 is activated by mitogen-treatment in confluent U-1242 MG cells to a lesser degree than in Swiss 3T3 cells. Since EGF, PDGF, TPA and serum caused high levels of MBP phosphotransferase activity in Swiss 3T3 cells, it is unlikely that the results obtained with U-1242 MG are related to the quality of the mitogens. Immunoblotting experiments indicate that the amount of immunoreactive proteins in U-1242 MG cells is comparable to
Swiss 3T3 cells (Figure 3.3). Therefore, the difference in relative MBP phosphotransferase activity is not related to differences in the amounts of Erk2 protein. Finally, the difference in phosphotransferase activity is not related to a slow time course for Erk2 activation since experiments using a gel shift assay, demonstrated that both U-1242 MG and Swiss 3T3 cells caused maximal stimulation of Erk2 within 5 to 10 minutes of EGF or PDGF treatment (data not shown). Based on these results we conclude that the MBP phosphotransferase activity of MAP kinase in U-1242 MG cells was lower than that of Swiss 3T3 cells due to a difference in either their catalytic or regulatory properties. The lack of a complete shift of Erk2 to its slower mobility form indicates that this lower mitogen-stimulated activity is, at least partially, due to an incomplete activation of the total Erk2 protein present in U-1242 MG cells.

Erk2 displays variable levels of phosphorylation in confluent serum-starved U-1242 MG cells, whereas in contact-inhibited Swiss 3T3 cells MAP kinases are unphosphorylated as evidenced by the lack of shifted bands on Western blot analysis. Unstimulated U-1242 MG cell lysates contained a shifted Erk2 band, although the amount of protein which was shifted varied somewhat from one experiment to another. This is consistent with the variable level of MAP kinase activity seen by the immune-complex kinase assay in unstimulated U-1242 MG cells which ranged from a level similar to unstimulated Swiss 3T3 cells to approximately three times that activity. The variability of the MAP kinase catalytic activity and amount of mobility-
shifted Erk2 in U-1242 MG cells may reflect the tendency for these cells to remain within the cell cycle both upon reaching confluence and when serum starved at either confluent or subconfluent density. This is evidenced by high basal levels of thymidine incorporation (Figure 3.6), increases in cell number, and the presence of many mitotic figures (data not shown).

We also found that activation of Erk2 in U-1242 MG cells is sensitive to culture conditions. In comparison to the incomplete mitogen-induced Erk2 activation seen in confluent U-1242 MG cells, these same mitogens caused a complete, or nearly complete shift of Erk2 when the cells were at subconfluent densities. Thus, it appears that while the signal transduction pathway which activates Erk2 in U-1242 MG is intact and capable of functioning normally, yet unidentified, density-dependent factors contribute to the regulation of MAP kinase in U-1242 MG cells.

Stimulation of Erk2 by growth factors did not correlate with an increase in DNA synthesis in U-1242 MG cells. Since Erk2 was partially activated by EGF, PDGF, TPA or serum in confluent U-1242 MG cells, we expected that these agents would cause an increase in DNA synthesis. However, none of these mitogens was able to stimulate thymidine incorporation beyond that seen in unstimulated control U-1242 MG cells. Therefore, we reasoned that the partial activation of Erk2 under these conditions might be insufficient to cause these cells to enter S phase. However, EGF, PDGF and TPA failed to stimulate DNA synthesis in subconfluent U-1242 MG cells as well, although they all led to a complete
band shift of Erk2. Only serum was capable of stimulating DNA synthesis in subconfluent U-1242 MG cells. Therefore, we conclude that activation of Erk2 alone is insufficient to cause mitogen-induced DNA synthesis in U-1242 MG glioma cells.

In U-1242 MG cells, the MAP kinase pathway may be more important for the continuous growth in culture, even in the absence of serum. As noted above, the immune-complex kinase assay and the band shift assay detected a variable level of MAP kinase activity in untreated U-1242 MG cells. The inhibitor of MEK activation PD98059 was able to lower basal [³H]thymidine incorporation by approximately 50%. Saltiel and co-workers have shown that PD98059 is specific for inhibition of the activation of MEK by its upstream regulator Raf, as it does not affect the activity of 18 other Ser/thr kinases, 4 tyrosine kinases or phosphatidylinositol 3'-kinase (Alessi et al., 1995; Dudley et al., 1995). It appears that PD98059 may prevent the interaction between MEK and Raf since it does not inhibit Raf kinase activity (Alessi et al., 1995). Although it can not be ruled out that PD98059 may still have other effects besides inhibition of the MAP kinase pathway (see Chapter 5), the PD98059 data supports the hypothesis that MAP kinase is important for continuous proliferation of U-1242 MG cells.

The basal level of Erk2 activity seen in unstimulated, serum starved U-1242 MG cells could result from autocrine stimulation, since U-1242 MG cells have been shown to express both PDGF and PDGF receptors (Nistér et al., 1988). (Vassbotn et al., 1994) have identified a glioma cell line which is
driven to proliferate by a PDGF autocrine pathway. Such an autocrine loop would be expected to lead to activation of MAP kinase activity. Alternatively, an activated oncogene upstream of MAP kinase could be responsible for the MAP kinase activity in unstimulated cells.

All the mitogens tested caused phosphorylation of Erk2 in subconfluent U-1242 MG cells, but only serum was able to stimulate DNA synthesis. Therefore, serum must cause DNA synthesis through the stimulation of additional signaling pathways, which may work either independently from or synergistically with the MAP kinase signaling cascade. It appears that EGF or PDGF receptor tyrosine kinases or TPA do not use these alternative or additional pathways, since EGF, PDGF or TPA did not stimulate DNA synthesis. Identification of this pathway could be an important step towards elucidating the molecular mechanisms responsible for uncontrolled glioma growth.

In summary, although the MAP kinase pathway appears to be important for U-1242 MG proliferation, Erk2 activation by growth factors does not necessarily lead to increased DNA synthesis. Since some glioma cell lines do respond to growth factors with increased DNA synthesis (Pollack et al., 1990a; Pollack et al., 1991), this study describes a novel aspect of growth regulation in U-1242 MG cells. In addition, these data provide a basis which is necessary for the following chapters, which will examine the involvement of Erk2 and additional signal transduction pathways in GM1-stimulated proliferation of these glioma cells.
Figure 3.1. Activation of MAP kinase in Swiss 3T3 and U-1242 MG cells by various mitogens. Cells were stimulated for 5 minutes with the indicated mitogens. Cell lysates were immunoprecipitated with antiserum 98. Immune complexes were assayed for MAP kinase activity with myelin basic protein (MBP) as the substrate. Results are expressed as fold increase in MBP kinase activity relative to unstimulated controls. Representative experiments are shown. Results of three separate experiments were similar.
Figure 3.2. in-gel MAP kinase assay. Cell lysates were immunoprecipitated with antiserum 98 and immunoprecipitates were separated on a polyacrylamide gel containing myelin basic protein (MBP). Proteins were denatured and then renatured and the kinase assay performed in the gel. Bands were visualized by autoradiography. Lanes 1 & 2 - Swiss 3T3 cells; Lanes 3 & 4 - U-1242 MG cells. Cells were either unstimulated (Lanes 1 & 3) or stimulated with PDGF (Lanes 2 & 4).

Figure 3.3. Western blotting of MAP kinase immunoprecipitates. Lysates (A) or anti-MAP kinase immunoprecipitates (B) from U-1242 MG cells treated with or without 50 ng/ml PDGF-BB for 5 min were separated on a 10% SDS polyacrylamide gel, transferred to nitrocellulose and probed with C16 anti-ERK1 antibody. Lanes 1 - unstimulated Swiss 3T3, Lanes 2 - PDGF-stimulated Swiss 3T3, Lanes 3 - unstimulated U-1242 MG, Lanes 4 - PDGF-stimulated U-1242 MG.
Figure 3.4. Phosphorylation of MAP kinase in Swiss 3T3 and U-1242 MG cells. Cell lysates were immunoblotted as described in the legend for Figure 3.3. Phosphorylation was determined by the presence of a mobility shifted band. The arrows in each panel indicate unshifted ERK1 (upper arrow) and unshifted ERK2 (lower arrow). Cells were treated as follows: Lanes 1 - unstimulated; Lanes 2 - EGF; Lanes 3 - PDGF; Lanes 4 - TPA; Lanes 5 - serum.
Figure 3.5. Phosphorylation of MAP kinase in confluent vs. subconfluent U-1242 MG cells. Cell lysates were analysed by western blotting as described in the legend for Figure 3.3. Cells were treated as follows: Lanes 1 - unstimulated; Lanes 2 - EGF; Lanes 3 - PDGF; Lanes 4 - TPA; Lanes 5 - serum.
Figure 3.6. Stimulation of DNA synthesis by mitogens in confluent vs. subconfluent U-1242 MG cells. Cells were plated at low density (5000/cm²) and grown either 2 days for subconfluence or one week for confluence. Mitogens were then added and cells were labeled with [³H]thymidine for 24 hours. Results are expressed as dpm incorporated into TCA precipitable material per well. Error bars represent standard deviations of four replicates. Repeat experiments gave similar results.
Figure 3.7. Effect of the MEK inhibitor PD98059 on DNA synthesis in U-1242 MG cells. Subconfluent U-1242 MG cells were treated with or without the indicated concentration of PD98059 or with 0.1% DMSO as vehicle control. $[^3]$H]thymidine incorporation was determined and the results are expressed as dpm incorporated into TCA precipitable material per well. Error bars represent standard deviations of four replicates. Repeat experiments gave similar results.
CHAPTER 4

GANGLIOSIDE GM1 ACTIVATES THE MITOGEN-ACTIVATED PROTEIN KINASE ERK2 AND P70 S6 KINASE IN U-1242 MG CELLS

ABSTRACT

Gangliosides are implicated in the regulation of cellular proliferation as evidenced by differences in ganglioside composition associated with malignant transformation and density of cells in culture, as well as their inhibitory effects when added to cells growing in culture. Exogenously added gangliosides have a bimodal effect on proliferation of U-1242 MG glioma cells, inhibiting DNA synthesis in growing cells and stimulating it in quiescent cells. We investigated the mechanisms involved in stimulation of DNA synthesis using [³H]thymidine incorporation experiments and immune complex kinase assays to identify the signal transduction pathways responsible. Treatment of quiescent U-1242 MG cells with GM1 caused activation of the MAP kinase isoform Erk2. Pretreatment with the specific
inhibitor of MAP kinase kinase activation PD98059 prevented the GM1-stimulated Erk2 activation and also prevented GM1-stimulated DNA synthesis. GM1 treatment stimulated another distinct signaling pathway leading to activation of p70 S6 kinase (p70^{S6k}), and this was prevented by pretreatment with rapamycin. Rapamycin also inhibited GM1-stimulated DNA synthesis. Platelet-derived growth factor also activated both Erk2 and p70^{S6k} but did not cause DNA synthesis, suggesting that GM1 may stimulate other cascades in addition to MAP kinase and p70^{S6k} pathways, which are involved in GM1-mediated DNA synthesis.

INTRODUCTION

Gangliosides are sialic acid-containing glycosphingolipids which are found in virtually all mammalian cells and are especially enriched in the central nervous system (Byrne et al., 1988; Ledeen, 1989; Stults et al., 1989). While their biological functions are not well understood, several lines of evidence suggest that they are involved in the regulation of cellular proliferation. This is discussed in some detail in the Introduction (Chapter 1). Briefly, there are differences in ganglioside composition of tumor cells in comparison to nontransformed cells which usually constitutes a simplification in oligosaccharide structure (Berra et al., 1985; Traylor and Hogan, 1980; Yates et al., 1979). Second, there is an increase in ganglioside complexity in cultured cells under conditions which prevent cell growth such
as contact inhibition or serum starvation (Hakomori, 1970). Third, ganglioside composition changes as density of cells in culture increases (Liepkalns et al., 1981).

Much of the data on gangliosides as regulators of cell growth comes from experiments in which exogenous gangliosides added to the culture medium were shown to inhibit growth factor mediated proliferation. Gangliosides added in this way prevent the activation of receptor tyrosine kinases such as the platelet-derived growth factor (PDGF) receptor (Bremer et al., 1984; Yates et al., 1993) and the epidermal growth factor (EGF) receptor (Bremer et al., 1986). The inhibitory mechanism is thought to involve binding of gangliosides to the receptor. This inhibits dimerization of the receptor monomers and prevents activation of tyrosine kinase activity (see Chapter 2) (Van Brocklyn et al., 1993).

In some cases gangliosides stimulate cell growth (Pettman et al., 1988; Saqr et al., 1995a; Spiegel and Fishman, 1987). Quiescent Swiss 3T3 cells can be stimulated to synthesize DNA by treatment with the B subunit of cholera toxin which binds specifically to the monosialoganglioside GM1 (Spiegel and Fishman, 1987). Saqr et al. (Saqr et al., 1995a) showed that addition of gangliosides caused an increase of DNA synthesis in U-1242 MG glioma cells, rendered relatively quiescent by serum starvation. The effects of the cholera toxin B subunit and GM1 are bimodal, since in proliferating cells both have the opposite effect of inhibiting DNA synthesis. GM1 also increased proliferation of rat astrocytes in serum-free medium (Pettman et
al., 1988). The mechanisms by which gangliosides stimulate cellular proliferation are entirely unknown.

Mitogenic agents cause cellular proliferation through the activation of multiple signal transduction pathways. As described above, a wide variety of mitogens stimulate a protein kinase cascade that involves the sequential activation of the serine/threonine kinases Raf, MAP kinase kinase (MAPKK) and the MAP kinase isoforms Erk1 and Erk2 (Crews and Erikson, 1993; Davis, 1993; Nishida and Gotoh, 1993; Pelech, 1993). Several lines of evidence (see Introduction) indicate that Erks are necessary for stimulation of cellular proliferation. The previous chapter provided evidence that Erks are important for growth of U-1242 MG cells. This suggests that stimulation of DNA synthesis in U-1242 MG cells by gangliosides may involve Erks.

Another common event in mitogenic signaling is the phosphorylation of ribosomal protein S6 by the p70/p85 S6 kinase, p70\(^{16k}\) (Ferrari and Thomas, 1994). This is thought to be necessary for increased translation of certain mRNAs containing a polypyrrimidine tract at their 5′ transcriptional start site (Jeffries et al., 1994), the products of which are presumably necessary for cell growth.

In this chapter the mechanism for ganglioside GM1 stimulation of DNA synthesis in U-1242 MG human glioma cells has been investigated, by examining the effects of GM1 on these two commonly studied, mitogenic signal transduction pathways.
MATERIALS AND METHODS

Materials

Ganglioside GM1 was from Fidia (Abano Terme, Italy). Asialo GM1, C2-ceramide, bacterial sphingomyelinase, and myelin basic protein (MBP) were from Sigma (St. Louis, MO). \[^{3}\text{H}]\text{thymidine was from American Radiolabeled Chemicals (St. Louis, MO).} \[^{32}\text{P}]\text{ATP was from Dupont, New England Nuclear (Boston, MA).} \text{Platelet-derived growth factor (rhPDGF-BB) was from R&D Systems (Minneapolis, MN). Minimum essential medium (MEM), essential and non-essential amino acids, vitamins and calf serum were from GIBCO (Grand Island, NY). PD98059 and rapamycin were from Calbiochem (La Jolla, CA). Antibodies to p70^{65k} (C18) and Jnk1 (C20) were from Santa Cruz Biotechnology (Santa Cruz, CA).}

Ganglioside GM1 was dissolved in chloroform:methanol 2:1 and quantitated by the resorcinol method (Miettien and Takki-Luukkainen, 1959; Svennerholm, 1957). For experiments the chloroform:methanol was evaporated under N\_2 and the ganglioside redissolved in serum-free MEM plus amino acids and vitamins (B0) by incubation at 37°C for 1 hour with vortexing and sonication every 20 min. Sulfatide was prepared as above except quantitation was by the orcinol method (Balazs et al., 1971). Asialo GM1 was dissolved in chloroform:methanol 2:1 and the proper amount prepared as for sulfatide.
C2-ceramide was dissolved in ethanol. Rapamycin was dissolved in 50% ethanol. PD98059 and wortmannin were dissolved in dimethyl sulfoxide (DMSO). Preparation and use of wortmannin, rapamycin and PD98059 was done in as low light as possible. Experimental controls were treated with appropriate vehicles (ethanol or DMSO).

Cell culture

U-1242 MG human glioma cells were obtained from Dr. Bengt Westermark (Uppsala, Sweden). They were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in Minimum essential medium plus essential and non-essential amino acids, vitamins and 10% calf serum (B10). Cells were passaged once per week at a ratio of 1:20.

[^3]H]thymidine incorporation

U-1242 MG cells were seeded in 24 well tissue culture plates (Corning) at 5,000 cells/cm² and incubated 2 days. The cells were then washed twice with phosphate buffered saline (PBS) and B0 was added (1 ml/well). Plates were then incubated for 2 additional days. The medium was then changed to 0.5 ml B0 or B0 plus ganglioside GM1 and the plates were incubated 16 hours. [^3]H]thymidine was then added to the wells to a final concentration of 0.5 μCi/ml and the cells incubated 24 hours. Wells were washed twice with cold PBS and DNA precipitated with cold 5% Trichloracetic acid (TCA). TCA insoluble material was dissolved in 0.5 ml of
0.25 M NaOH and 50 μl samples were added to 5 ml Aquasol scintillation cocktail (Packard Instruments, Meriden, CT). Radioactivity was quantitated by scintillation counting. Each condition was done in four replicates and the results expressed as the average plus and minus standard deviation.

**Immune complex kinase assays**

Cells were seeded at 5,000/cm² in 100 mm plates and after 2 days were serum starved for 48 hours. Subconfluent cells were stimulated as above. They were then lysed by the addition of 0.5 ml lysis buffer containing 1% Triton X-100, 20 mM 4(-2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 1 mM dithiothreitol (DTT), 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM ethylene glycol-bis(b-aminoethyl ether)N,N,N′,N′-tetaacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na₃VO₄, 0.1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), and 1 μg/ml aprotinin for 10 minutes on ice. Lysates were centrifuged 15 min at 4°C. Anti-MAP kinase anti-serum 98 (5 μl) was then added and lysates were incubated 2 hours at 4°C with rocking. Protein A Sepharose beads (Pierce, Rockford, IL) (40 μl of a 50% suspension) were then added and the incubation continued for an additional 30 minutes. The beads were then pelleted, washed three times in lysis buffer and twice in 20 mM HEPES pH 7.4, 1 mM DTT. The kinase assay was begun by resuspending the beads in 50 μl of 20 mM HEPES pH 7.4, 10 mM MgCl₂, 50 μM ATP, 0.5 mg/ml MBP
and 5000 dpm/pmol [γ-32P]ATP, and incubating 30 minutes at 30°C. The reaction was stopped by the addition of 20 µl of 5 X concentrated Laemmli sample buffer (Laemmli, 1970) and the samples were boiled 5 min, separated on 12.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Nitrocellulose membranes were stained with Ponceau S (Sigma) to visualize substrate bands and then exposed to film for autoradiography. Radioactivity was quantitated by cutting radioactive bands from the membrane and scintillation counting.

Immune complex kinase assays for p70 S6k and Jnk1 were performed as above except 1 µg of antibody to p70 S6k or Jnk1 (Santa Cruz) was used and 2.5 µg 40S ribosomal subunits (a kind gift of Dr. Hwa-Chain Robert Wang) or 3.5 µg GST-cJun-79 (a kind gift of Dr. Silvio Gutkind) was used as substrate for p70 S6k and Jnk1 respectively.

RESULTS

Ganglioside GMI stimulates DNA synthesis in relatively quiescent U-1242 MG cells.

Gangliosides have a stimulatory effect on DNA synthesis in U-1242 MG cells that have been rendered relatively quiescent by serum starvation (Saqr et al., 1995a). I verified this effect by performing [3H]thymidine incorporation experiments on subconfluent U-1242 MG cells which had been
serum starved 2 days then treated with variable amounts of GM1. Figure 4.1A shows a dose-response for the effect of GM1 on DNA synthesis. There was approximately a 3 fold increase in DNA synthesis in response to 5 μM GM1. The peak response, a 5 fold increase, was seen at 10 μM GM1. There was no further increase at higher concentrations. Therefore, subsequent experiments were performed using 10 μM GM1.

To determine the specificity of the thymidine response to GM1, subconfluent U-1242 MG cells were treated with other sphingolipids in the same way as with GM1 and [³H]thymidine incorporation was measured. Figure 4.1B shows that in contrast to GM1, other sphingolipids (asialo GM1, sulfatide, C2-ceramide) and treatment with bacterial sphingomyelinase to increase intracellular ceramide were ineffective. Sialic acid alone also had no effect on [³H]thymidine incorporation.

**GM1 activates MAP kinase in U-1242 MG cells.**

For many mitogens, stimulation of DNA synthesis is preceded by the rapid activation of the MAP kinases (Crews and Erikson, 1993; Davis, 1993; Nishida and Gotoh, 1993; Pelech, 1993). To determine whether activation of MAP kinase might also be an early event following GM1 treatment, phosphotransferase activity of the MAP kinase isoform Erk2 was examined. Serum-starved, subconfluent U-1242 MG cells were treated with 10 μM GM1 then at various times Erk2 phosphotransferase activity was measured by an immune complex kinase assay.
Results shown in Figure 4.2A reveal that 10 μM GM1 activated Erk2 phosphotransferase activity several fold above basal levels. The activity peaked after 15 min of treatment with GM1 and returned to near basal levels after 1 hour. It should be noted that the relative level of Erk2 phosphotransferase activity was almost the same as that observed in serum-treated cells. Thus, GM1 is nearly as potent as serum in its ability to activate Erk2 in these cells.

Several other sphingolipids were tested for their ability to activate Erk2 in U-1242 MG cells. Figure 4.2B shows that whereas GM1 potently activated Erk2, of the other sphingolipids tested only C2-ceramide had any effect on Erk2 activity. Treatment with C2-ceramide at 10 μM was only about half as potent as GM1 at the same concentration. Although C2-ceramide slightly activated Erk2, sphingomyelinase treatment was without effect. Neither asialoGM1 nor sulfatide stimulated Erk2 activity above basal levels.

The pharmacological inhibitor PD98059 specifically inhibits the activation of MAP kinase kinase (MAPKK) and subsequent activation of MAP kinase (Alessi et al., 1995). Pretreatment with PD98059 prevented the GM1-stimulated increase in Erk2 phosphotransferase activity towards MBP substrate in a dose-responsive fashion with complete inhibition at 50 μM (Figure 4.3A). This suggests that GM1 activates MAP kinase through a pathway involving a MAP kinase kinase.
PD98059 inhibits GM1-stimulated DNA synthesis.

To determine whether PD98059 could also prevent the stimulation of DNA synthesis by GM1, U-1242 MG cells were treated with various concentrations of PD98059 for 30 minutes then GM1 was added for 16 hours, and [³H]thymidine incorporation was measured (Figure 4.3B). The GM1-stimulated increase in DNA synthesis was completely abolished by pretreatment with 25 μM PD98059, and this was not due to loss of cell viability as determined by trypan blue staining (data not shown). The concentration dependence of the inhibition of DNA synthesis by PD98059 closely parallels that for inhibition of Erk2 activation (Figure 4.3A). These results suggest that early MAP kinase activation may be involved in GM1-mediated increased DNA synthesis in U-1242 MG cells.

The effects of GM1 on Erk2 activation and DNA synthesis were compared to that of PDGF, which has been shown to activate the Erk signaling cascade. PDGF activated Erk2 as potently as did GM1 (Figure 4.4A), and this activation was also prevented by PD98059. However, in contrast to GM1, PDGF had no effect on DNA synthesis under these conditions (Figure 4.4B).
GM1 activation of p70\textsuperscript{65k} may also contribute to increased DNA synthesis.

Increased phosphorylation of ribosomal protein S6 by p70\textsuperscript{65k} is a common response of cells following treatment with mitogens such as PDGF or serum (Ferrari and Thomas, 1994). Therefore, GM1, PDGF and serum were compared for increased p70\textsuperscript{65k} catalytic activity. Cell lysates prepared from untreated as well as GM1- and PDGF-treated U-1242 MG cells were submitted to immunoprecipitation with antibodies specific for p70\textsuperscript{65k} and the immunoprecipitates tested for phosphotransferase activity using purified 40S ribosomal subunits as substrate. Figure 4.5A shows that relative to untreated cells GM1 treatment caused a clear but small increase in S6 phosphotransferase activity. PDGF and serum also increased p70\textsuperscript{65k} catalytic activity. Time course experiments indicated that the peak activation of p70\textsuperscript{65k} by GM1 occurred at 30 min (data not shown). Thus, p70\textsuperscript{65k} is activated by GM1 treatment at a later time compared to Erk2.

To assess the possible contribution of p70\textsuperscript{65k} activation to the mitogenic effects of GM1 the effects of rapamycin on GM1-stimulated p70\textsuperscript{65k} activity and [$^3$H]thymidine incorporation were examined. Rapamycin pretreatment inhibited the activation of p70\textsuperscript{65k} in response to subsequent GM1, PDGF or serum treatment (Figure 4.5A). Rapamycin was also able to prevent more than 50% of the increase in DNA synthesis seen in response to GM1 (Figure 4.5B). There was still about a two fold increase in DNA synthesis in response to GM1 after rapamycin treatment. While PDGF
treatment of U-1242 MG cells led to significant Erk2 and p70s6k activation, this same treatment failed to cause any increase in DNA synthesis (Figure 4.4).

**PD98059 and rapamycin inhibit separate pathways activated by GM1.**

To verify that the inhibitory effects of PD98059 and rapamycin were specific for Erk2 and p70s6k respectively, the effects of both inhibitors were tested on each of the two enzymes using immune complex kinase assays. Figure 4.6A shows that rapamycin has no effect on the GM1-stimulated activation of Erk2, whereas Erk2 activation is completely prevented by PD98059. Conversely, Figure 4.6B shows that PD98059 does not prevent activation of p70s6k at concentrations which abolish the GM1-stimulated Erk2 activation and increase in DNA synthesis (Figure 4.3). As shown in Figure 4.5, rapamycin completely inhibited GM1-stimulated activation of p70s6k. Thus, PD98059 and rapamycin inhibit separate signaling pathways activated by GM1 treatment.

**Serum, but not GM1, activates jun N-terminal kinase in U-1242 MG.**

The jun N-terminal kinase (Jnk1) is related to the Erk family of MAP kinases (Davis, 1994). It shares homology with Erks and is also activated by phosphorylation of the conserved threonine and tyrosine residues located in kinase subdomain VIII (Davis, 1994). Jnk1 is activated by a variety of stimuli, including several mitogens (Coso et al., 1995; Shapiro et al., 1996)
and many cellular stresses (Adler et al., 1995; Kyriakis et al., 1994; Pombo et al., 1994). To determine whether Jnk1 activity was stimulated by GM1, immune complex kinase assays were performed for Jnk1 on U-1242 MG cells treated with GM1.

Figure 4.7 shows that serum, which is a potent mitogen for U-1242 MG cells (Saqr et al., 1995a), caused an increase in Jnk1 phosphotransferase activity toward the c-Jun substrate. GM1 had no significant effect on Jnk1 activity. Addition of C2-ceramide or exogenous sphingomyelinase to U-1242 MG cells was also ineffective in activating Jnk1. PDGF, which is not mitogenic for these cells under these conditions, caused a small increase in Jnk1 activity. These results indicate that Jnk1 is not activated by GM1 treatment of these cells within the same time frame as Erk2. In separate time course experiments, treatment of U-1242 MG cells with GM1 for up to 2 hours did not lead to significant activation of Jnk1 (data not shown).

DISCUSSION

Previous studies have shown that gangliosides stimulate growth of some cells under specific conditions, but the underlying molecular mechanisms are unknown. In this chapter the effect of GM1 on two commonly studied mitogenic signal transduction pathways has been
examined in the human glioma cell line U-1242 MG. The results
demonstrate that both pathways are activated by GM1 in these cells and
suggest that they are important for the mitogenic effect of GM1.

GM1 treatment of U-1242 MG cells led to the rapid and transient
increase in Erk2 phosphotransferase activity. The increase in Erk2 activity
caused by GM1 was nearly equal to that of serum and shows that GM1 is a
potent activator of Erk2. In U-1242 MG cells pretreated with the MAPKK
inhibitor PD98059, GM1 failed to cause Erk2 activation. These results
argue against direct stimulation of Erk2 by GM1 and provide evidence that
GM1 stimulates a signaling cascade involving MAPKK or a MAPKK-related
protein kinase that subsequently activates Erk2.

Pretreatment with PD98059 also prevented the GM1-mediated
increase in DNA synthesis. Together with the biochemical data, this
suggests that Erk2 activation is involved in the GM1-mediated proliferation
of U-1242 MG cells. Since Erk2 activity peaks and returns to basal levels
within 1 hour after GM1 treatment, it is possible that the PD98059 blocks
GM1-stimulated DNA synthesis by preventing the early activation of Erk2.
It is also possible that PD98059 inhibits another, undefined pathway which
is involved in GM1-stimulated DNA synthesis, however the close correlation
between the concentrations of PD98059 needed to prevent Erk2 activation
and DNA synthesis, suggest that Erk2 activation is the important pathway
affected by PD98059. In addition PD98059 has been shown not to inhibit
several other kinases including Raf, cAMP-dependent protein kinase.
Activation of p70 S6 kinase occurs independently of Erk2 stimulation (Ming et al., 1994) and causes increased phosphorylation of the ribosomal protein S6 in proliferating cells (Ferrari and Thomas, 1994). As this signaling pathway is distinct from the Erk cascade we tested whether GM1 might also cause activation of p70s6k. Treatment of U-1242 MG cells with GM1 led to an increase in p70s6k activity which was completely suppressed by pretreatment with the immunosuppressant drug, rapamycin. We also observed a basal rapamycin-insensitive phosphorylation of ribosomal S6 protein by a kinase that immunoprecipitated with anti-p70s6k antibody. Greater than 50% of the increase in [3H]thymidine incorporation is prevented by rapamycin pretreatment, suggesting that activation of p70s6k is necessary for maximal stimulation of DNA synthesis by GM1. Since rapamycin did not influence Erk2 activity, and PD98059 did not affect p70s6k activity, we conclude that mitogenic concentrations of GM1 stimulate two distinct signaling cascades in U-1242 MG cells.

U-1242 MG cells have PDGF receptors that are activated by PDGF-BB (Saqr et al., 1995b; Van Brocklyn et al., 1993; Yates et al., 1992). Interestingly, PDGF treatment of U-1242 MG cells led to Erk2 and p70s6k activation, but this same treatment failed to increase DNA synthesis in these cells. Thus, GM1-stimulated DNA synthesis must involve other
mechanisms in addition to, or separate from, activation of Erk2 and p70s6k, otherwise PDGF treatment also would have increased DNA synthesis. Possibly, GM1 stimulates other signaling pathways in addition to the cascades involving Erk2 and p70s6k.

The jun N-terminal kinase (Jnk) is reported to be stimulated by several mitogens through pathways different from that of the Erks and p70s6k (Coso et al., 1995; Shapiro et al., 1996; Vojtek and Cooper, 1995; Westwick et al., 1994). Therefore, we examined whether GM1 treatment would lead to the activation of the Jnk1 isoform. Our results clearly demonstrate that serum, but not GM1 (or PDGF), enhanced Jnk1 activity. This indicates that while Jnk1 can be activated under our experimental conditions its activity is not affected by GM1, and therefore probably does not mediate the proliferative effects of GM1. On the other hand, these results highlight the specificity of GM1 for stimulating discrete signaling cascades in U-1242 MG cells.

Based on our [3H]-thymidine incorporation studies GM1 but not structurally related glycolipids, asialo GM1, and sulfatide, stimulated proliferation of U-1242 MG cells. Consistent with these results we observed little if any activation of Erk2 by these agents. Gangliosides are metabolized to ceramide (van Echten and Sandhoff, 1993). Ceramide has been shown to act as a second messengers in several cell types (Kolesnick and Golde, 1994; Obeid and Hannun, 1995), and to activate Erk2 (Raines et al., 1993). In addition short-chain ceramides, and exogenously added sphingomyelinase,
stimulate proliferation of some cells (Augé et al., 1996; Olivera et al., 1992). We, therefore, tested whether ceramide might mimic the effects of GM1 on Erk2 and DNA synthesis in U-1242 MG cells. Neither a cell-permeable ceramide analog, C2-ceramide, nor treatment with bacterial sphingomyelinase (to release endogenous ceramide from sphingomyelin) caused significant increases in $[^3H]$-thymidine incorporation. While C2-ceramide treatment did stimulate Erk2 activation, this was only about half as potent as that of GM1 at the same concentration. We conclude that ceramide has a negligible if any contribution to the effects of GM1 in these cells.

It should be emphasized that the growth inhibitory effects of GM1 on U-1242 MG cells, as described by Saqr et al. (Saqr et al., 1995a) and in Chapter 2 are, and the stimulatory effects investigated in this chapter occur under different growth conditions and by different mechanisms. The inhibition of DNA synthesis occurs in cells which have been stimulated to proliferate by addition of serum more rapidly than they would in serum free medium. We have hypothesized that this is due to inhibition of dimerization of growth factor receptors. Conversely, stimulation of DNA synthesis by gangliosides occurs in cells which have been deprived of serum, and are therefore proliferating at a slower, basal rate. Based on the data presented in this chapter, we propose that this is due to the activation of mitogenic signal transduction cascades including Erk2 and p70$^{56k}$. This difference in the regulation of growth by gangliosides and its dependence on
the cell culture conditions is what we refer to as the bimodal effect of gangliosides. These studies have provided the first insights into the molecular basis for the bimodal effect.

In summary, the results shown in this chapter demonstrate that treatment of U-1242 MG cells with mitogenic concentrations of GM1 causes activation of at least two distinct signaling pathways, the Erk and p70S6k cascades. These pathways may be important for the mitogenic effect of GM1 on these cells. One or more additional GM1-mediated signaling pathway may also be needed to drive the proliferation of U-1242 MG cells. The results also support the view that the entire ganglioside GM1 molecule is important for mediating its proliferative effects. Thus, gangliosides are likely to be important modulators of cellular signaling pathways, and this may be especially important in the brain where these glycolipids are concentrated.
Figure 4.1. Stimulation of DNA synthesis in U-1242 MG cells by GM1. Subconfluent U-1242 MG cells were serum starved for 48 hours then treated with: (A) the indicated concentration of GM1 or (B) 10 μM GM1 or other sphingolipids or sialic acid. [3H]thymidine incorporation into DNA was determined as described in Experimental Procedures. Error bars represent standard deviations from four replicates. Similar results were obtained in at least three independent experiments.
Figure 4.2. Activation of Erk2 by GM1 in U-1242 MG cells  

U-1242 MG cells were treated with: (A) 10 μM GM1 for the indicated time or (B) 10 μM GM1 or other sphingolipids for 15 min. Inset - Autoradiograph showing phosphorylated myelin basic protein (MBP) (arrow). Lane 1 - unstimulated; Lane 2 - 10 μM GM1; Lane 3 - 10 μM AsialoGM1 (GA1); Lane 4 - 10 μM sulfatide; Lane 5 - 10 μM C2-ceramide; Lane 6 - 0.1 units/ml sphingomyelinase. Cells were lysed and Erk2 was immunoprecipitated from cell lysates. Immunoprecipitates were assayed for phosphotransferase activity using MBP as substrate. Results are expressed as fold increase in 32P incorporated into MBP as determined by scintillation counting. Similar results were obtained in three independent experiments.
Figure 4.3. PD98059 inhibits GM1-stimulated Erk2 activation and DNA synthesis. U-1242 MG cells were pretreated with or without PD98059 at the indicated concentration for 30 min after which they were stimulated with 10 μM GM1 for 15 min (Panel A). Cells were lysed and Erk2 activity was measured as described in the legend for Figure 4.2. (A) pmol phosphate incorporated into myelin basic protein (MBP) as determined by scintillation counting of bands seen in inset. Inset - Autoradiograph showing phosphorylated MBP (arrow). All lanes except Lane 1 were stimulated with 10 μM GM1. Lanes 3 - 8 were pretreated with PD98059 at the following concentrations: Lane 3 - 0.1 μM; Lane 4 - 1 μM; Lane 5 - 10 μM; Lane 6 - 25 μM; Lane 7 - 50 μM; Lane 8 - 70 μM. (B) Cells were stimulated with 10 μM GM1 for 16 hours after which [3H]thymidine was added and incorporation into DNA was determined as described in Experimental Procedures. Error bars represent standard deviations from four replicates. Similar results were obtained in three independent experiments.
Figure 4.4. PDGF activates Erk2 similarly to GM1 but does not increase DNA synthesis. U-1242 MG cells were pretreated with or without 70 μM PD98059 for 30 min after which they were stimulated with 10 μM GM1 or 50 ng/ml PDGF for 15 min (Panel A) or 16 hours (Panel B). (A) Cells were lysed and Erk2 activity was measured as described in the legend for Figure 4.2. (B) [3H]thymidine was added and incorporation into DNA was determined as described in Experimental Procedures. Error bars represent standard deviations from four replicates. Similar results were obtained in three independent experiments.
Figure 4.5. Rapamycin prevents GM1-stimulated activation of p70^{S6k} and most of GM1-stimulated DNA synthesis. U-1242 MG cells were pretreated with or without 50 ng/ml rapamycin for 30 min. (A) Autoradiograph from immune complex S6 kinase assay. Cells were stimulated with 10 μM GM1 for 30 min, 50 ng/ml PDGF or 10% calf serum for 15 min, lysed and p70^{S6k} was immunoprecipitated from cell lysates. Immunoprecipitates were assayed for phosphotransferase activity using 40S ribosomal subunits as substrate. (B) Cells were stimulated with 10 μM GM1 for 16 hours after which [³H]thymidine was added and incorporation into DNA was determined as described in Experimental Procedures. Error bars represent standard deviations from four replicates. Similar results were obtained in three independent experiments.
Figure 4.6. **PD98059 and rapamycin inhibit separate pathways.** Both panels are autoradiographs of gels from immune complex kinase assays. (A) U-1242 MG cells were pretreated with or without 70 μM PD98059 or 50 ng/ml rapamycin for 30 min then stimulated with or without 10 μM GM1 for 15 min. Cells were lysed and Erk2 activity was measured as described in the legend for Figure 2. Arrow indicates phosphorylated myelin basic protein (MBP). Lanes 1-3 were unstimulated. Lanes 4-6 were stimulated with 10 μM GM1. Lanes 1 & 4 - no inhibitor; Lanes 2 & 5 - rapamycin; Lanes 3 & 6 - PD98059. (B) Cells were pretreated with or without the indicated concentration of PD98059 or 50 ng/ml rapamycin for 30 min then stimulated with or without 10 μM GM1 or 10% calf serum for 15 min. Cells were lysed and p70<sup>60k</sup> activity was measured as described in the legend for Figure 4.4. Lane 1 - Preimmune serum control immunoprecipitation; Lane 2 - unstimulated; Lanes 3 - 8 were stimulated with 10 μM GM1. Lanes 4 - 7 were pretreated with PD98059 at the following concentrations: Lane 4 - 1 μM; Lane 5 - 10 μM; Lane 6 - 25 μM; Lane 7 - 50 μM. Lane 8 - 50 ng/ml rapamycin.
Figure 4.7. GM1 does not activate Jnk1 in U-1242MG cells. U-1242 MG cells were treated with 10 μM GM1, 10 μM C2-ceramide, or 0.1 units/ml sphingomyelinase for 30 minutes, or 10% calf serum or 50 ng/ml PDGF for 15 minutes. Cells were lysed and Jnk1 was immunoprecipitated from cell lysates. Immunoprecipitates were assayed for phosphotransferase activity towards GST-cJun79 as described in Experimental Procedures. Inset - autoradiograph of gel from immune complex assay. Results are expressed as fold increase in $^{32}$P incorporated into GST-cJun79 as determined by scintillation counting. Results are means plus or minus standard deviations for at least three independent experiments.
CHAPTER 5

STUDIES ON THE MECHANISM OF ACTIVATION
OF ERK2 BY GANGLIOSIDE GM1

ABSTRACT

Treatment of quiescent U-1242 MG glioma cells with ganglioside GM1 causes increased DNA synthesis. Mitogenic concentrations of GM1 activate the mitogen-activated protein kinase Erk2 and p70 S6 kinase (p70\text{\text{S6}}). The mechanism for activation of Erk2 by GM1 was investigated. GM1 treatment of U-1242 MG cells did not cause an increase in protein tyrosine phosphorylation that could be detected by anti-phosphotyrosine Western blots or immunoprecipitation. Two inhibitors of Protein Kinase C, chelerythrine and GF 109203X, and prolonged TPA treatment failed to block activation of Erk2 by GM1. Erk2 activation was inhibited by forskolin treatment, however GM1 had no effect on cAMP levels. The MAP kinase kinase kinase c-Raf-1 was activated by GM1. GM1-stimulation of Erk2, p70\text{\text{S6}} and c-Raf-1 were blocked by pretreatment with wortmannin, but the
increase in DNA synthesis was not. These results suggest that GM1 activates Erk2 through a pathway that utilizes c-Raf-1 but is independent of protein tyrosine kinases, PKC or changes in cAMP levels.

INTRODUCTION

Several studies in recent years have focused on the regulation of protein phosphorylation by gangliosides. As discussed in some detail in the Introduction, gangliosides have been shown to modulate the activities of Ca\(^{2+}\)/calmodulin-dependent kinase II (Fukunaga et al., 1990), phosphorylase b kinase (Chan, 1989), protein kinase C (Kim et al., 1986; Kreutter et al., 1987; Momoi, 1986), cyclic-AMP-dependent protein kinase (Yates et al., 1989) and calcineurin (Bassi et al., 1991; Yates et al., 1988). However, these studies have been performed on broken cell preparations, purified or partially purified kinases rather than whole cells. Thus, the biological relevance of these effects must be interpreted with caution.

The experiments described in the previous chapter demonstrated activation of two kinases by the monosialoganglioside GM1 in whole cells. Erk2 and p70\(^{65k}\) were activated through separate signal transduction pathways and their activation correlated with the stimulation of DNA synthesis by GM1. Prevention of either GM1-stimulated Erk2 or p70\(^{65k}\) activation by PD98059 or rapamycin respectively correlated with inhibition
of GM1-stimulated DNA synthesis, suggesting that these kinases were important for this biological response to GM1.

Although both Erk2 and p70^{6k} are activated by a wide variety of agents (Ferrari and Thomas, 1994; Pelech and Sanghera, 1992) the pathway leading to Erk activation has been extensively studied and is well understood (Seger and Krebs, 1995), while that leading to p70^{6k} activation less clear (Ferrari and Thomas, 1994). Therefore, efforts were focused on how GM1 activates Erk2 by examining the effect of GM1 on upstream signaling events that lead to Erk activation.

**MATERIALS AND METHODS**

**Materials**

Materials which were also used in Chapter IV are from the same sources as noted in Chapter IV. Gangliosides were prepared as in Chapter IV.

Forskolin and wortmannin were from Sigma (St. Louis, MO). Forskolin was dissolved as a 10 mM stock in ethanol. Wortmannin was dissolved as a 1 mM stock in DMSO and protected from light as much as possible. Anti-cAMP antibody, and [^{125}I]succinyl-cAMP were generously provided by Dr. Richard Fertel. The sources of all other materials are as noted below.
Cell culture, $[^3]H$thymidine incorporation and Immune complex kinase assays

U-1242 MG human glioma cells were maintained as described in Chapter IV. $[^3]H$thymidine incorporation assays, and immune complex kinase assays for Erk2 and p70$^{65k}$ were performed as described in Chapter IV.

Anti-phosphotyrosine immunoprecipitation

Cells were seeded at 5,000/cm$^2$ and after 2 days were serum starved for 48 hours. Cells were stimulated by the addition of B0 containing 10 μM GM1 or 50 ng/ml PDGF for the indicated time at 37°C. They were then lysed by the addition of 0.5 ml lysis buffer containing 1% Triton X-100, 20 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 1 mM dithiothreitol (DTT), 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tertaacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na$_3$VO$_4$, 0.1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), and 1 μg/ml aprotinin. Following 10 minutes on ice, lysates were centrifuged 10 min at 4°C. The supernatants were incubated overnight at 4°C with 5 μl/sample of a cocktail of anti-phosphotyrosine antibodies consisting of PY20:PY72:4G10 (30:30:1). Rabbit anti-mouse IgG-conjugated Immunobeads (BioRad, 40 μl of a 50% suspension in lysis buffer) were added and the incubation continued for 2
additional hours at 4°C. The beads were pelleted by brief centrifugation and washed three times in 1 ml lysis buffer.

Western Blot Analysis

Cells were grown and serum-starved as detailed above in 6 well tissue culture plates. They were then stimulated with 10 μM GM1 (Fidia) or 50 ng/ml PDGF (R&D Systems). Cells were then washed in PBS and lysed by the addition of 1% sodium dodecyl sulfate (SDS) containing 1 mM Na₃VO₄, 5 mM EDTA. Cell lysates or anti-phosphotyrosine immunoprecipitates were boiled in Laemmli sample buffer (Laemmli, 1970) 5 min and separated by SDS-PAGE on 12.5% gels (acrylamide:bis 37.5:1). Proteins were then transferred to nitrocellulose (Micron Separations) using an Idea mini-blotter at 400 mAmpps for 1 hour. Membranes were blocked in 3% gelatin in Tris-Buffered Saline (TBS) for 30 min at room temperature for 4G10 blots, or 1% BSA in 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20 overnight at 4°C for RC20HRP blots. They were then incubated 2 hours with either 4G10 (Upstate Biotechnology) or 1 hour with RC20HRP (Santa Cruz Biotechnology) antiphosphotyrosine antibody. The membranes were then washed 5 times for 10 minutes each in TBS plus 0.5% Tween 20. For 4G10 blots, membranes were incubated an additional hour with 1:2,500 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase, then washed again as above. Enhanced chemiluminescence substrate (Amersham) was added for 1 minute and the membrane was exposed to film.
Phosphatidylinositol 3-kinase assays

Anti-phosphotyrosine immunoprecipitates were washed three times in 10 mM HEPES pH 7.4 to remove detergents. They were then resuspended in 40 μl assay buffer (30 mM HEPES pH 7.4, 30 mM MgCl₂, 1 mM EDTA, 50 μM ATP) and placed on ice. Phosphatidylinositol (PI) substrate was prepared by drying 100 μl PI stock (10 mg/ml in CHCl₃) in Speed Vac, resuspending in 100 μl 30 mM HEPES pH 7.4 and sonicating 5 minutes. PI was mixed 1:1 (vol:vol) with [γ-³²P]ATP at 1 μCi/μl in 30 mM HEPES pH 7.4. To initiate the reaction, 20 μl substrate mixture was added to each sample and reactions were incubated 30 min at room temperature. The reaction was terminated by addition of 100 μl 0.1 M HCl. The substrate was extracted with 300 μl chloroform:methanol (1:1). The organic phase was dried and redissolved in 50 μl chloroform:methanol (1:1).

A silica G-60 HPTLC plate was prepared by immersing it in CDTA buffer (4.55 g 1,2-cyclohexanediaminetetraacetic acid (CDTA), 330 ml ethanol, 3 ml 10 M NaOH, 165 ml H₂O). The plate was allowed to dry and baked 10 min at 80°C. Solvent was prepared by dissolving 12 g boric acid in a mixture of 75 ml methanol, 60 ml chloroform and 45 ml pyridine. Once dissolved, 7.5 ml water, 3 ml formic acid, 0.375 g butylated hydroxytoluene, and 75 μl ethoxyquin were added. Samples were spotted and the plate developed. The plate was allowed to dry and exposed to film for autoradiography.
Preparation of MEK1-K97

Plasmid encoding a recombinant hexahistidine tagged MEK1 was a gift of Dr. Gary Johnson of the University of Colorado at Boulder. This recombinant carries a K97 mutation which renders the enzyme catalytically inactive, but can still be used as a substrate for Raf isoforms. Recombinant protein was purified from 0.5 L cultures according to the method of Lange-Carter (Gardner et al., 1994) using TALON resin (Clontech, Palo Alto, CA). Purified protein was dialyzed into 50% glycerol in PBS and stored at -20°C.

Immune complex Raf assays

Cells were grown, serum starved and stimulated as above. They were then lysed in 0.5 ml extraction buffer (1% Triton X-100, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 20 μg/ml aprotinin, 1 mM PMSF, 2 mM Na3VO4). Lysates were centrifuged 10 min at 4°C and the supernatants were incubated 2 hours with 5 μl anti-c-Raf-1 or anti-B-Raf antibodies (Santa Cruz Biotechnology). Protein A agarose beads (Pierce - 40 μl of a 50% suspension in extraction buffer) were added and the incubation continued for an additional hour. The beads were then pelleted by brief centrifugation and washed twice in 1 ml cold extraction buffer, twice in PAN buffer (10 mM PIPES pH 7.0, 20 μg/ml aprotinin, 100 mM NaCl) plus 0.5% NP-40 and twice in PAN buffer. The beads were resuspended in 50 μl kinase buffer (20 mM PIPES pH 7.0, 10 mM MnCl₂, 20 μg/ml
aprotinin, 50 μM ATP, 5000 dpm/pmol [γ-32P]ATP, 1 μg MEK-K97) and incubated 30 min at 30°C. The reaction was stopped by the addition of 13 μl of 5 X concentrated Laemmli sample buffer (Laemmli, 1970) and the samples were boiled 5 min. Supernatants were separated by SDS-PAGE and phosphorylated MEK-K97 was visualized by autoradiography.

cAMP radioimmunoassay

Cells were grown and serum starved as above. They were stimulated with 10 μM GM1 for the indicated time or 10 μM forskolin for 30 min. TCA was added to a final concentration of 10% and the plates allowed to sit 30 min at room temperature. The precipitates were then scraped off the plates and centrifuged 10 min at room temperature in a microcentrifuge. Supernatants were extracted by the addition of 3 volumes of water-saturated diethyl ether. The extraction was repeated twice. Tubes containing the aqueous phase were placed in a hot water bath for 30 min in a fume hood to allow any remaining ether to evaporate. The pH was adjusted by the addition of 0.1 volumes 1 M sodium acetate pH 6.0. Samples were diluted 1:5 in 50 mM sodium acetate pH 6.3 and 100 μl was placed in a fresh tube. Acetylation was begun by adding 10 μl acetic anhydride:triethylamine (2:5) while vortexing. The acetylation was allowed to continue for 10 min and 50 μl 0.25 bovine gamma globulin (BGG), 50 μl [125I]succinyl-cAMP in 0.25 BGG (16,000 cpm/sample), and 50 μl anti-cAMP antibody were added. The reactions were allowed to sit overnight at 4°C.
The antibody was precipitated by the addition of 2 ml 60% saturated ammonium sulfate. Samples were centrifuged 20 min at 3500 g. The supernatant was discarded and the radioactivity counted. Concentrations of cAMP were determined by comparison to a standard curve which was generated using standards ranging from 0.15 to 5000 fmol cAMP/100 μl.

RESULTS

Lack of effect of GM1 treatment on tyrosine phosphorylation

To determine if GM1 treatment of U-1242 MG cells activated a protein tyrosine kinase which might form part of the pathway responsible for GM1 stimulation of Erk2 activity, western blotting was performed on lysates from cells treated with GM1 for various times. Figure 5.1A shows that no change in the pattern of protein tyrosine phosphorylation was observed in cells treated with GM1 for up to 2 hours.

To increase the sensitivity of phosphotyrosine detection immunoprecipitation with a cocktail of three different monoclonal antibodies to phosphotyrosine was performed on lysates from a larger number of cells. Approximately 2.5 x 10⁶ cells were used for immunoprecipitation as compared to approximately 1 x 10⁵ cells for direct western blotting. The immunoprecipitates were then probed on western blots with a different phosphotyrosine antibody. Figure 5.1B shows that several tyrosine phosphorylated bands were observed in immunoprecipitates.
from PDGF-treated cells. The apparent molecular weights of several of these proteins are similar to those of proteins which are known to tyrosine phosphorylated in response to PDGF. The band migrating at 170 kDa is likely to be the PDGF receptor which we have previously identified by western blotting (see Chapter 2). The band migrating at approximately 85 kDa may be the p85 subunit of Phosphatidylinositol 3′-kinase (PI 3′-kinase). Thus, this technique is able to detect tyrosine phosphorylated proteins. However, immunoprecipitates from cells treated with GM1 for up to 15 minutes were indistinguishable from untreated cells, indicating that GM1 does not cause tyrosine phosphorylation of any proteins that could be detected by this technique.

Lack of effect of PKC inhibition on GM1-stimulated Erk2 activation

PKC isoforms are important for activation of the MAP kinase pathway by several agents (Hill et al., 1995; Kazlauskas and Cooper, 1988; L'Allemand et al., 1991; Seufferlein and Rozengurt, 1995; Young et al., 1996). Therefore, the effect of PKC inhibition on GM1-stimulated Erk2 activation was examined. Figure 5.2 shows that two PKC inhibitors, chelerythrine chloride and the bisindolylmaleimide GF 109203X had no effect on GM1-stimulated Erk2 activity when used at their reported IC50s, 1μM for chelerythrine chloride (Herbert et al., 1990) and 2 μM for GF 109203X (Toullec et al., 1991). A separate experiment showed that treatment of U-1242 MG cells for 24 hours with a high concentration of TPA, to
downregulate phorbol ester-sensitive PKC isoforms, prevented activation of Erk2 by a subsequent TPA challenge (data not shown). However, prolonged TPA treatment caused only a slight decrease in GM1-stimulated Erk2 activity (Figure 5.2).

Thymidine incorporation experiments using several doses of the two PKC inhibitors showed a 50% inhibition of GM1-stimulated DNA synthesis by chelerythrine at 1 μM and no significant effect of GF 109203X (data not shown). However, considerable cytotoxicity was noted at 3 μM chelerythrine. Thus, it is unclear whether the moderate inhibition by 1 μM chelerythrine is due to PKC involvement or a mild toxicity at this concentration. The lack of effect of GF 109203X suggests that PKC may not be involved in GM1-stimulation of DNA synthesis.

Activation of c-Raf-1 but not B-Raf by GM1

To further clarify the signaling mechanism by which GM1 activates Erk2 the effect of GM1 on the activities of the kinases c-Raf-1 and B-Raf was investigated. c-Raf-1 was immunoprecipitated from lysates of subconfluent U-1242 MG cells which had been treated with or without 10 μM GM1, 50 ng/ml PDGF or 10% calf serum. Immunoprecipitates were then tested for the ability to phosphorylate a kinase-inactive mutant of MEK1 (MEK1-K97). Figure 5.3A shows that GM1 caused an increase in the activity of c-Raf-1 compared to unstimulated cells. PDGF activated this kinase more potently than GM1, however, serum was completely ineffective.
The kinase B-Raf is also capable of activating the MAP kinase pathway (Papine et al., 1995; Reuter et al., 1995). Therefore, immune complex kinase assays using antibody specific for B-Raf were performed on lysates from GM1- as well as PDGF- or serum-treated U-1242 MG cells. Figure 5.3B shows that none of the agents tested had a strong effect on B-Raf activity. PDGF appeared to cause a very slight activation of B-Raf, however GM1 was completely ineffective in two separate experiments. Interestingly, serum, which activates Erk2 in U-1242 MG cells, failed to activate either c-Raf-1 or B-Raf, suggesting that serum may activate Erk2 through another MAP kinase kinase kinase such as A-Raf or MEK kinase.

cAMP antagonizes GM1-stimulated mitogenic signaling

Several reports have shown that increased intracellular levels of cAMP inhibit mitogen-stimulated activation of the MAP kinase signaling pathway by preventing the interaction of c-Raf-1 with the small GTP-binding protein p21 ras (Graves et al., 1993; Sevetson et al., 1993; Wu et al., 1993). This is mediated by phosphorylation of c-Raf-1 by cAMP-dependent protein kinase (Wu et al., 1993). In addition, gangliosides have been shown to stimulate cyclic nucleotide phosphodiesterase at nanomolar concentrations in vitro (Yates et al., 1989). It is therefore possible that GM1 could be acting by lowering cellular cAMP levels, thus releasing c-Raf-1 from a negative regulation by PKA.
To test this possibility the effect of the adenylate cyclase activator forskolin on GM1-stimulated signaling was tested. Figure 5.4A shows that forskolin inhibited basal as well as GM1-stimulated Erk2 activity in a dose responsive manner. At high concentrations, forskolin overcame GM1-stimulation of Erk2 activity completely. Forskolin was also able to block GM1-stimulated activation of p70^{66k}, although it had no effect on basal p70^{66k} activity (Figure 5.4B). As expected for an agent that blocks GM1-stimulation of Erk2 and p70^{66k}, forskolin also prevented GM1-stimulated increased DNA synthesis (Figure 5.4 C).

To determine whether GM1 treatment of U-1242 MG cells altered levels of cAMP, radioimmunoassays for cAMP were performed on untreated as well as GM1- and forskolin-treated cells. Figure 5.4D shows that whereas forskolin increases cAMP levels in U-1242 MG cells, treatment with GM1 for up to 1 hour causes no significant change in cAMP levels.

Effect of wortmannin on GM1 signaling

Several recent reports have shown that some agents activate the MAP kinase pathway in a wortmannin sensitive manner, suggesting the possible involvement of phosphatidylinositol 3-kinase (PI 3'-kinase) (Ferby et al., 1996; Hawes et al., 1996; Karnitz et al., 1995; Von Willebrand et al., 1996). Therefore, the effect of wortmannin on GM1 signaling in U-1242 MG cells was examined. Figure 5.5A shows that wortmannin inhibited GM1-stimulated activation of Erk2 in a dose-responsive fashion, whereas it had
only a small effect on PDGF-stimulated Erk2 activation even at 100 nM. Consistent with this result, wortmannin completely blocked GM1-stimulated activation of c-Raf-1, but only partially blocked activation of c-Raf-1 by PDGF (Figure 5.5B).

Wortmannin pretreatment of U-1242 MG cells also blocked GM1-stimulated activation of p70^{60k} at a concentration similar to that for Erk2 inhibition. Surprisingly, pretreatment of U-1242 MG cells with up to 500 nM wortmannin had no significant effect on GM1-stimulated DNA synthesis (Figure 5.5D).

To determine if GM1 activated PI 3'-kinase in U-1242 MG cells antiphosphotyrosine immunoprecipitates were tested for their abilities to phosphorylate phosphatidylinositol. Figure 5.5E shows that although PDGF activated a phosphotyrosine associated PI 3'-kinase, treatment with GM1 for up to 15 minutes did not.

**PD98059 inhibits GM1-stimulated DNA synthesis when added after GM1**

The lack of effect of wortmannin on GM1-stimulated DNA synthesis, taken together with its complete inhibition of GM1-stimulated Erk2 activation, suggests that PD98059 inhibits DNA synthesis by a mechanism other than blocking the early activation of Erk2 (i.e. during the first hour of GM1 treatment). To determine if PD98059 might be acting by inhibiting a later GM1-stimulated event, [3H]thymidine incorporation experiments were
performed in which PD98059 was added at various times after GM1. Figure 5.6A shows that up to 14 hours after addition of GM1, addition of PD98059 is still able to block GM1-stimulated DNA synthesis.

Since GM1-stimulated U-1242 MG cells do not begin to synthesize DNA for 24 hours after GM1 treatment (Saqr et al., 1995a), the time course of PD98059 addition was extended to 23.5 hours after GM1 addition. Figure 5.6B shows that even at the latest time tested PD98059 is able to inhibit GM1-stimulated DNA synthesis.

DISCUSSION

In the previous chapter it was shown that the activation of Erk2 by GM1 was sensitive to the inhibitor PD98059 which is known to prevent the activation of MEK by Raf (Alessi et al., 1995), suggesting that GM1 activates the MAP kinase pathway by acting at, or upstream of a Raf isoform. Therefore, the main focus of the experiments in this chapter was to elucidate further details of GM1 signaling upstream of MAP kinase.

Many agents activate the MAP kinase pathway by stimulation of a protein tyrosine kinase (Crews and Erikson, 1993; Davis, 1993; Nishida and Gotoh, 1993; Pelech, 1993). Surprisingly, western blotting of lysates from GM1-treated cells revealed no change in tyrosine phosphorylation compared to untreated cells. Immunoprecipitation from a larger number of cells also
revealed no change in tyrosine phosphorylation, although this method detected several tyrosine phosphorylated proteins in response to PDGF. It remains possible that GM1 causes a weak tyrosine phosphorylation event which could not be detected by these methods. However, GM1 and PDGF lead to similar levels of Erk2 activation, yet PDGF potently activates tyrosine phosphorylation, suggesting that GM1 activation of Erk2 may not involve an upstream tyrosine kinase. In addition, preincubation with the tyrosine kinase inhibitor tyrphostin B42 had no effect on GM1-stimulated Erk2 activity (data not shown).

Activation of PKC by phorbol esters is known to lead to activation of the MAP kinase pathway (Hoshi et al., 1988; Troppmair et al., 1994). This is thought to be a result of a direct activation of Raf by PKC (Kolch et al., 1993). It was therefore of interest to determine whether GM1 might be activating the MAP kinase pathway through activation of PKC. Three different strategies were used to inhibit PKC. Prolonged treatment with phorbol ester to downregulate PKC, and two different pharmacological inhibitors, chelerythrine chloride and the bisindolylmaleimide GF 109203X, all failed to prevent the activation of Erk2 by GM1. This indicates that GM1 activates Erk2 by a PKC-independent mechanism.

The kinase c-Raf-1 is ubiquitously expressed and functions as a MAP kinase kinase kinase in many different cell types. However, the related enzyme B-raf is expressed in neural tissue (Storm et al., 1990) and is thought to be the major regulator of the MAP kinase pathway in PC12 cells.
We, therefore, examined the effect of GM1 on both c-Raf-1 and B-Raf. Raf catalytic assays revealed that GM1 activates the c-Raf-1 isoform but not the B-Raf isoform. Since c-Raf-1 is known to be inhibited by increases in cAMP levels through phosphorylation by PKA (Wu et al., 1993), lower cAMP levels could lead to activation of c-Raf-1. The results show that increasing cAMP levels with the adenylate cyclase activator forskolin antagonizes GM1 signaling in U-1242 MG cells, consistent with the possibility that GM1 might signal by lowering cAMP. However, quantitative cAMP assays revealed no significant effect of GM1 on cAMP concentration. Therefore, although cAMP is capable of modulating GM1 signaling, GM1 does not act by altering cAMP concentration.

PI 3'-kinase is a ubiquitous signaling enzyme which has been shown to be necessary for stimulation of mitogenesis by several agents (Varticovski et al., 1994), and is specifically inhibited by wortmannin at nM concentrations (Ui et al., 1995). Wortmannin pretreatment of U-1242 MG cells inhibited GM1-stimulated activation of Erk2, c-Raf-1 and p70S6k. However, wortmannin was much less potent at preventing PDGF-stimulated Erk2 activation. In addition, PDGF strongly activated a phosphotyrosine associated PI 3'-kinase. This enzyme is likely to be the p85/p110 PI 3'-kinase, which is known to be regulated by interaction of the p85 subunit with phosphotyrosine residues via its SH2 domain. Consistent with the lack of effect of GM1 on tyrosine phosphorylation, GM1 did not activate a
phosphotyrosine-associated PI 3'-kinase. Therefore, it appears that GM1 signals to c-Raf-1, Erk2, and p70\textsuperscript{66k} through a wortmannin-sensitive mechanism which does not involve the "classical" p85/p110 PI 3'-kinase.

Surprisingly, wortmannin had no significant effect on GM1-stimulated DNA synthesis even at 500 nM. This suggests that the activation of Erk2 at early times after GM1 treatment, which is potently inhibited by either wortmannin or PD98059 is not crucial for the stimulation of DNA synthesis by GM1. Since it has been shown that GM1-stimulated U-1242 MG cells do not enter S phase for at least 24 hours after GM1 treatment (Saqr et al., 1995a), it is possible that an important signaling event occurs after prolonged treatment with GM1. Since PD98059 has a strong inhibitory effect on GM1-stimulated DNA synthesis, experiments were done to determine whether the crucial PD98059-sensitive event might be occurring at later times following GM1 stimulation. The results clearly show that PD98059 is capable of inhibiting GM1-stimulated DNA synthesis even when it is added 23.5 hours after GM1. This indicates that a crucial event for GM1-stimulation of DNA synthesis occurs at very late times after GM1 treatment and that this event is sensitive to PD98059. Further experiments are necessary to determine whether this late event is an activation of a MAP kinase or another pathway which is also sensitive to PD98059.

Exogenously added gangliosides are slowly incorporated into the plasma membrane of cells in culture (Saqr et al., 1993). The long delay
before the crucial PD98059-sensitive event could be the result of a gradual uptake of GM1 by U-1242 MG cells. Alternative possibilities include a slow metabolism of added gangliosides to a mitogenic sphingolipid, such as sphingosine 1-phosphate, or late expression of a growth factor which then acts in an autocrine fashion.

In summary, the activation of Erk2 by GM1 treatment of U-1242 MG cells does not appear to involve an upstream tyrosine kinase, PKC or modulation of cAMP levels. In contrast to PDGF, GM1 signals to both Erk2 and p70^{6k} via a wortmannin-sensitive pathway. However, these early events are not necessary for GM1-stimulated DNA synthesis. Rather, a much later event, which is sensitive to PD98059 but not wortmannin, is crucial for the proliferative response.
**Figure 5.1.** GM1 causes no detectable increase in tyrosine phosphorylation. A) U-1242 MG cells were treated with 10 μM GM1 for the indicated time. Whole cell lysates were resolved by SDS-PAGE and western blotting was performed using a monoclonal antibody specific for phosphotyrosine (4G10). Similar results were obtained in two independent experiments. B) U-1242 MG cells were treated with 10 μM GM1 for the indicated time or 50 ng/ml PDGF for 5 min. Immunoprecipitation was performed using a cocktail of antibodies to phosphotyrosine (PY20:PY72:4G10, 30:30:1). A different phosphotyrosine specific antibody (RC20HRP) was used to detect tyrosine phosphorylated proteins by western blotting. Filled arrows indicate positions of molecular weight standards. Unfilled arrows indicate positions of PDGF-stimulated phosphotyrosine containing proteins.
Figure 5.2. Activation of Erk2 by GM1 is PKC-independent.
U-1242 MG cells were pretreated for 30 min with DMSO vehicle (Lanes 1), 1 µM chelerythrine chloride (Lanes 2) or 2 µM GF109203X (Lanes 3), or for 24 hours with 1 µg/ml TPA (Lanes 4). Cells were then treated with or without 10 µM GM1 for 15 min and Erk2 activity was measured by immune complex kinase assay. A) Autoradiograph showing phosphorylated myelin basic protein (MBP) (arrow). B) Radioactivity incorporated into MBP was determined by scintillation counting. Similar results were obtained in two independent experiments.
Figure 5.3. GM1 activates c-Raf-1 but not B-Raf.
Autoradiographs of gels from immune complex Raf kinase assays. U-1242 MG cells were treated with 10 µM GM1 for 10 min (A), for the indicated time (B) or with 50 ng/ml PDGF for 10 min. Kinase activity toward MEK-K97 was measured by immune complex kinase assay using antibodies to c-Raf-1 (A) of B-Raf (B).
Figure 5.4. Effect of forskolin on GM1-stimulated mitogenic signaling. U-1242 MG cells were pretreated with the indicated concentration of forskolin for 30 min (A, B and C) and then stimulated with 10 μM GM1 for 15 min (A), 30 min (B) or 16 hrs (C). A) Erk2 activity was determined by immune complex kinase assay. B) p70S6k activity was determined by immune complex kinase assay. C) [%H]thymidine incorporation was determined. D) U-1242 MG cells were treated with 10 μM GM1 for the indicated time or 10 μM forskolin for 30 min. cAMP concentrations were determined by radioimmunoassay. Error bars represent standard deviations from duplicate samples.
Figure 5.5. GM1 activation of the MAP kinase pathway and p70^S6k, but not DNA synthesis, is inhibited by wortmannin. Panels A, B, C and E are autoradiographs from kinase assays. A) U-1242 MG cells were pretreated for 30 min with the indicated concentration of wortmannin or with vehicle. Cells were stimulated with 10 μM GM1 or 50 ng/ml PDGF for 15 min and Erk2 activity was measured by immune complex kinase assay. The arrow indicates phosphorylated myelin basic protein (MBP). Radioactivity was quantitated by scintillation counting. B) Cells were pretreated for 30 min with 100 nM wortmannin or vehicle, then stimulated with 10 μM GM1 or 50 ng/ml PDGF for 10 min and c-Raf-1 activity was determined by immune complex kinase assay. C) Cells were pretreated for 30 min with the indicated concentration of wortmannin or with vehicle. Cells were stimulated with 10 μM GM1 for 30 min and p70^S6k activity was measured by immune complex kinase assay. The arrow indicates phosphorylated S6. Radioactivity was quantitated by scintillation counting. D) Cells were pretreated for 30 min with the indicated concentration of wortmannin or with vehicle, then stimulated with 10 μM GM1 and [3H]thymidine incorporation was determined. E) Cells were stimulated with 10 μM GM1 for the indicated time or 50 ng/ml PDGF for 5 min. Anti-phosphotyrosine immunoprecipitates were assayed for the ability to phosphorylate phosphatidylinositol (PI). As described in Methods, 3-phosphorylated PI was separated by TLC on silica G-60 plates.
Figure 5.6. PD98059 inhibits GM1-stimulated DNA synthesis when added after GM1.
(A) U-1242 MG cells were stimulated with 10 μM GM1 for 16 hrs. At the indicated times after GM1 addition medium was changed to 10 μM GM1 plus 50 μM PD98059. [3H]Thymidine incorporation was measured. The -30 min time indicates 30 min pretreatment with PD98059.
(B) U-1242 MG cells were stimulated with 10 μM GM1 for 24 hrs. At the indicated times after GM1 addition medium was changed to 10 μM GM1 plus 25 μM PD98059. [3H]Thymidine incorporation was measured.
CHAPTER 6

FINAL CONCLUSIONS AND FUTURE STUDIES

This dissertation has examined the role of gangliosides in regulating cellular proliferation and mitogenic signal transduction. Because of the prevalence of gangliosides in the central nervous system and evidence implicating them in glioma cell growth we have concentrated on a human malignant glioma cell line, U-1242 MG. However, we were also specifically interested in the effects of gangliosides on signal transduction by the mitogen PDGF. We, therefore, also made use of the murine fibroblast cell line Swiss 3T3, which is known to express a large number of PDGF receptors and has been well characterized for stimulation of mitogenic signaling by PDGF.

We wished to determine whether effects which had been noted for gangliosides on signal transduction enzymes in vitro were capable of occurring in whole cells. Since gangliosides are thought to be located predominantly in the outer leaflet of the plasma membrane it is questionable whether modulation of the activities of intracellular enzymes
is biologically relevant. Even in the case of growth factor receptors, which would probably be more accessible to gangliosides, dependence of the *in vitro* effects on assay conditions called their relevance into question. In addition, without the ability to demonstrate an effect of gangliosides in whole, living cells, the effects can not be correlated with a biological event.

We were able to examine both growth inhibitory and stimulatory effects of gangliosides on whole cells. Gangliosides, which are known to inhibit PDGF-stimulated proliferation of Swiss 3T3 cells, were shown to prevent PDGF-stimulated tyrosine phosphorylation of PDGF receptors. Our understanding of how gangliosides modulate growth factor receptors was extended further by the demonstration that gangliosides inhibit PDGF receptor activation by preventing dimerization of receptor monomers.

Dimerization is a common mechanism for activation of receptor tyrosine kinases. Gangliosides have been shown to inhibit activation of other dimerizable receptors as well (Bremer and Hakomori, 1982; Bremer et al., 1986). It is clear that different gangliosides have different efficacies for the various receptors affected. In addition, ganglioside GM1 can enhance NGF-stimulated activation of its receptor, Trk (Mutoh et al., 1995; Rabin and Mocchetti, 1995). This appears to be due to increased Trk dimerization (Farooqui, et al., manuscript in preparation). Thus modulation of receptor dimerization may be a general mechanism for regulation of signal transduction by gangliosides.
By altering the activities of the glycosyltransferases responsible for ganglioside biosynthesis a cell could change its responsiveness to available growth factors. In addition, changes in gangliosides expressed on tumor cells could contribute to increased tumor cell proliferation by allowing increased activation of mitogenic growth factor receptors. This possibility is consistent with the pattern of change in ganglioside expression typically seen in gliomas, a simplification of ganglioside structure, since more complex gangliosides were more effective at inhibiting PDGF receptor activation (Chapter 2).

Growth factors such as PDGF signal by activation of pathways which often involve cascades of protein phosphorylation reactions. The MAP kinase pathway is the best studied of these mitogenic signaling cascades. This pathway is thought to be necessary, and at least in some cases, sufficient to stimulate proliferation. Regarding the role of the MAP kinase pathway in the growth of U-1242 MG glioma cells, we have found the following:

1. U-1242 MG cells contain a moderate amount of active Erk2 even when serum starved.
2. The MAP kinase kinase inhibitor PD98059 decreases basal DNA synthesis in serum starved U-1242 MG cells.
3. Erk2 activity is increased in U-1242 MG cells by treatment with growth factors, phorbol esters, serum and GM1.
4. Not all of the agents that activate Erk2 stimulate DNA synthesis.

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5. PD98059 blocks Erk2 activation by PDGF or GM1.

6. PD98059 also prevents GM1-stimulated DNA synthesis, but can do so when added long after Erk2 activity has returned to basal levels.

7. The PI 3'-kinase inhibitor wortmannin blocks Erk2 activation by GM1 but does not inhibit GM1-stimulated DNA synthesis.

The first two observations suggest that a constitutive signaling to the MAP kinase Erk2 may be involved in the continuous proliferation of this transformed cell line. However, it is clear that activation of Erk2 is not sufficient to stimulate further growth, since under the conditions where PDGF activates Erk2 it does not increase DNA synthesis. Combined with the inhibition of both Erk2 activation and GM1-stimulated DNA synthesis by PD98059, the data to this point suggested that Erk2 activation is necessary but insufficient for increased DNA synthesis in U-1242 MG cells.

Contrary to the above interpretation, the lack of effect of wortmannin on GM1-stimulated DNA synthesis, while it clearly inhibits Erk2 activation, indicates that Erk2 activation is not required. Also, if Erk2 activation is necessary, one would expect to lose the inhibitory effect of PD98059 when the inhibitor was added after the time course of Erk2 activation by GM1. This is clearly not the case. Two possibilities exist that could explain these data. 1) Erk2 activation by GM1 is not required for increased proliferation, suggesting that PD98059 inhibits some other event necessary for GM1-stimulated DNA synthesis. The basal level of Erk2 activity present in
unstimulated U-1242 MG cells may be sufficient to support the proliferation of these cells. Further increases may require a separate, yet unidentified pathway which is also affected by PD98059. 2) PD98059 may be inhibiting a later activation of a MAP kinase which occurs just before the cells enter S phase. Although MAP kinase assays were performed at several later times, we can not rule out that a transient activation occurred that was not detected at the time points chosen.

The wortmannin data raise the same questions which are discussed above for Erk2 concerning p70\textsuperscript{sk} activation. In light of the fact that wortmannin blocks GM1-activation of p70\textsuperscript{sk}, what is the relevance of the inhibition of GM1-stimulated DNA synthesis by rapamycin? Similar possibilities exist to those described above for PD98059. p70\textsuperscript{sk} may be required at a later time to support increased protein synthesis before the cells enter S phase.

Consistent with the idea that the inhibitors act at a later time, stimulation of DNA synthesis by gangliosides requires a lag phase of at least 24 hours before the cells begin to incorporate thymidine (Saqr et al., 1995a). This is considerably longer than the time required after serum treatment of U-1242 MG cells. Possible explanations for this long lag include the following: 1) As exogenously added gangliosides are slowly incorporated into cells in culture (Saqr et al., 1993) several hours may be required to build up sufficient amounts of GM1 in U-1242 MG cells to stimulate the critical pathway(s). 2) Early events triggered by GM1 may
lead to the expression of a growth factor and/or its receptor, activation of which then stimulates the cells in an autocrine fashion.

We also attempted to determine the mechanism by which GM1 activates Erk2. While several possibilities, such as tyrosine kinase activation, protein kinase C activation, and changes in cAMP levels, were eliminated or at least shown to be unlikely, two important clues were discovered. First, GM1 activates the kinase c-Raf-1 which is known to be located upstream of Erks in the pathway activated by many agents. Second, GM1-stimulated Erk2 activation is sensitive to wortmannin. This suggests that PI 3'-kinase may be involved, however GM1 did not activate a PI 3'-kinase activity in phosphotyrosine immunoprecipitates, whereas PDGF did. Therefore, the wortmannin-sensitive GM1-activated enzyme is not the conventional p85/p110 PI 3'-kinase which is known to be activated by association with tyrosine phosphorylated proteins.

Figure 6.1 shows the proposed pathways activated by GM1 treatment of U-1242 MG cells. Two of the proteins labeled with question marks, FRAP and MEK, have not been investigated in these studies, but are included as possible mediators of the effects of rapamycin and PD98059 respectively. It is thought that FRAP (FKBP12-rapamycin associated protein) is the enzyme that is specifically inhibited by rapamycin (Brown et al., 1994; Choi et al., 1996). FRAP itself is a kinase which regulates the activity of p70^S6k in a rapamycin-sensitive manner (Brown et al., 1995). Also, PD98059 is thought to be a specific inhibitor of MEK activation (Alessi et al., 1995). It is
therefore reasonable to propose that FRAP and MEK may be involved in GM1 signaling.

The inhibitory effect of wortmannin on both Erk2 and p70\(^{65k}\) activation suggests that a PI 3'-kinase, or a related enzyme, may be involved in GM1 signaling at an early stage. Further experiments are required to determine if this is the case or if wortmannin is acting through some other undefined mechanism.

In summary, we have identified several mitogenic signal transduction systems which can be modulated by gangliosides. We have shown that gangliosides can alter signals at the cell surface by affecting growth factor receptor activation, and within the cell by activating mitogenic signal transduction pathways (see Figure 6.1). Although further work is necessary to determine the exact contribution of these effects to the regulation of cellular proliferation by gangliosides, our studies have contributed to the knowledge of ganglioside biology by demonstrating that gangliosides can modulate specific signal transduction pathways in whole, living cells and that this can be correlated with a biological outcome such as cell growth.
Figure 6.1. Proposed signaling pathways activated by GM1, showing points at which inhibitors are likely to act.

Future Studies

The results of this work as discussed above suggest a number of further experiments to define better how gangliosides modulate cell growth including the following:

1. The inhibition of receptor dimerization by gangliosides suggests a physical interaction between the PDGF receptor and the gangliosides. Preliminary work in Dr. Yates' laboratory indicates that GM1 binds specifically to the PDGF receptor. Efforts are now underway to define the GM1 binding site on the PDGF receptor through the use of recombinant mutated PDGF receptors.

2. The necessity of MAP kinases for basal and for GM1-stimulated DNA synthesis in U-1242 MG cells could be more clearly defined by use of alternate means of inhibiting MAP kinases. These include transfection of dominant negatively acting mutant MAP kinases, or the use of antisense
oligonucleotides. These approaches would avoid the potential problem of non-specificity, which is always an issue for pharmacological inhibitors.

3. Although the wortmannin sensitivity suggests the involvement of a PI 3'-kinase in GM1-stimulated Erk2 activation, further experiments are needed to demonstrate activation of such an enzyme. Labeling of U-1242 MG cells with either [3H]inositol or [32P]orthophosphate and assaying levels of 3-phosphorylated inositides would establish activation of such an enzyme.

4. The involvement of Raf-1 in the activation of Erk2 by GM1 suggests that ras may also be important. This can be tested by transfection with a dominant negative mutant of ras.

5. The critical events for GM1-stimulation of DNA synthesis should be defined. This can be done by first determining at what time after addition of GM1 PD98059 needs to be present to block the stimulation. This would then be the time at which to investigate the critical event which is blocked by PD98059. Assay of MAP kinases would then determine whether Erks are necessary at this time or whether other pathways must be sought.

6. The possibility of activation of an autocrine pathway by GM1 could be investigated by treating U-1242 MG cells with conditioned media from GM1-stimulated cells and determining whether the cells enter S phase after a shorter lag period.
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