THE EXPRESSION OF PROTEIN KINASES AND THE ROLE OF EXTRACELLULAR SIGNAL-REGULATED PROTEIN KINASES 1 AND 2 IN OLIGODENDROCYTES

by

ROCHELLE HEISEL

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Department of Medicine

The University of British Columbia
Vancouver, Canada

Date Aug. 24/01

ABSTRACT

Oligodendrocytes (OL), the myelinating cells of the central nervous system, extend processes to contact axons and wrap them in an insulative layer of myelin. This series of studies was undertaken to examine the role of extracellular signal-regulated protein kinases (ERKs) 1 and 2 in OL process extension. First, it was determined that stimulation of mature primary bovine OL with the phorbol ester PMA could induce both process extension and ERK1/2 activation. Furthermore, application of the MEK1 inhibitor PD 98059 was able to both block PMA-induced process extension and reduce ERK1/2 phosphotransferase activity. Thus it appears that a threshold of ERK1/2 phosphotransferase activity is required for primary OL process extension.

To continue to elucidate the signalling cascades involved in OL process extension, the Central-Glial 4 (CG-4) cell line was assessed for suitability as an OL model. CG-4 are bipotential cells capable of differentiating into either astrocytic or oligodendrocytic (CG-4 OL) cells. A multi-kinase Western blot profile was conducted to compare the kinase expression patterns of primary rat OL to CG-4 OL. Overall, the expression of a wide variety of kinases, including conventional protein kinase C (PKC) isoforms, mitogen-activated protein kinases, protein kinase A and protein kinase B were very similar between the two cell types. However, some differences in kinase expression were detected. Increased expression of focal adhesion kinase, PKC-ε and cyclin-dependent kinase (CDK) 7 in CG-4 cells could be a function of the self-renewal capacity of this cell line. Increased expression of Pak-α, PKC-δ and CDK5 in primary OL

could explain why these primary cells can achieve a greater degree of differentiation than CG-4 OL.

After verifying the suitability of the CG-4 cell line as an OL model, further process extension studies were undertaken. It was found that transient ERK1/2 activation is required to prevent bipolar CG-4 cells from acquiring a multipolar phenotype. This transient ERK1/2 activation was provided by addition of medium containing B-104 mitogens. In B-104 mitogen-free medium, ERK1/2 was not activated and the CG-4 cells acquired a multipolar phenotype. Furthermore, PMA was able to activate ERK1/2 in lieu of B-104 mitogens, while at the same time inhibiting the formation of a multipolar phenotype. To verify a role for ERK1/2 activation in the inhibition of a multipolar phenotype, CG-4 cells were exposed to B-104 mitogens in the presence of the MEK1/2 inhibitors PD 998059 or UO-126. Surprisingly, even pretreatment of the cells with either MEK inhibitor could not induce the formation of multipolar processes. Western blots, however, indicated that neither inhibitor was able to completely abolish ERK1/2 activity. Therefore, it is possible that the MEK1/2 inhibitors were unable to reduce ERK1/2 activity below the level necessary to inhibit multipolar process formation.

In summary, ERK1/2 activation can both induce process extensions from primary OL and inhibit the formation of a multipolar phenotype in CG-4 OL. This could indicate that the CG-4 cell line does not make a suitable model for OL signal transduction studies. Therefore, caution must be taken when applying the results of signal transduction experiments conducted on CG-4 cells to primary OL.

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5% HS	Dulbecco's Modified Eagle Mediim plus 5% horse serum
70/30	70% N1 + 30 % B-104-conditioned medium
ADB	assay dilution buffer
AEC	3-amino-9-ethyl-carbazole
ATP	adenosine triphosphate
BSA	bovine serum albumin
bFGF	basic fibroblast growth factor
CG-4	Central-Glial 4 cell line
CG-4 OL	oligodendrocytic CG-4
cm	centimeter
CNP	2,3-Cyclic nucleotide 3-phosphohydrolase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminotetraacetic acid
ERK	extracellular signal-regulated protein kinase
GalC	galactocerebroside
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
Grb-2	Growth factor receptor binding-2
GTP	guanosine triphosphate
HCl	hydrochloric acid
HOG	high osmolarity glycerol response kinase
hr	hour

IP_3	inositol (1,4,5) tris-phosphate
IL-1	interleukin-1
JNK	c-Jun N-terminal kinase
LIF	leukemia inhibitory factor
M	molar
mA	milliampere
MAP-2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MAPKAP-2	MAPK-activated protein kinase-2
MARCKS	myristoylated alanine-rich C-kinase substrates
MBP	myelin basic protein
MEK	MAPK/ERK kinase
MGDG	monogalactosyl diglyceride
μg	microgram
μl	microlitre
μm	micromillimeter
μM	micromolar
min	minute
ml	milliliter
mm	millimeter
MMP-9	metalloproteinase 9
MOPS	3-[N-morpholino]ethanesulfonic acid
MS	multiple sclerosis
N1	defined CG-4 feeding medium
NaCl	sodium chloride
NGF	nerve growth factor
NGS	normal goat serum
nM	Nanomolar
NT-3	neutrophin-3
O2A	oligodendrocyte type 2-astrocyte
OL	Oligodendroctye

Pak	p21-associated protein kinase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
Penstrep	penicillin and streptomycin solution
PI 3-K	phosphatidylinositol 3-kinase
PICK's	protein's that interact with C-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKI	protein kinase inhibitor
PL	poly-L-lysine
PLC	phospholipase C
PLP	Proteolipid
PMA	phorbol 12,13-myristate acetate
PMSF	phenyl methylsulphonyl fluoride
PS	Phosphatidylserine
RACK's	receptors for activated-C kinase
R-mAb	Ranscht monoclonal antibody
rpm	revolutions per minute
RT	room temperature
SAPK	stress-activated protein kinase
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SH	src-homology
SOS	son-of-sevenless
STATs	signal transducers and activators of transcription
TBS	Tris buffered saline
TBST	Tris buffered saline + Tween-20
TNF	tumour necrosis factor
Tris	tris (hydroxylmethyl) methylamine
Tween 20	polyoxyethylene-20-sorbitan monolaurate

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. . . my husband, Olaf Heisel, who makes me so happy!

CHAPTER 1: GENERAL INTRODUCTION

1.1 An Introduction to Oligodendrocytes and Central-Glial 4 Cells

In the developing central nervous system (CNS), pluripotent stem cells have the capability of differentiating into cells of either neuronal or glial lineage. Cells of neuronal lineage develop into nerve cells, while cells of glial lineage can develop into astrocytes or oligodendrocytes (OL). While glial cells were originally thought to be merely supporting cells for neurons, they are now known to be highly specialized cell types. For example, astrocytes provide framework and trophic support for neurons, and can also buffer excess potassium. As the myelinating cells of the CNS, OL also play a very important physiological role. These cells are responsible for wrapping nerve cell axons in an insulative coating of myelin, thus allowing for the efficient conduction of nerve impulses. Disruption of OL and the myelin they create can lead to severe pathological consequences, such as the paralysis, numbness, and loss of vision associated with the disease multiple sclerosis (MS). The significance of OL is reinforced by studies on MS, which have shown that spontaneous remyelination by surviving OL can alleviate symptoms (Rodriguez, 1992; Prineas et al., 1993). Unfortunately, this spontaneous remyelination is incomplete. Thus, in order to treat diseases associated with OL dysfunction, it is crucial to understand the functional biology of these cells.

Studies of OL functional biology have historically relied on the use of primary OL cultures. However, due the post-mitotic nature of mature OL, scientists have searched for an OL cell line capable of self-renewal. Relatively recently, a cell line known as Central-Glial 4 (CG-4) has emerged as an OL model (Louis et al., 1992). CG-4 cells have become a popular choice for a number of reasons. First, this cell line initially arose as a spontaneous mutation from a primary culture of rat OL progenitor cells. As such, it is not considered to be a transformed cell line, and it shows a normal karyotype after 25 passages. Second, phenotypic marker analysis has shown that CG-4 cells can display OL-specific markers, such as myelin basic protein (MBP). Third, transplantation studies have shown that CG-4 cells can myelinate demyelinated axons (Duncan et al., 1996). Therefore, using both CG-4 cultures and primary OL cultures, the knowledge base on OL functional biology has steadily increased. The characteristics of primary OL and CG-4 cell models will be reviewed below.

1.1.1 The Biology of Primary Oligodendrocytes: OL Progenitor Cells

The classical pathway of OL maturation is best characterized for the developing rat CNS. However, this developmental pathway may not adequately define the steps of OL maturation in the CNS of humans and other mammalian species. Despite this potential species variation, the well-defined rat OL developmental pathway provides a useful framework with which to characterize the various stages of OL maturation.

For neonatal rat brain cells committed to the glial lineage, the first main step of OL development begins with a bipotential progenitor known as an oligodendrocyte type 2-astrocyte (O2A). The O2A cell is a proliferative, bipolar and motile progenitor that has the capacity to differentiate into either OL or type 2-astrocytes (Raff et al., 1983; Small et al., 1987). It can be identified with a monoclonal antibody, A2B5, which recognizes the gangliosides GQ1b, GD3, GT1, and GD2 (Kundu et al., 1983). *In vitro*, addition of serum to the culture medium favours the differentiation of O2A cells into astrocytes. Conversely, the use of serum-free medium induces O2A cells to develop into mature OL (Raff et al., 1983). Addition of mitogens such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), however, act to keep O2A cells in their proliferative, bipotential progenitor form and to prevent differentiation (Gard and Pfeiffer, 1993; Grinspan et al., 1993; 1996). For instance, injection of PDGF into the cerebrospinal fluid of neonatal rats has been shown to delay O2A differentiation, and anti-PDGF antibodies have been shown to block O2A mitosis (Dutly and Schwab, 1991; Butt et al., 1997). Furthermore, decreases in PDGF-signalling have been shown to correspond to an exit of O2A cells from the proliferative cell cycle (Calver et al., 1998). Once these cells exit the cell cycle, they are able to spontaneously differentiate into mature OL (Dutly and Schwab, 1991). While O2A cells are commonly isolated from neonatal rat tissue, accounts of isolation of similar cells from human fetal tissue indicate that there is at least some species similarity in OL development (Kennedy and Fok-Seang, 1986; Weidenheim et al., 1994; Rivkin et al., 1995).

As well as the discovery of O2A cells in neonatal rat brain, O2A cells have also been discovered in the adult rat brain (Ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). The function of the adult rat O2A is most likely to maintain a pool of cells capable of both self-renewal and generation of new mature OL by asymmetrical division (Noble et al., 1992; Wren et al., 1992). This is in contrast to neonatal rat O2A cells, which divide symmetrically and are therefore not capable of self-renewal once differentiation has occurred.

To date there is only one account of isolation of a proliferative human oligodendrocyte progenitor from adult tissue. This cell was shown to be a uni-, bi- or tri- polar cell that was positive for A2B5 staining. It was able to differentiate into astrocytes in serum-rich medium, and into OL in serum-deprived medium (Scolding et al., 1995; 1999).

1.1.2 The Biology of Primary Oligodendrocytes: Mature OL

O2A cells committed to the OL lineage next mature into pro-OL that are still proliferative and bipotential but no longer motile (Levi et al., 1987; Gard and Pfeiffer, 1993; Warrington et al., 1993). These cells also lose their A2B5 reactivity, and can instead be identified by an O4 antibody that recognizes sulfatide (Trotter and Schachner, 1989; Gard and Pfeiffer, 1990). In human fetal tissue, a sub-set of precursor OL that do not react with O4 but that do react with the Ranscht monoclonal antibody (R-mAb) have been identified (Satoh and Kim, 1994). The R-mAb recognizes galactocerebroside (GalC) as well as sulfatides

(Ranscht et al., 1982). These O4-/R-mAb+ precursors have not been identified in neonatal rat tissue. Furthermore, O4+ cells isolated from the adult human CNS lack the bipotential and proliferative capacities of rat-derived O4+ cells (Armstrong et al., 1992). These differences underline the existence of species variation between rat and human OL.

Finally, pro-OL derived from neonatal rat brain differentiate into post-mitotic, multipolar OL that express cell surface markers which are recognized by O4, anti-GalC, and anti-2,3-cyclic nucleotide 3-phosphohydrolase (CNP) antibodies. Fully mature OL will then produce the rest of the myelin proteins, such as myelin basic protein (MBP) and proteolipid protein (PLP) (Ranscht et al., 1982; Bansal and Pfeiffer, 1992; Bansal et al., 1992). Mature OL are commonly isolated not only from rat, but also from cow, pig, sheep, and human tissue (Szuchet et al., 1980; Kim et al., 1983; Smyrnis et al., 1986; Kim, 1990; Stariha et al., 1997).

As mentioned above, mature OL function as the myelinating cells of the CNS. The first step in myelination involves the extension of processes from OL, as this extension allows OL to contact unmyelinated axons. OL myelinate axons of 1 μm or more, and one OL can myelinate as many as 50 axons (Compston et al., 1997). Signals such as substratum adhesions and cell-cell contact between specific cell adhesion molecules on OL and neurons probably stimulate myelin sheath production once contact between OL and axons has been made (Yim et al., 1986; Poltorak et al., 1987). Myelin itself is not merely a lipid-rich insulative coating, but it also possesses specific enzymes, such as CNP, and a variety of

ion channels. Once an OL has created a functional myelin sheath, it continues to support the metabolic activities of the sheath (Compston et al., 1997).

There appears to be a direct correlation between OL cultured *in vitro* and OL found *in vivo*, making primary OL cultures a useful model with which to study OL function. For instance, both *in vitro* and *in vivo* OL show extension of processes. They also both express the major myelin lipids and proteins, such as MBP and PLP (Yim et al., 1986; Vartanian et al., 1992). As well, myelin lipids and proteins are produced *in vitro* along a similar time course to those produced *in vivo* (Zeller et al., 1985; Dubois-Dalcq et al., 1986). *In vitro* studies on OL functional biology, however, are hampered by the post-mitotic nature of mature cells. This lack of proliferation makes it inherently difficult to produce sufficient quantities for experimentation. Although proliferating O2A cells can be cultured and induced to differentiate into mature OL, these progenitor cells do not divide indefinitely and can be cultured only in relatively small amounts from rat brain. Therefore, there is also a cell quantity problem associated with primary O2A cultures. To overcome this problem, scientists have begun to use a self-renewing cell line known as the CG-4 cell line to conduct studies on OL.

1.1.3 Discovery and Characterization of the Central Glial-4 (CG-4) Cell Line

The CG-4 cell line arose as a spontaneous mutation from rat O2A primary cultures (Louis et al., 1992). These O2A primary cells were initially cultured in the usual fashion, and most cells showed the classical differentiation to mature

OL after approximately 5-6 weeks in culture. However, it was noted that a sub-set of cells did not differentiate but rather continued to proliferate. This sub-set of continuously self-renewing cells was subsequently found to be a bipotential cell line capable of differentiating into either OL-like cells or astrocyte-like cells. This cell line is named CG-4, and has been shown to resemble cells of O2A lineage.

CG-4 cells were first characterized by immunocytochemisty (Louis et al., 1992). In their progenitor, bipolar state, 95% of CG-4 cells were found to stain with A2B5. Furthermore, only 2-3% of the cells stained for GalC, and less than 1% stained for glial fibrillary acidic protein (GFAP). As GalC is an OL marker, and GFAP is an astrocyte marker, these immunocytochemical results confirmed that the cells had not yet differentiated into either OL or astrocytes. To maintain CG-4 cells in their proliferative, bipotential state, serum-free medium containing mitogens provided by the B-104 neuroblastoma cell line was used. Further analysis determined that bFGF and PDGF could replace this B-104 conditioning.

The bipotential CG-4 cells were shown to differentiate into OL-like cells upon removal of the B-104 conditioning (Louis et al., 1992). After 48 hr in serum-free medium with no B-104 conditioning, the CG-4 cells took on the morphological characteristics of mature OL. These OL-like cells were multi-polar, and lost their capacity for proliferation. An immunocytochemical analysis showed that all of these differentiated CG-4 cells expressed GalC, over 50% expressed MBP, and only approximately 2% still stained with A2B5.

To differentiate the bipotential CG-4 cells into astrocyte-like cells, medium containing 20% fetal calf serum (FCS) was used (Louis et al., 1992). After

approximately 1 week of culturing in this serum-containing medium, the CG-4 cells took on the morphological characteristics of astrocytes. Furthermore, they remained highly proliferative and 50% stained for the astrocyte marker GFAP.

1.1.4 A Comparison of the Properties of Primary OL and CG-4 Cells

Striking parallels can be found between primary cultures and CG-4 cultures when comparing O2A cells to bipotential CG-4 cells (Table 1). First, both O2A and bipotential CG-4 cells stain with the A2B5 antibody. Second, both cell types can differentiate either into mature OL in serum-free medium or into astrocytes in serum-containing medium. O2A and bipotential CG-4 cells also both contain nestin, which is a progenitor cell marker that is lost upon differentiation (Gallo and Armstrong, 1995). As noted above, bFGF and PDGF are instrumental in keeping O2A cells in their proliferative state. Removal of these mitogens in both O2A and CG-4 cultures allows the cells to exit the cell cycle and to begin differentiation towards OL. Furthermore, O2A and CG-4 cells can both differentiate into astrocytes in the presence of mitogens such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) (Raff et al., 1983; Hughes et al., 1988; Lillien et al., 1990; Kahn and De Vellis, 1994; Mayer et al., 1994; Vos et al., 1996). The signal transduction pathways activated by CNTF also appear to be similar in both cell types, as they both involve signal transducers and activators of transcription (STATs) (Kahn et al., 1997; Dell'Albani et al., 1998).

Property	Primary O2A Cells and Bipotential CG-4 Cells	Primary Mature OL and CG-4 OL
Phenotypic marker expression	A2B5, nestin	Galc, MBP, CNP
Effects of serum-containing medium	Differentiation to astrocytes	N/A
Effects of serum-free medium	Differentiation to oligodendrocytes	N/A
Effects of CNTF and LIF	Increased differentiation to astrocytes	Increased survival with removal of trophic factors
Effects of bFGF	Maintenance of cells in a bipotential, proliferative state	Primary OL: stimulation of process extension in concert with astrocyte extracellular matrix CG-4 OL: N/T
Thyroid Receptor Expression	Thyroid receptor-α	Thyroid receptor-α and -β
Predominant glutamate receptor expression	AMPA and Kainate	AMPA and Kainate
Krox-24 expression	High expression	Low expression
Migration ability	Good migration on pleiotrophin and myelin substrates	Generally considered to be non-migratory cells
Myelination potential	Must differentiate into mature OL prior to affecting myelination	Can myelinate

Table 1: **The Shared Properties of Primary OL and the CG-4 Cell Line**

N/A = not applicable

N/T = not tested

As well as possessing similar differentiation pathways, O2A and bipotential CG-4 cells also possess similar functions (Table 1). For instance, one important function of O2A cells is their ability to migrate, as this ability gives them the potential to move into areas of demyelination and affect repair. *In vitro* tests conducted on myelin and pleiotrophin coated dishes have found that O2A and bipotential CG-4 cells can migrate on these CNS substrates (Amberger et al., 1997; Rumsby et al., 1999). Perhaps most significantly, O2A and CG-4 cells are both able to remyelinate demyelinated axons in irradiated tissue (Groves et al., 1993; Franklin et al., 1995).

Similarities between the primary OL culture model and CG-4 culture model do not only exist between O2A cells and bipotential CG-4 cells. In fact, there are also many parallels between mature primary OL and CG-4-derived OL (CG-4 OL). For instance, CNTF and LIF have been shown to increase the survival of both primary OL and CG-4 OL in response to removal of trophic factors (Barres et al., 1993; Kahn and De Vellis, 1994; D'Souza et al., 1996; Vos et al., 1996; Jiang et al., 1999). Both similarities and differences, however, were discovered between the two cell types in an extensive study comparing the expression of glycolipids and myelin-associated glycoprotein in primary OL and CG-4 cells (Yim et al., 1995; Schnaar et al., 1996). These studies found that while the levels of GalC and sulfatide increased during differentiation of bipotential CG-4 cells towards CG-4 OL, the final levels of these proteins were still lower than the levels of GalC and sulfatide found in mature, primary OL. As well, although both cell types expressed a high amount of the ganglioside GD3, CG-4 OL expressed

much less GM3 and more GD1b and GT1b than primary OL. Finally, CG-4 OL also expressed lower levels of myelin-related glycolipids and myelin-associated glycoprotein than primary OL. This decrease in OL-related proteins in CG-4 OL as compared to primary OL indicates that CG-4 OL perhaps do not achieve as high a degree of differentiation as primary OL. However, the fact that they still express relevant OL proteins indicates that the CG-4 cell line could be a reasonable OL model.

CG-4 cells also share the expression of various non-OL related proteins with primary OL. These proteins are often found in both the mature and immature CG-4 and primary OL differentiation stages. For instance, both CG-4 cells and primary OL show developmental expression of thyroid receptors, which is significant as thyroid hormones play a role in the regulation of myelination (Baas et al., 1994; Pombo et al., 1999). The developmental expression of the krox-24 differentiation regulator protein is also similar in both CG-4 cells and in primary OL, indicating that OL differentiation can be studied using CG-4 cell cultures (Sock et al., 1997). Both cell types also express functional ionotropic glutamate receptors of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate sub-types (Pende et al., 1994; Yoshioka et al., 1995; Meucci et al., 1996; Yoshioka et al., 1996). This glutamate receptor expression makes CG-4 cells useful for studies on calcium regulation and excitotoxicity in OL. Finally, neural cell adhesion molecules, cadherins, and beta catenin are similarily expressed in both cell types (Hughson et al., 1998). Therefore, while

there are differences between primary OL and CG-4 cells, the two systems appear to be similar enough to both be used as OL model systems.

1.2 Signal Transduction

Signal transduction involves the transmission of signals from the plasma membrane of a cell to intracellular protein intermediates. Protein intermediates called protein kinases phosphorylate substrates and thereby influence cellular functions such as proliferation and differentiation. Protein phosphatases reverse these kinase-mediated phosphorylation events. To understand which signalling pathways govern the survival, death, proliferation, differentiation, process extension and myelination capacities of OL, scientists have employed both the primary OL culture and CG-4 culture models. Currently, many studies of OL intracellular signalling events have focused on intracellular signalling enzymes known as protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs). These kinases will be reviewed below.

1.2.1 General Protein Kinase C Signalling

The term PKC encompasses a family of widely expressed protein-serine/threonine kinases. In the CNS, PKC has been linked to events such as modulation of ion channels, neurotransmitter release, long-term potentiation, and differentiation (Spinelli and Ishii, 1983; Baraban et al., 1985; Madison et al.,

1986; Malenka et al., 1986; 1987). PKC signalling in OL has been linked to proliferation, process extension, and myelination capacity (Yong et al., 1988; Bhat, 1989; Asotra and Macklin, 1993; Yong et al., 1994). Direct substrates for PKC isoforms can include the cytosolic myristoylated alanine-rich C kinase substrates (MARCKs), the cytoskeletal proteins vinculin and talin, and the kinase Raf (Morrison et al., 1988; Stumpo et al., 1989; Simons and Elias, 1993; Perez-Moreno et al., 1998). Activation of Raf can link PKC signalling to MAPK signalling, as will be discussed later under MAPKs.

There are three main sub-groups of PKC isoforms; namely, the conventional, novel, and atypical sub-groups [reviewed in: (Newton, 1995; 1997; Kanashiro and Khalil, 1998; Ron and Kazanietz, 1999)]. All three sub-groups are composed of a regulatory N-terminal region and a catalytic C-terminal region. They also share similar conserved domains known as the C1, C2, C3, and C4 domains (Fig. 1).

The C1 conserved domain is characterized by two cysteine-rich zinc finger repeats. These repeats represent a binding site for diacylglycerol (DAG) and phorbol esters. An autoinhibitory pseudosubstrate domain can be found just N-terminal to C1. The C2 domain recognizes acidic lipids, such as phosphatidylserine (PS), and can bind calcium in some isoforms. The C3 domain contains the ATP binding site, and the C4 domain contains the substrate binding site. Thus, C1 and C2 are part of the regulatory region, while C3 and C4 are part of the catalytic region (Fig. 1).

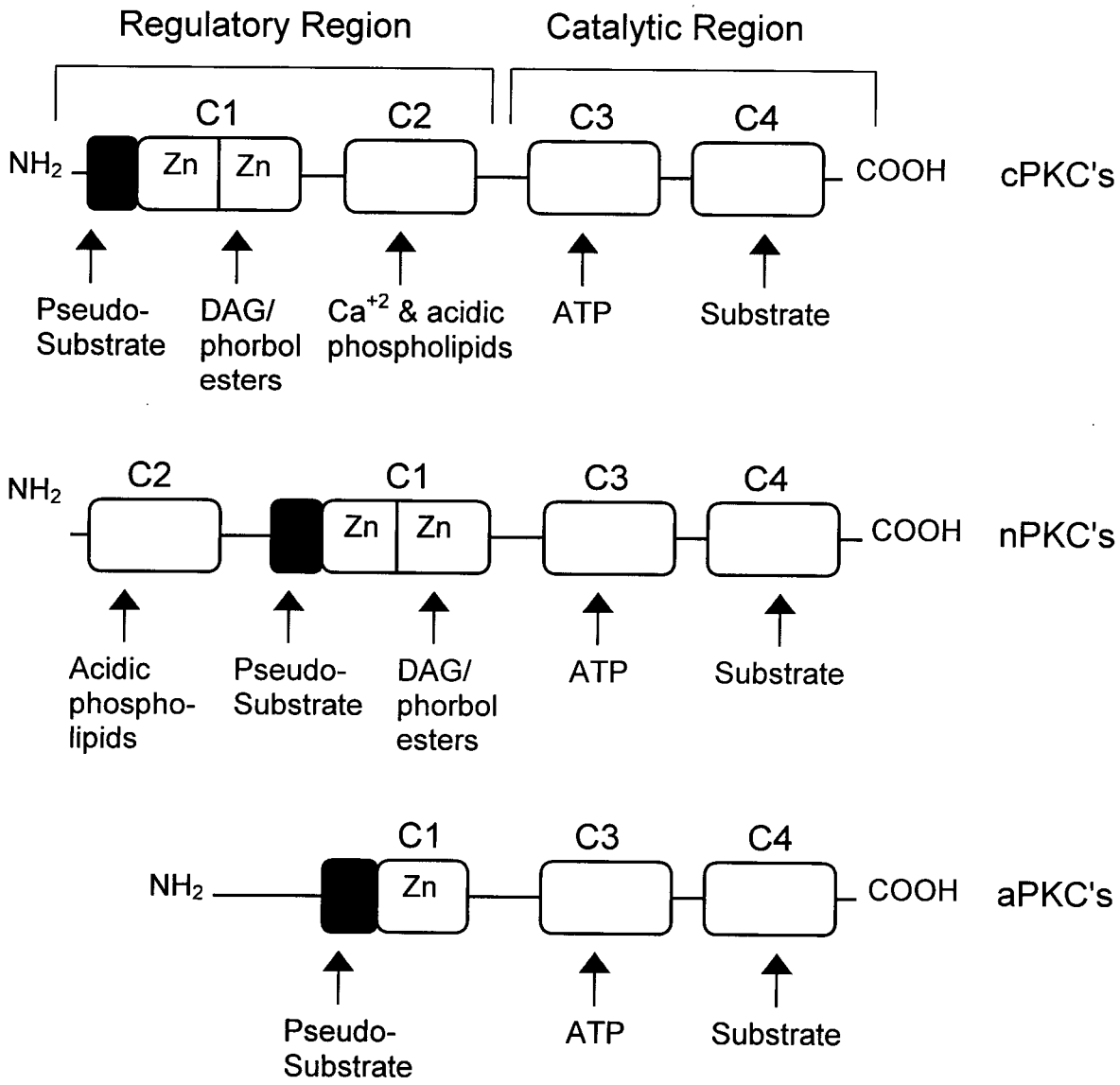

Figure 1: The Conserved Domains of PKC Isoforms

The conventional PKC's (cPKC's) have four conserved domains. The first conserved domain (C1) has two zinc finger repeats in a cystein-rich motif. This domain binds DAG/phorbol esters, and can also be found in the novel PKC's (nPKC's). The C1 domain in the atypical PKC's (aPKC's), however, has only one zinc-finger repeat, and therefore does not bind DAG /phorbol esters. The second conserved domain (C2) binds calcium and acidic phospholipids in cPKC's, but does not bind calcium in nPKC's and does not exist in aPKC's. The C3 and C4 domains bind ATP and substrate, respectively, and can be found in all PKC isoforms.

In OL signalling pathways, the conventional PKC (cPKC) isoforms have been proven to play an important role. The conventional PKC's include the α, βI, βII, and γ isoforms. These isoforms are regulated by phospholipids, calcium, and DAG/phorbol esters (Ogawa et al., 1981; Castagna et al., 1982; Kikkawa et al., 1983; Nishizuka, 1983; Blumberg et al., 1984). The proposed mechanism of cPKC activation has been previously reviewed by Newton (Newton, 1997). This mechanism involves the binding of DAG to C1 and PS to C2, localizing PKC to the cell membrane and causing a conformational change that removes the autoinhibitory sequence from the active site. Although binding of either DAG or PS is sufficient for weak PKC-membrane interactions, binding of both is required for high affinity interactions and removal of the autoinhibitory sequence. Binding of calcium to C2 is thought to increase the affinity of PKC for PS, thus decreasing the amount of DAG required for full activation. Phorbol esters can compete with DAG for binding to C1, and are thus also able to target PKC to the membrane for activation.

Since DAG is a pivotal molecule in the activation of the conventional PKC's, signalling pathways which lead to the production of DAG can result in the activation of PKC [reviewed in: (Nishizuka, 1992; Haeffner, 1993)]. For instance, activation of receptor tyrosine kinases or G-protein associated receptors can lead to the activation of phospholipase C (PLC). Activation of PLC in turn leads to the break down phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and DAG. Phospholipase D can also contribute to the production of DAG by orchestrating the breakdown of phosphatidylcholine, which

is subsequently dephosphorylated. Once DAG is produced, as mentioned, it is instrumental in targeting PKC to the membrane for activation.

PKC binding proteins are also thought to play a role in PKC signalling [reviewed in (Faux and Scott, 1996; Ron and Kazanietz, 1999)]. Briefly, it is thought that PKC binding proteins help to compartmentalize the different PKC isoforms so that they can be brought into close proximity with their target substrates. PKC binding proteins include receptors for activated C-kinase (RACK's), proteins that interact with C-kinase (PICK's), and substrates that interact with C-kinase (STICK's).

Finally, PKC can also be regulated by phosphorylation events. A protein known as PDK-1 has been identified as a kinase able to phosphorylate various PKC isoforms, including α and βII (Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998). This phosphorylation event is followed by autophosphorylation of PKC on two C-terminal sites. It is proposed that these phosphorylation and autophosphorylation events are required to create and maintain a catalytically competent conformation of PKC. They may also play a role in PKC localization (Bornancin and Parker, 1996; 1997; Newton, 1997).

In brief, the other two remaining sub-groups of PKC isoforms are the novel and atypical sub-groups. The novel sub-group (nPKC) consists of isoforms δ, ε, φ, and η. These nPKCs, unlike the conventional PKCs, are calcium independent. Like the conventional PKC's, however, they can be activated by PS in the presence of DAG or phorbol esters. The atypical PKC sub-group includes the ζ, ι, and λ isoforms. These atypical isoforms have only one cysteine

rich repeat in the C1 domain and appear to be DAG/phorbol ester independent. They also appear to be calcium independent, but can be at least partially activated by PS.

1.2.2 PKC Signalling Cascades in Primary OL

The effects of PKC activation have been studied in both immature and mature OL primary cultures. In general, PKC activation appears to promote proliferation in progenitor OL and to promote dedifferentiation and process extension in mature OL. However, studies of PKC in OL are often contradictory.

Progenitor OL have been shown to increase proliferation in response to bFGF, while use of the H-7 PKC inhibitor has been shown to abolish this bFGF-induced proliferation (Radhakrishna and Almazan, 1994). Other studies have shown that short-term treatment of O4+ OL with phorbol 12,13-myristate acetate (PMA), a biologically active phorbol ester, can also increase proliferation. Once again, this increase in proliferation was blocked by the use of the H-7 PKC inhibitor (Bhat, 1992). One mediator of PKC-induced proliferation appears to be c-fos. Induction of c-fos in OL occurs along with induction of bFGF-stimulated proliferation, while the use of H-7 blocks both bFGF-induced proliferation and c-fos gene induction (Bhat et al., 1992; Radhakrishna and Almazan, 1994). One possible explanation for these results is that bFGF engages growth factor receptors that activate PLC, which in turn could lead to activation of PKC and eventual activation of extracellular-signal regulated protein kinases (ERKs) 1 and

2. These ERKs could then translocate to the nucleus, inducing c-fos gene expression and promoting the transcriptional events required for cell proliferation. Another potential mediator of PKC-induced proliferation is the MARCKS protein, as PMA-treatment of progenitor OL has been shown to cause MARCKS phosphorylation. In OL, MARCKS can be found in both the cytoplasm and process extensions (Bhat, 1991; 1995).

These experiments indicate a role for PKC activation in the proliferation of immature OL. However, other studies have shown that long-term exposure of progenitor OL to PKC activators causes either no increase or a decrease in proliferation (Deloulme et al., 1992; Liu and Almazan, 1995). A possible explanation for these results is the phenomenon of PKC down-regulation. Down-regulation of PKC activity can occur either with the use of PKC inhibitors or with prolonged exposure (upwards of 1 hr) to PKC activators. In the latter scenario, prolonged phorbol ester treatments have the potential to down-regulate PKC activity, despite the fact that short-term phorbol ester treatments normally up-regulate PKC activity. Although Western blot studies have shown that progenitor OL do not down-regulate PKC upon long-term exposure to phorbol esters, they have also shown that OL changing from A2B2 expression to O4 expression do begin to down-regulate PKC (Asotra and Macklin, 1993; 1994). Asotra and Macklin speculate that perhaps this differential modulation of PKC activity between immature and differentiating OL could be attributed to differences in calpain-like activity. Since calpain has been shown to mediate phorbol ester-induced PKC degradation, they hypothesize that a lack of calpain-like activity in

immature OL may protect these cells from phorbol ester-induced PKC down-regulation. Asotra and Macklin also show that the change from A2B5+ immature OL to O4+ differentiating OL occurs over two culture days, thus making the change to OL which are susceptible to PKC down-regulation very rapid. Under experimental conditions, growth factors are often removed from the culture medium prior to treatment of progenitor OL with PKC activators. As mentioned above, removal of growth factors has been shown to induce OL differentiation. Therefore, it is possible that the removal of growth factors prior to exposure of progenitor OL to PKC activators is enough to make these cells susceptible to PKC down-regulation.

In contrast to its stimulation of immature OL proliferation, PKC appears to inhibit immature OL differentiation. Two separate studies on A2B5+ OL progenitors have reported a decrease in the percentage of A2B5+ OL differentiating into mature OL after activation of PKC (Baron et al., 1998; Heinrich et al., 1999). First, A2B5+ OL progenitors were assessed for the development of a CNP+ phenotype in the presence or absence of PMA. The results showed that OL were most likely to differentiate from A2B5+ bipolar cells to CNP+ multipolar cells in the absence of PMA. Furthermore, the PKC inhibitor BIM negated the effects of PMA treatment on these cells (Baron et al., 1998; 1999). Subsequent studies used the neurotrophic factor NT-3 to induce differentiation of bipolar progenitors toward multipolar GalC+ OL. In these studies, it was shown that PKC inhibitors (staurosporine and chelerythrine chloride) could increase NT-3-induced morphological differentiation (Heinrich et al., 1999). It has also been

demonstrated that PMA treatment can cause transient reversion of OL from an A2B5+/O4+ phenotype to an even more immature A2B5+/O4- phenotype (Avossa and Pfeiffer, 1993). Finally, the presence of PMA in mixed rat brain cultures has been shown to prevent the developmental expression of MBP and PLP in OL (Baron et al., 2000a).

From the results of these experiments, it seems reasonable to hypothesize that PKC exerts its mitogenic effects on A2B5+ OL progenitors at least partially by preventing proliferative OL progenitors from differentiating into post-mitotic, mature OL. Thus, there appears to be a dual and complementary role for PKC activation in these cells.

While PKC prevents the differentiation of immature OL, studies have shown that PKC activation can be linked to differentiation events in mature OL. For instance, most studies conducted on mature OL corroborate a role for PKC activation in enhancement of process extensions. From early studies, it was noted that treatment of mature OL with PMA induced process extension over basal levels. As well, the use of a variety of PKC inhibitors was shown to block this PMA-induced extension (Yong et al., 1988; Althaus et al., 1991; Yong et al., 1994). Further analysis of the phorbol ester-induced process extension response used Ca^{+2}-dependence and specific pharmacological agonists to determine that the α-isoform of PKC contributes most to these process extension signalling pathways (Yong et al. 1994). Soon, PKC activators other than phorbol esters were found to induce OL process extension. For instance, it was discovered that monogalactosyl diglyceride (MGDG) could enhance OL process re-growth in

adult porcine OL, while at the same time stimulating PKC-α activity (Schmidt-Schultz and Althaus, 1994). However, a definitive link between MGDG-induced process extension and PKC activity has yet to be established. It has also been reported that a combination of bFGF and astrocyte extracellular matrix can enhance OL process re-growth in adult human OL. Furthermore, selective PKC inhibitors (calphostin C and CGP 41251) have been shown to abolish this bFGF/astrocyte extracellular matrix-induced enhancement of processes (Oh et al., 1997). Finally, metalloproteinase 9 (MMP-9) has been presented as a downstream effector of the PKC-induced process extension response. In these experiments, phorbol ester treatment was shown to induce both process extension and MMP-9 activity. The use of a PKC inhibitor, calphostin C, was able to both block process extension and decrease MMP-9 activity. Perhaps most convincingly, inactivation of MMP-9 was shown to inhibit the phorbol ester-induced extension of processes (Uhm et al., 1998).

Since most studies on mature OL process extension involve exposure of OL to phorbol esters for upwards of 24 hr, the question of PKC down-regulation must be addressed. It has been shown that PKC activity actually increases 400-500% in mature OL treated with phorbol dibutyrate (PDB) for 48 hr, thus indicating a lack of PKC down-regulation in mature OL. Furthermore, PKC enzyme activity assays have shown that PKC activity does not decrease even after 12 days of PDB treatment (Yong et al., 1994). It should be noted, however, that contrary results have been obtained. Through immunocytochemical analysis, down-regulation of PKC-α and other PKC isoforms in both GalC+ and

MBP+ OL has been observed (Asotra and Macklin, 1993; 1994). One possible explanation for these varied results is species specificity; the experiments indicating that PKC is not downregulated involved human OL, whereas the experiments indicating that PKC is downregulated involved rat OL.

Not only does PKC activation play a role in the process extension of mature OL, it also plays a role in their myelination capacity. On the one hand, studies have shown that phorbol ester-induced PKC activation in mature OL can lead to increased phosphorylation of MBP, as well as increased MBP synthesis (Vartanian et al., 1986; Yong et al., 1994). On the other hand, a contradictory study points to a role for PKC in demyelination and OL dedifferentiation (Pouly et al., 1997). The main differences between these studies are the varied culture methods employed. The studies pointing to a role for PKC in myelination were conducted on primary cultures enriched for mature OL, while the study pointing to a role for PKC in demyelination was conducted on aggregating brain cultures. While aggregating brain cultures have the advantage of allowing neural cell interactions that more closely approximate *in vivo* conditions, their heterogeneous nature makes interpretation difficult. For instance, it is not possible to determine if the noted demyelination was a result of phorbol esters acting directly on OL. It is possible that other neural cells were also affected by the phorbol ester, and that these cells then mediated the demyelination. As well, without an analysis of cell marker expression, it is not possible to ascertain if the decreased OL differentiation seen in the aggregating cultures was truly a result of mature OL dedifferentiation. It is also possible that a lack of immature OL

differentiation led to the decreased expression of myelin proteins observed in these phorbol ester-treated cultures.

1.2.3 General MAPK Signalling

The mitogen-activated protein kinases (MAPKs) are a family of structurally related protein-serine/threonine kinases that are characterized by a requirement for dual-phosphorylation on both tyrosine and threonine residues for full activation. The MAPK family includes the extracellular-signal regulated protein kinases (ERKs), the stress-activated c-Jun N-terminal kinases (JNKs), and the high osmolarity glycerol response kinase (p38) [reviewed in: (Cano and Mahadevan, 1995; Pelech and Charest, 1995; Widmann et al., 1999)]. Studies of MAPKs in OL have determined unique roles for the varied members of this family. These roles will be discussed in the next section, and range from proliferation and differentiation to survival and apoptosis.

i) ERK1/2

The best-characterized members of the MAPK family are the 44 kDa ERK1 and the 42 kDa ERK2. Although they will not be discussed here, ERKs 3,4,5 and 6 also exist. Classical activation of ERKs 1 and 2 involves binding of a growth factor to its tyrosine-kinase receptor. Growth factor receptors involved in ERK1/2 activation include the PDGF receptor and the Trk A nerve growth factor receptor. Dimerization of the receptor follows ligand binding, leading to

autophosphorylation and receptor activation (Bishayee et al., 1986; 1989; Heldin et al., 1989). Receptor activation ultimately leads to the sequential activation of the following kinases: Ras, Raf-1, MEK1/2, ERK1/2 (Fig. 2). This sequential cascade utilizes interactions among many adapter proteins. First, the activated receptor recruits an adapter protein such as growth factor receptor binding protein-2 (Grb-2). Grb-2 is recruited by virtue of its src homology-2 (SH2) domain, which is a recognition domain containing a specific conserved protein pattern that helps mediate protein-protein interactions (Matuoka et al., 1993; Schlessinger, 1994). Next, Grb-2 interacts with a guanine nucleotide exchanger known as Son-of-sevenless (SOS), also via its SH domain. These interactions draw SOS to the membrane, where it can interact with Ras and activate it by catalyzing the exchange of GDP for GTP (Chardin et al., 1993). Activated Ras can then contribute to activation of Raf-1 by localizing Raf-1 to the plasma membrane and exposing the Raf-1 kinase domain. Although the exact mechanism of Raf-1 activation remains unclear, PKC is one of the proteins that is thought to play a role (Morrison and Cutler, 1997).

Activation of Raf-1 in turn leads to activation of MAPK/ERK kinase (MEK) via phosphorylation (Morrison et al., 1988; Van Aelst et al., 1993; Williams and Roberts, 1994). MEK1 and MEK2 are dual-specificity kinases that activate ERK1 and ERK2 by phosphorylating both tyrosine and threonine residues in a TEY motif (Zhang et al., 1994). It is thought that tyrosine phosphorylation of ERK1/2 allows for substrate binding, while threonine phosphorylation allows for the correct alignment of catalytic residues (Marshall, 1994). Once ERK1 and ERK2

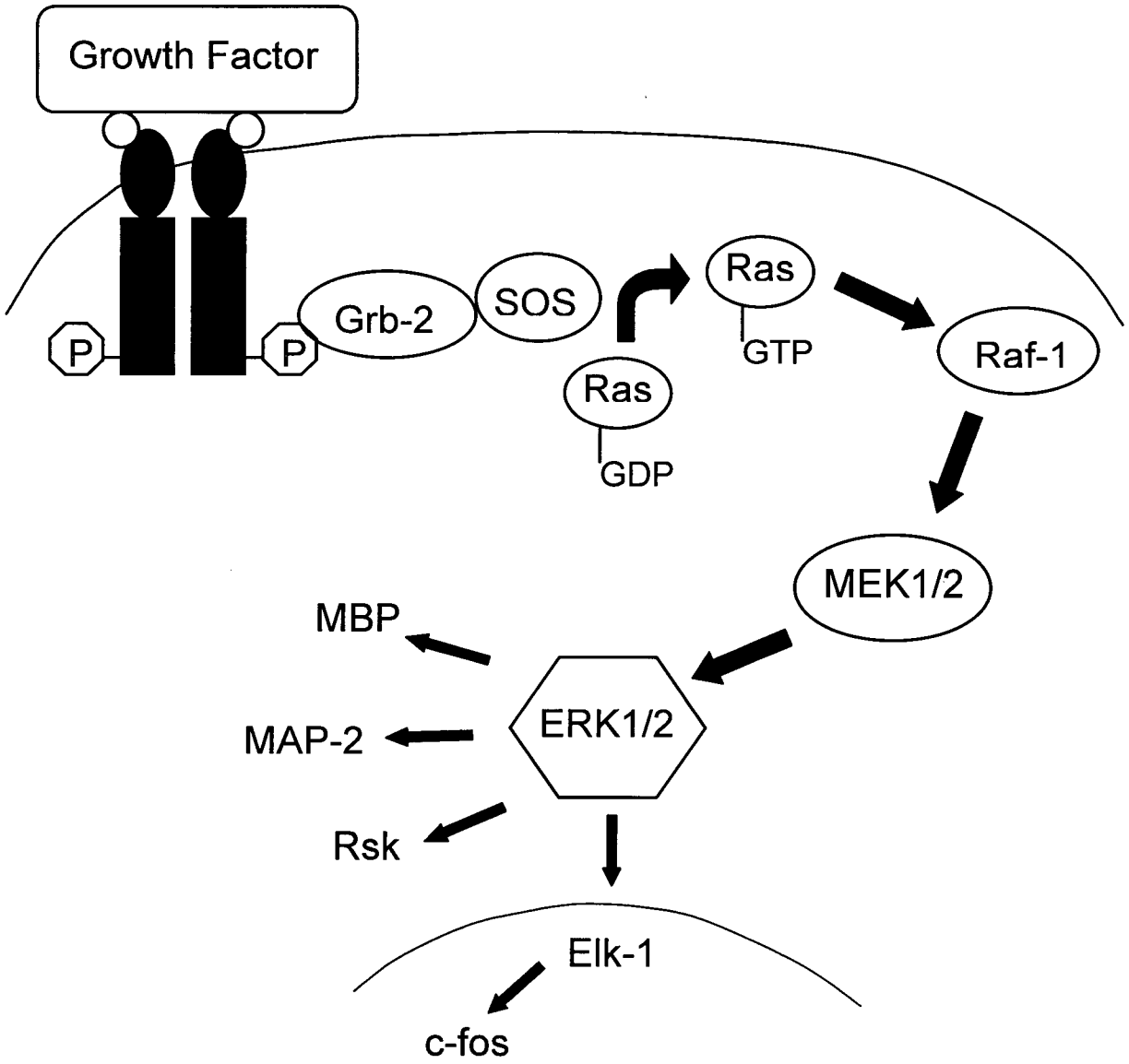

Figure 2: General Schematic of ERK1/2 Signalling via Growth Factor Activation

Growth factor receptor dimerization and autophosphorylation (P) follows ligand binding. This in turn leads to recruitment of an adapter molecule (Grb-2) to the receptor, which subsequently leads to the recruitment a guanine nucleotide exchange factor (SOS). Sos then converts Ras-GDP to Ras-GTP, and a sequential cascade of kinase phoshorylations leads from Raf-1 to MEK1/2 to ERK1/2 activation. Cytoplasmic substrates of ERK1/2 can include MBP, MAP-2, and Rsk, while translocation of ERK1/2 to the nucleus can cause c-fos gene induction via Elk-1 phosphorylation.

have been activated, they can phosphorylate a variety of substrates. Cytosolic substrates of ERK1/2 include MBP, microtubule-associated protein-2 (MAP-2), and kinases of the Rsk family (Sanghera et al., 1990a; 1990b; Gotoh et al., 1991; Blenis, 1993). Translocation of activated ERK1/2 to the nucleus can lead to phosphorylation of the transcription factor Elk-1, which in turn can lead to the induction of the c-fos gene (Gille et al., 1995; Hodge et al., 1998). Cellular responses to ERK1/2 activation include increased survival and proliferation.

Since there are no commercially available inhibitors of ERK1 and ERK2, experiments on these kinases often rely on inhibitors of MEK1 and MEK2. Two such MEK inhibitors are PD 98059 and UO-126 (Dudley et al., 1995; Favata et al., 1998). Both inhibitors appear to act on MEK1/2 through allosteric mechanisms, and do not compete with ATP or ERK1/2 for binding.

ii) JNK

The JNK members of the MAPK family are kinases that become activated in response to cellular stresses. In fact, JNKs are sometimes referred to as stress-activated protein kinases, or SAPKs. The stresses that can activate JNKs include exposure to anisomycin, UV-irradiation, and ceramide (Westwick et al., 1995; Meier et al., 1996). As well, tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), which engage receptors of the TNF-α superfamily, have been shown to activate JNK (Westwick et al., 1994; Rosette and Karin, 1996). Another member of this receptor superfamily that can lead to JNK activation is the p75 low affinity neurotrophin receptor. The kinases directly upstream of JNK, known

as MEK4 and MEK7, activate JNK by dual phosphorylation of tyrosine and threonine in a TPY motif (Derijard et al., 1995). Unlike activation of MEK 1/2 in the ERK1/2 cascade, the Ras-Raf-1 signalling cascade does not lead to activation of MEK4 or MEK7 in the JNK cascade. Rather, a MAPK/ERK kinase kinase (MEKK) is thought to be upstream of MEK4 and MEK7, and p21-activated kinase (Pak) has also been shown to act upstream of MEK4 (Bagrodia et al., 1995; Frost et al., 1996; Fanger et al., 1997; Tibbles and Woodgett, 1999). The classical substrate of JNK is the N-terminus of c-Jun, and cellular responses to JNK activation include apoptosis and cessation of cell growth.

iii) p38

A third member of the mammalian MAPK family, p38, has a counter-part in yeast known as high osmolarity glycerol response kinase (HOG). Not surprisingly, therefore, p38 is classically activated by osmotic stress. There is also evidence of overlap between p38 and JNK cascades, as ceramide, TNF-α, and IL-1 have been shown to activate both JNK and p38 (Raingeaud et al., 1995). The kinases directly upstream of p38, MEK 3/6, activate p38 via dual phosphorylation of tyrosine and threonine in a TGY motif (Derijard et al., 1995; Han et al., 1995; Stein et al., 1996). Signals downstream of p38 are often transduced through a kinase known as MAPK-activated protein kinase-2 (MAPKAPK-2), which in turn phosphorylates substrates such as the small heat-shock proteins (Guay et al., 1997; Larsen et al., 1997). Activation of p38 can also lead to induction of the ATF-2 nuclear transcription factor (Raingeaud et al.,

1996). Cellular responses to p38 activation can include apoptosis or proliferation (Craxton et al., 1998; Hida et al., 1999).

1.2.4 ERK1/2 Signalling in Primary OL and CG-4

Studies on the role of ERK1/2 in OL have predominantly employed primary cultures. These studies show a general trend whereby ERK1/2 activation leads to increased OL proliferation and survival. For instance, circumstantial evidence linking progenitor proliferation to activation of ERK1/2 has shown that treatment of cells with NT-3 increases both cell proliferation and ERK1/2 phosphorylation (Cohen et al., 1996). In parallel experiments, however, it was noted that NGF treatment could also phosphorylate ERK1/2 but could not induce OL proliferation. Since treatment of progenitor OL with NGF was less effective at sustaining ERK1/2 phosphorylation than NT-3, and since NGF did not stimulate progenitor proliferation, it could be concluded that a certain level of ERK1/2 activation must be reached before OL are induced to proliferate. Other studies have led to speculation that the ERK2 isoform, rather than the ERK1 isoform, is preferentially activated by NT-3 in progenitor OL (Kumar et al., 1998).

It is widely accepted, that, under normal *in vitro* and *in vivo* conditions, mature OL do not proliferate (Althaus et al., 1984; Kim and Yong, 1990; Althaus et al., 1991; Keirstead and Blakemore, 1997; 1999). However, there is some evidence that a select sub-population of mature OL can be induced to proliferate. Althaus et al. (1992; 1997) were able to induce proliferation of a sub-set of

mature OL using nerve growth factor (NGF) treatment, and subsequently showed that such NGF treatment also activates ERK1 in OL. Furthermore, the complement complex C5b-9 has also been shown to increase both mature OL proliferation and ERK1 activation (Rus et al., 1997). This complement-induced increase in proliferation was blocked with the use of the PD 98059 MEK1 inhibitor, thus strengthening the link between increased ERK activation and OL proliferation. Since ERK1 has been implicated in the proliferation of mature OL, while ERK2 has been implicated in the proliferation of O2A cells, it seems that ERK1/2 isoforms may be developmentally regulated in OL (Althaus et al., 1992; Kumar et al., 1998).

Experiments done on primary, mature OL have shown a correlation between ERK1/2 activation and increased survival. For instance, NT-3 or NGF treatment of mature OL has been shown to increase both ERK1/2 phosphorylation and OL survival (Cohen et al., 1996). Further experiments were conducted in which the effect of ERK1/2 activity on cell survival was compared to the effect of JNK activity on cell survival. In a first set of experiments, NGF was used to selectively activate JNK in OL that expressed p75 but not TrkA. In this case, cell death occurred (Casaccia-Bonnefil et al., 1996). However, in OL expressing both p75 and TrkA, ERKs 1 and 2 were activated to a much greater extent than JNK. In this experiment, the cells survived (Yoon et al., 1998). These complimentary studies indicate that JNK activation induces cell death, while ERK activation promotes cell survival.

As of yet, few studies of ERKs in CG-4 cells have been undertaken. However, ERK1/2 expression in CG-4 has been noted, and CG-4 cells cultured in serum-free medium for 24 hr show an upregulation of ERK1/2 phosphorylation in response to phorbol ester treatment (Bhat and Zhang, 1996). There have also been studies undertaken on the relationship of ERK1/2 activation to cell survival in CG-4 cells. First, increased ERK1/2 activation has been correlated to increased survival of CG-4 OL after treatment with prosaposin, a neurotrophic factor (Hiraiwa et al., 1997). While these results compliment primary culture data that also show increased OL survival after neurotrophin-induced ERK1/2 activation, other CG-4 culture experiments appear to contradict primary culture results. For instance, CG-4 OL exposed to H_2O_2 have been shown to demonstrate increased MAPK activity and increased cytotoxicity. Use of the PD 98059 MEK1 inhibitor was able to block this cytotoxicity, implicating ERK1/2 in the cytotoxic response (Bhat and Zhang, 1999). However, as mentioned above, ERK1/2 activation appears to promote cell survival in primary OL. It is still unclear whether ERK1/2 responses differ between primary OL and CG-4 OL, or whether ERK1/2 activation mediates H_2O_2 cytotoxicity in primary OL as well as in CG-4 OL.

1.2.5 JNK and p38 Signalling in Primary OL and CG-4

Both JNK and p38 have the distinction of being considered stress-related kinases, and are therefore often associated with cell death. In the case of JNK,

studies on human OL have shown that TNF-α treatment can cause activation of both OL apoptosis and activation of JNK (Ladiwala et al., 1998; 1999). As well, studies on rat OL have used NGF to show activation of both OL apoptosis and JNK (Casaccia-Bonnefil et al., 1996). Other OL studies have confirmed the activation of JNK by TNF-α, as well as by ceramide, sphingosine and sphingomyelinase. However, these studies did not assess the potential apoptotic effects of this JNK activation on OL (Zhang et al., 1996; 1998).

A case can also be made for a role for p38 in OL apoptosis. In experiments that used ceramide to induce OL apoptosis, p38 was activated (Hida et al., 1999). Furthermore, use of the p38 inhibitor SB203580 decreased this ceramide-induced apoptosis, while transfection of cells with dominant negative p38 attenuated the apoptotic response. Interestingly, no activation of JNK was noted with ceramide treatment. Furthermore, transfection of cells with dominant negative c-Jun did not lead to attenuation of ceramide-induced apoptosis. These responses contradict a role for JNK in ceramide-induced apoptosis, while at the same time indicating a role for p38. More experiments are needed to define the function of these two kinases in the OL apoptotic response. One possibility is that JNK predominantly mediates TNF-α responses, while p38 predominantly mediates ceramide responses. Since the kinetics of JNK activation have been shown to be different in response to TNF-α than in response to ceramide, it is conceivable that the kinetics of activation and not just activation itself play a role in determining whether or not OL will undergo apoptosis (Zhang et al., 1996).

While studies of JNK in CG-4 cells have not yet been undertaken, studies of p38 in CG-4 cells tend to corroborate an apoptotic role for this kinase in OL. For instance, it has been shown that p38 activity is linked to CG-4 cytotoxicity via induction of nitric oxide synthase (Bhat et al., 1999).

The similarities noted in the MAPK signalling pathways between primary OL and CG-4 cells, as well as similarities in their differentiation pathways and protein expression patterns, indicate that both primary OL and CG-4 cells can potentially be used to further elucidate the signal transduction pathways involved in OL functional biology.

1.3 Rationale and Objectives

Studies indicate that, after the onset of pathological demyelination in diseases such as MS, surviving mature OL do not, in fact remyelinate. Rather, it seems that a surviving pool of progenitor OL contribute to the remyelination process by migrating into the area of demyelination and proliferating (Keirstead and Blakemore, 1997; 1999). Presumably, they then differentiate into new mature OL, which in turn attempt to remyelinate the axons. Since the remyelination effected by the progenitor OL population in MS is incomplete, it would be beneficial if the surviving mature OL could somehow also be induced to contribute to the remyelination process.

To determine how to stimulate remyelination from mature OL, experiments have been undertaken to elucidate the signal transduction pathways involved in

OL process extension. The experimental endpoint of process extension is relevant because axons must be contacted by OL before they can be myelinated. As well, this endpoint is easily monitored, since OL lose their processes during the culture procedure and must re-grow them *in vitro*. Since the use of primary culture often results in a limited number of OL, the CG-4 culture model has also been used to facilitate research on OL signal transduction.

Signal transduction studies on OL functional biology have thus far included the PKC and MAPK signalling cascades. PKC has been shown to play a role in immature OL proliferation, as well as in mature OL process extension. Specifically, treatment of mature primary OL with phorbol esters has been shown to both activate PKC and induce OL process extension (Yong et al., 1988; Althaus et al., 1991; Yong et al., 1994). MAPKs have been shown to contribute to survival and death signals in OL, and may potentially also be downstream of PKC in process extension signalling cascades.

The objectives of the experiments in this thesis were therefore three-fold:

1 - The first objective was to use primary OL cultures to uncover any potential role for the ERK1/2 members of the MAPK family in OL process extension. This involved examination of the specific phosphotransferase activity levels of ERK1/2 in the presence or absence of phorbol ester induction of OL process extension. As well, the effects of the MEK1 inhibitor PD 98059 on both OL process extension and ERK1/2 activity were assessed.

2 - The second objective was to identify a suitable cell line model for use in OL signal transduction studies. In particular, the utility of the CG-4 cell line was

examined through a series of multi-kinase Western blots. These blots were used to compare and contrast the expression profile of various kinases in primary OL and in CG-4 cells. Since the CG-4 cell line initially arose as a spontaneous mutation from primary rat O2A cultures, primary rat OL were employed in these experiments.

3 - The third objective was to use the CG-4 cell line to further investigate the involvement of ERK1/2 in OL process extension. Specifically, the effects of the phorbol ester PMA, the MEK1/2 inhibitors PD 98059 and UO-126, and the PKC inhibitor Ro-32 on the development of a multipolar phenotype were assessed.

In conclusion, the objectives for this thesis were to use both the primary OL culture model and the CG-4 culture model to outline the role of ERKs 1 and 2 in OL process extension.

CHAPTER 2: MATERIAL AND METHODS

2.1 General Materials

2.1.1 Chemical Reagents

Acrylamide	Fisher
[γ-^{32}P] adenosine triphosphate	Amersham/Pharmacia
Albumin, bovine	Sigma
3-amino-9-ethyl-carbazole (AEC) tablets	Sigma
Avidin-Biotin Complex kit	Vector
Bio-Rad Protein Assay Dye Reagent	Bio-Rad
Biotin	Sigma
Bis-acrylamide	Fisher
3-bromo-4-chloro-3-indoyl phosphate	Sigma
Bromophenol blue	ICN
BSA protein standard	Biorad
Dimethylsulfoxide	Sigma
Dithiothreitol (DTT)	BDH
DNase	Sigma
Dulbecco's Modified Eagle Medium	Sigma
Enhanced chemiluminescence kit (ECL)	Amersham

Ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA)	Fisher/ICN
Ethylene diamine tetraacetate disodium salt (EDTA)	Fisher/ICN
Fetal calf serum	Hyclone
Fibronectin	Sigma
Gentamicin	Gibco-BRL
Glycerol	Anachemia
β-glycerophosphate	ICN/Fisher
Glycine	ICN/Fisher
Horse serum	Hyclone
Insulin	Sigma
Leupeptin	Roche
MBP substrate	Kinetek/Sigma
MBP synthetic peptide	Kinetek
β-mercaptoethanol	Fisher
Methanol	Fisher
β-methyl aspartic acid	Sigma
Mono Q	Pharmacia
3-[N-mopholino]ethanesulfonic acid (MOPS)	Sigma/ICN
Nitro blue terazolium	Sigma
Noniodet P-40	BDH
Normal goat serum	Sigma
PD 98059	Calbiochem

Penstrep (10^4 units/ml penicillin, 10 mg/ml streptomycin)	Stemcell
Percoll	Pharmacia
Phenyl methylsulphonyl fluoride (PMSF)	Sigma
Phorbol 12,13-myristate acetate	Sigma
Phosphate Buffered Saline	Oxoid
Phosphoric acid	Fisher
Poly-L-lysine	Sigma
Ponceau S	Sigma
Progesterone	Sigma
Protein kinase inhibitor peptide	Sigma
Putrescine	Sigma
Ro-32	Calbiochem
Scintillation fluid	Fisher
Skim milk	Safeway
Sodium azide	Fisher
Sodium chloride (NaCl)	Fisher
Sodium dodecylsulphate (SDS)	Fisher
Sodium orthovanadate (Na_3VO_4)	Fisher
Sodium selenite	Sigma
Transferrin (human)	Sigma
Tris hydroxylmethyl aminomethane hydrochloride (Tris-HCl)	Fisher

Tris (hydroxylmethyl) methylamine (Tris)	Fisher
Triton X-100	Pharmacia
Trypsin	Sigma
Tween-20 (polyoxyethylene-20-sorbitan monolaurate)	Fisher
UO-126	Calbiochem

2.1.2 Laboratory Supplies

3MM filter paper	VWR
Bottle top filters, 0.22 μm pore size	Nalgene
Culture Dishes: Cell+	Sarstedt
Disposable Cell Scrapers	Sarstedt
MAPK assay kit	Upstate Biotechnology
Nitrocellulose membrane	VWR
Plastic coverslips, Aclar	Allied Signal
Syringe filters, 0.22 μm pore size	Gelman
Syringes	BD

2.1.3 Antibody Reagents

Anti-CNP	Sigma
Anti-ERK (ERK1-CT)	Upstate Biotechnology

Anti-phosphotyrosine (4G10)	Upstate Biotechnology
Anti-PKC-α	Transduction Labs
Anti-PKC-β (C16)	Santa Cruz
Biotinylated anti-mouse IgG	Vector
Goat anti-mouse IgG alkaline phosphatase conjugate	BioRad
Goat anti-mouse IgG-horse radish peroxidase conjugate	BioRad
Goat anti-rabbit IgG alkaline phosphatase conjugate	BioRad
Goat anti-rabbit IgG-horse radish peroxidase conjugate	Calbiochem
Multiblot primary antibodies	Provided by Kinexus

2.2 General Methods

2.2.1 Primary Bovine OL Cultures

Bovine OL cultures were prepared following the procedures described previously (Kim et al., 1983; Kim, 1990). Fresh adult bovine brains were obtained from an abattoir and transported on ice in a phosphate buffered saline solution (PBS). The PBS also contained 10^4 units/ml penicillin and 10 mg/ml streptomycin in a 1:100 dilution (Penstrep). After cleaning off the blood vessels and meninges, the brains were manually chopped into pieces of approximately ~3x3x3 mm. The pieces were then suspended in a PBS + penicillin/streptomycin

solution containing 0.25% trypsin and 20 μg/ml DNase. They were placed on a cell shaker in a 37 °C incubator and incubated for approximately 1 hr. Following trypsinization, the dissociated cells were passed through a nylon filter with a pore size of 100 μm and centrifuged at 1400 rpm for 10 min. The cell pellets were then resuspended in PBS, and 20 ml of cell suspension was layered over 9 ml of Percoll plus 1 ml of 10x PBS. After centrifugation for 30 min at 15 000 rpm, the cell and Percoll mixture was separated into five distinct layers. The top layer consisted of mainly debris, the second layer of myelin, the third layer of neural cells, the fourth layer of blood cells, and the fifth layer of remaining Percoll. The debris and myelin layers were removed by aspiration, and the third layer of cells was collected. These cells were diluted with PBS and centrifuged for 10 min at 1400 rpm. The pellets were then resuspended in PBS and centrifuged for 10 min at 1200 rpm. Finally, the pellets were resuspended in feeding medium consisting of Dulbecco's Modified Eagle Medium plus 5% horse serum, 0.04 mg/ml gentamicin, and penicillin/streptomycin.

After a final centrifugation for 10 min at 1200 rpm, the cells were resuspended in feeding medium (5% HS) and plated on 10-cm-diameter plastic Petri dishes. Twenty-four hours later, most OL had not attached to the plastic dishes, while most other cells (i.e. astrocytes and microglia) had attached. The floating OL were therefore collected and replated. This replating procedure was carried out at least four times to obtain an enriched culture of OL. OL were maintained in a 37 °C incubator with a 5% CO_2 content.

2.2.2 Primary Rat OL Cultures

Rat brains were removed from 21-25 day old Sprague-Dawley or Wistar rats that were sacrificed by exposure to carbon dioxide. The brains were then processed in a similar fashion to bovine brains, except that the trypsinization time for rat material was 30 min as opposed to a trypsinization time of 1 hr for bovine material.

2.2.3 Bipotential CG-4 Cultures

The CG-4 cell line was originally provided by Dr. J.C. Lewis of Amgen. CG-4 cells were maintained as bipotential cultures, then differentiated to either OL-like or astrocyte-like cells as needed. To maintain CG-4 cells in a bipotential state, the cells were fed with 70% defined medium supplemented with 30% B-104 conditioned medium (70/30). The defined medium (N1) was made by adding 50 μg/ml transferrin, 5 μg/ml insulin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, and 10 ng/ml biotin to DMEM. Gentamicin and penicillin-streptomycin were also added to the medium. The cells were maintained in 10-cm-diameter round culture dishes coated with poly-L-lysine (PL) and overlayed with fibronectin. For PL coating, dishes were incubated in PL for 1 hr at 37 °C, washed three times with distilled water, and left to air dry. After drying, the dishes were overlayed with 50 μg of human fibronectin/dish and incubated for 24

hr at 37 °C. The dishes were rinsed once with DMEM before being used for CG-4 plating.

i) B-104 Conditioning

Prior to the creation of conditioned medium, B-104 cultures were maintained in DMEM containing 10% heat inactivated fetal calf serum (FCS). Once the cells were confluent, they were rinsed three times with PBS and incubated in N1 medium plus 2 mM glutamine for 72 hr. Subsequently, the conditioned medium was collected and filtered (0.22 μm). The medium was either used immediately for CG-4 cultures or stored at -20 °C.

2.2.4 Differentiation of CG-4

Bipotential CG-4 cells were differentiated into OL-like cells (CG-4 OL) by removal of B-104 conditioning from the medium. Cells fed with 100% N1 medium differentiated in CG-4 OL after 24-48 hr. Bipotential CG-4 cells were induced to differentiate into astrocyte-like cells by incubation in N1 medium supplemented with 20% FCS. The differentiation into astrocyte-like cells occurred over a one week period.

2.2.5 Treatment Protocols

Prior to treatments and morphological monitoring, bovine OL were adhered to 12-mm-diameter round Aclar coverslips coated with PL. Coverslips were coated by incubation for 1 hr in PL, then washed twice in distilled water and left to air-dry. OL suspended in 5% HS medium were then dropped onto the coverslips and allowed to settle for 24 hr.

CG-4 cells were maintained in 70/30 medium prior to treatments. They were adhered to PL+ fibronectin coated dishes as described above.

All treatment reactions were stopped by flooding the OL or CG-4 cells with ice cold PBS prior to cell lysis.

i) Phorbol Ester Treatments

Phorbol 12,13-myristate acetate (PMA) stocks of 1 mM were made by dissolving PMA powder in DMSO. The 1 mM stocks were then stored at -20 °C until just prior to use, when they were further diluted to 100 μM in DMSO. After a final dilution in feeding medium, the solutions were filtered through a 0.22 μm pore size filter.

OL were exposed to 100 nM PMA for 24 hr. They were then monitored morphologically using a phase contrast microscope. Parallel OL cultures were exposed to 100 nM PMA for 15 min prior to lysis.

CG-4 in N1 medium were exposed to 1, 5, 10, 20, or 40 nM concentrations of PMA and observed under a phase contrast microscope over

the next 24-72 hr. Parallel CG-4 cultures were exposed to 1, 5, 10, 20, or 40 nM concentrations of PMA in N1 medium for 15 min prior to lysis.

ii) Ro-32 Treatments

Ro-32 was dissolved in DMSO and filtered through a 0.22 μm pore size filter to make 1 mM stock solutions. Solutions were stored at -20 °C prior to use. CG-4 cells to be lysed were exposed to 1, 2.5, 5, 10 or 20 μM concentrations of Ro-32 in N1 medium for 30 min, 1hr, or 2 hr. Parallel cultures were exposed to 1, 2.5, 5, 10 or 20 μM concentrations of Ro-32 in N1 medium and observed under a phase contrast microscope over the next 24-72 hr.

CG-4 cells in 70/30 medium were also treated with 5 μM Ro-32, and either observed over the next 24-72 hr or lysed after 30 min. In some cases, the cells were pretreated with Ro-32. First, the 70/30 medium was collected after 48 hr. Second, 5 μM Ro-32 was added to the collected medium, and the medium was then replaced. Twenty minutes later, the medium was aspirated off and replaced with fresh 70/30 medium containing fresh 5 μM Ro-32.

iii) UO-126 and PD 98059 Treatments

Both UO-126 and PD 98059 were dissolved in DMSO to make 1 mM stock solutions. Stock solutions were stored at -20 °C prior to use. After further dilution in feeding medium to reach the appropriate concentration, the solutions were filtered through a 0.22 μm pore size filter.

OL were incubated in 12.5, 25, 50, or 100 μM concentrations of PD 98059 for 15 min prior to exposure to PMA. The optimal dose of PD 98059 was assessed morphologically by determining the lowest dose to completely inhibit PMA-induced process extension without causing cytotoxicity. To do this, OL were exposed to PD 98059 for 15 min prior to addition of 100 nM PMA. The extent of process inhibition as compared to cells treated with 100 nM PMA alone was monitored using a phase-contrast microscope.

CG-4 cells to be lysed were incubated in 12.5, 25, 50, or 100 μM concentrations of UO-126 or PD 98059 in 70/30 medium for 30 min. Also, selected cultures were pretreated with the inhibitors for 20 min prior to the 30 min treatment just described. To pretreat cells, 70/30 medium was collected from the cultures after 48 hr. UO-126 or PD 98059 was then added, and the medium was replaced. After 20 min, the medium was aspirated off and replaced with fresh 70/30 medium containing the appropriate inhibitor. Once again, parallel cultures were used for morphological monitoring

2.2.6 Cell Lysis

i) Cytosolic Lysates

OL in suspension were flooded with ice cold PBS, collected, and pelleted by centrifugation at 1200 rpm for 10 min. The supernatant was aspirated off and the pellet resuspended in a homogenization buffer consisting of 50 mM β-glycerol phosphate, 20 mM MOPS, 5 μM EGTA, 5 μM β-methyl aspartic, 2 mM EDTA,

and 2 mM Na_3V0_4. This buffer was stored at 4 °C, while 1 μg/ml leupeptin and 5 mM PMSF were added directly before use. The resuspended pellets were then sonicated on ice and centrifuged at 13 000 rpm for 30 min at 4 °C. The cytosolic proteins were collected with the supernatant, the protein concentrations determined, and the proteins loaded onto a Mono Q column.

Adherent CG-4 cells were prepared for lysis by washing in ice cold PBS. The cells were then collected by scrapping in a lysis buffer (75 mM β-glycerolphosphate, 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 5 mM PMSF). After collection, the cells were sonicated and centrifuged as described for OL. The lysates were stored at -70 °C until use.

ii) Membrane Lysates

To obtain membrane proteins, CG-4 cells were initially processed as described for cytosolic proteins. After sonication and centrifugation, cytosolic proteins were collected with the supernatant and kept on ice. In the meantime, the remaining pellets were resuspended in lysis buffer containing 1% Triton X-100. The resuspended pellets were then centrifuged at 100 000 rpm for 10 min in an ultracentrifuge. After centrifugation, the membrane proteins were collected with the supernatant. The cytosolic and membrane fractions were kept on ice while protein determinations were performed. The samples were then equalized for protein content (0.5-1 mg/ml) and boiled in 5x concentrated sample buffer. Samples were stored at -70 °C until use.

2.2.7 Determination of Protein Concentration

Protein concentrations were determined using the method of Bradford (1976). First, a 1.42 mg/ml BSA standard was diluted with dH_2O to a total volume of 100 μl/tube in order to create a series of standard tubes ranging from 0 to 30 μg BSA/tube. Second, 5 μl of each unknown sample was diluted with dH_2O to a total volume of 100 μl/tube. To each tube was added 2.5 ml of Bio-Rad Protein Assay Dye Reagent. After gently vortexing to mix and allowing to stand for approximately 5 min, the samples absorbances were read at a wavelength of 595 nM using a spectrophotometer. The concentrations of the samples were then determined using a linear regression plot.

2.2.8 Mono Q Fractionation

Cytosolic extracts from OL were fractionated by fast protein liquid chromatography (FPLC; Pharmacia) on a Resource Q column (Pelech et al., 1991). The column was equilibrated with Mono Q buffer (10 mM MOPS, pH 7.2; 25 mM β-glycerol phosphate, 5 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol (DTT), 2 mM Na_3V0_4) prior to loading. Samples containing approximately 1 mg of protein were loaded onto the column, which was eluted at a flow rate of 0.8 ml/min with a 15-ml linear 0-0.8 M NaCl gradient. Fractions of 0.25 ml were collected and further analyzed by kinase phosphotransferase assays and Western blotting.

2.2.9 ERK1/2 Kinase Assays

The ERK1/2 activity in the Mono Q fractions of OL cytosolic lysates was assayed for phosphotransferase activity using a MAPK assay kit. The phosphotransferase activity was assessed using either 1 mg/ml MBP or 1 mg/ml synthetic MBP peptide. The synthetic peptide is an ERK substrate designed around the Thr-97 substrate recognition sequence for ERK1 in MBP (Clark-Lewis et al., 1991). Five times concentrated MBP substrates were made up in a pH 7.2 assay dilution buffer (ADB) containing 1 M β-glycerophosphate, 1 M MOPS, 0.25 M EGTA, 0.1 M EDTA, 1 M magnesium chloride, 0.25 M DTT, and 5 mM β-methyl aspartic acid. The 30 μl total reaction volume contained 10 μl of sample. The reaction mix also contained 0.5 μM protein kinase inhibitor peptide (PKI) and 50 μM [γ-^{32}P] adenosine triphosphate (ATP). The reactions were carried out for 10 min and stopped by spotting 10 μl into wells containing filter paper. The wells were washed in 1% H_3PO_4 (v/v) to remove excess ATP. The filter papers were subsequently punched out of the wells and into scintillation vials containing scintillation fluid. The vials were then quantitated for radioactivity in a scintillation counter.

2.2.10 SDS-polyacrylamide Gel Electrophoresis

Mono Q fractions of OL lysates were subjected to protein separation via sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

according to the general methods of Laemmli (1970). Each lane was loaded with 90 μl containing the Mono Q fraction sample diluted with 5x concentrated SDS-sample buffer (250 mM Tris-HCl (pH 6.8), 10% SDS (w/v), 25% glycerol (v/v), 10% β-mercaptoethanol, 0.02% bromophenol blue (w/v)). The sample mixes were boiled for 5 min prior to loading onto gels. The samples were then electrophoresed through 4% stacking and 11% separating gels made with an acrylamide to bis-acrylamide ratio of 37.5:1. Electrophoresis was conducted at 8 mA for 15 hr in a running buffer consisting of 25 mM Tris, 192 mM glycine, and 3.4 mM SDS.

In general, the SDS-PAGE methods for CG-4 cells were the same as just described for OL. However, for better bandshift resolution, samples were electrophoresed through 13% low-bis separating gels as opposed to the regular 11% separating gels. These low-bis gels consisted of an acrylamide to bis-acrylamide ratio of 150:1. Protein samples of 30-70 μg were loaded onto large gels of 1.5 mm thickness. They were then electrophoresed through the stacking gel at 30 mA and the separating gel at 45 mA for approximately 3 hr. Once the dye front reached the bottom of the gel, electrophoresis was continued for another 50 min at 45 mA. Protein samples of 20-30 μg were loaded onto mini-gels of 1.5 mm thickness. Electrophoresis was performed at 30 mA through the stacking gel and at 35 mA through the separating gel for approximately 90 min. Once the dye front reached the bottom of the gel, electrophoresis was continued for another 40 min at 35 mA. Both large and mini-gels were cooled during the gel running procedure.

2.2.11 Western Blotting

i) Oligodendrocytes

Following SDS-PAGE, the separating gels were soaked in transfer buffer (20 mM Tris, 120 mM glycine, 20% methanol (v/v), pH 8.6) prior to layering on a nitrocellulose membrane. The gel and membrane were sandwiched between 3 MM filter papers and sponges, which were also soaked in transfer buffer prior to layering. The proteins were then electrophoretically transferred from the gel to the membrane for 3 hr at 300 mA on ice using a Hoeffer transfer cell system. To assess the transfer of proteins from gel to membrane, the membranes were incubated in a Ponceau S protein stain for 2-5 min following transfer. The membranes were then destained by rinsing in distilled water followed by rinsing in TBS (50 nM Tris-HCl, 150 mM NaCl, pH 7.5). Membranes were blocked for 30 min using 5% skim milk (w/v) in TBS. Following blocking, the membranes were rinsed briefly in TBST (0.05% Tween-20 (v/v) in TBS) and then incubated in an anti-ERK1/2 primary antibody overnight at 4 ^{o}C. In general, primary antibodies were diluted to an optimal concentration in TBST containing 0.05% sodium azide (w/v). Blots were rinsed 4x in TBST after primary antibody incubation. Each rinse lasted 5-10 min, with agitation. The blots were then incubated in an alkaline phosphatase-conjugated secondary antibody for 2 hr at room temperature. After rinsing 4x in TBST, the blots were developed with nitro blue terazolium and 5-bromo-4-chloro-3-indoyl phosphate in an alkaline phosphatase buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$ $6H_20$, pH 9.5)

For probing with the 4G10 anti-phosphotyrosine antibody, membranes were blocked for at least 6 hr using 3% BSA (w/v) in low-salt TBS (20 mM Tris, pH 7.5, and 50 mM NaCl). All rinses were done in low-salt TBS containing 0.05% Nonidet P-40. Development of the blots was otherwise as described above.

ii) CG-4

The general procedures for CG-4 Western blots were the same as described above for primary OL, with the following alterations. Large gels were transferred overnight at 100 mA at 4 °C, while mini gels were transferred at 300 mA for 45 min on ice. Membranes were blocked for 45 min in a TBS solution containing 2.5% skim milk and 1.5% BSA. After incubations in primary and secondary antibodies, the membranes were developed using an enhanced chemi-luminescence kit. The results were acquired with the use of a Fluoro S Max Multi-imaging system.

2.2.12 Immunoprecipitations

OL lysates containing 500 μg of protein were incubated in an equal volume of 3% NETF (3% NP-40 (v/v) in 100 m NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 50 mM NaF) plus 10 μl antibody and 30 μl Protein A Sepharose slurry. They were rotated for 3 hr at 4 °C prior to washing twice in 3% NETF and twice in KII buffer (12.5 mM sodium-β-D-glycerophosphate, 12.5 mM MOPS (pH

7.2), 5 mM EGTA, 20 mM $MgCl_2$, 50 mM NaF and 0.25 mM DTT (pH 7.2)). The samples were then boiled for 5 min in 5x sample buffer and stored at -70 °C until used for Western blotting.

2.2.13 Immunocytochemistry

CG-4 OL and primary OL adhered to coverslips were fixed and permeabilized in methanol for 10 min at -20 °C. The cells were then washed in PBS and incubated in PBS + 5% normal goat serum (NGS) for 45 min at room temperature (RT). Next, the coverslips were incubated overnight in a monoclonal anti-CNP antibody (1:100) at 4 °C. After washing 3x in PBS, the coverslips were then incubated for 1 hr at RT in a biotinylated anti-mouse IgG secondary antibody (1:100). They were then washed 3x in PBS prior to incubation in an avidin-biotin complex solution for 30 min at RT. Colour development was done using 3-amino-9-ethyl-carbazole (AEC) plus hydrogen peroxide. Coverslips were inverted and mounted onto slides using gelvatol.

CHAPTER 3: THE ROLE OF ERKS 1 AND 2 IN PRIMARY OL PROCESS EXTENSION

3.1 Introduction

Based on findings that treatment of OL with phorbol esters can induce OL process extension via PKC activation, and that PKC can be found upstream of ERK1/2 in many cell systems, it is possible that ERKs 1 and 2 are involved in mediating OL process extension. This potential role for ERK1/2 also takes into account that process extension precedes myelination, and that MBP is a well-known ERK1/2 substrate as well as a major component of myelin. Furthermore, activation of ERK1/2 has already been shown to play a role in the extension of neurites from neurons, PC12 cells, and neuroblastoma cells (Kim et al., 1997; Encinas et al., 1999; Perron and Bixby, 1999; Schmid et al., 1999; Walowitz and Roth, 1999).

The following experiments were conducted to assess the activation state of ERKs 1 and 2 in OL induced to extend processes as compared to control OL. Mature bovine OL were used in these experiments in order to generate a sufficient quantity of cells. Bovine OL were also chosen for their slow basal rate of process re-growth, allowing for easily observable differences between treated and untreated cultures. Process extension is therefore defined in this thesis as the appearance of thin branches extending from the cell bodies of OL, as opposed to a complete lack of branches.

3.2 Results

To assess the potential role of ERK1/2 in phorbol ester-induced process extension, three parallel OL cultures were set-up. The first set of OL were left untreated, the second set were treated with 100 nM PMA or PDB, and the third set were pre-treated with 50 μM PD 98059 for 15 min prior to the addition of 100 nM PMA or PDB. Observation of the cultures over the next 24-72 hr showed that untreated cells did not extend processes, while cells treated with 100 nM phorbol ester showed intricate process extensions (Fig. 3). It was found that 100 nM PDB (data not shown) or 100 nM PMA (Fig. 3) could both produce this effect. Furthermore, OL pre-treated with 50 μM PD 98059 prior to addition of phorbol ester did not extend processes (Fig. 3).

A dose-response was conducted using PD 98059 concentrations of 12.5, 25, 50, and 100 μM. Cells were pre-treated with PD 98059 prior to addition of PMA, then observed over the next week. It was determined that 50 μM of the MEK inhibitor was the lowest concentration sufficient to abolish the induction of processes (Fig. 4). Furthermore, as these inhibitor-treated cells were still phase bright under a phase-contrast microscope even one week after treatment, 50 μM was deemed to be a non-toxic inhibitor dose (Fig. 4). OL treated with 50 μM PD98059 alone also showed no process extensions or toxic effects.

To verify that activation of ERK1/2 is required for phorbol ester-induced process extension, OL were lysed and the cytosolic lysates were subjected to Mono Q fractionation. Eluted protein fractions were then subjected to

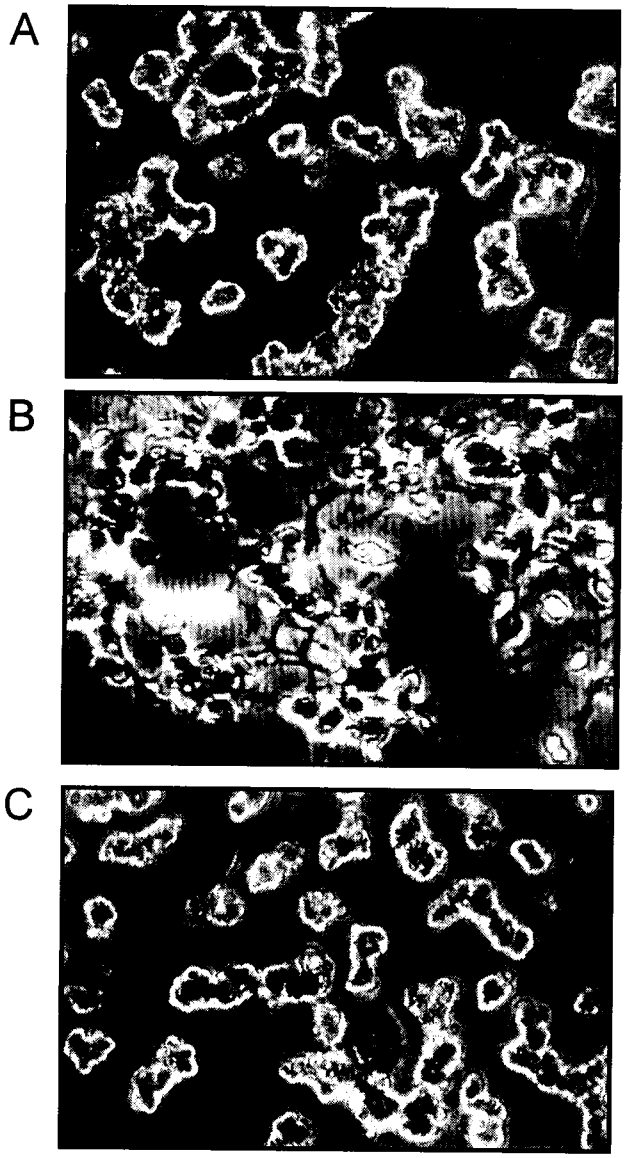

Figure 3: The Effects of PMA and PD 98059 on Primary Bovine Oligodendrocytes

Photographs were taken 24 hr after treatment using a phase contrast microscope.

A = untreated OL

B = OL treated with 100 nM PMA for 24 hr

C = OL pretreated with 50 μM PD 98059 for 15 min prior to treatment with 50 μM PD 98059 + 100 nM PMA for 24 hr.

Note the extensions visible after PMA treatment, but not after PD 98059 + PMA treatment. This data is representative of five experiments. Bar = 20 μm

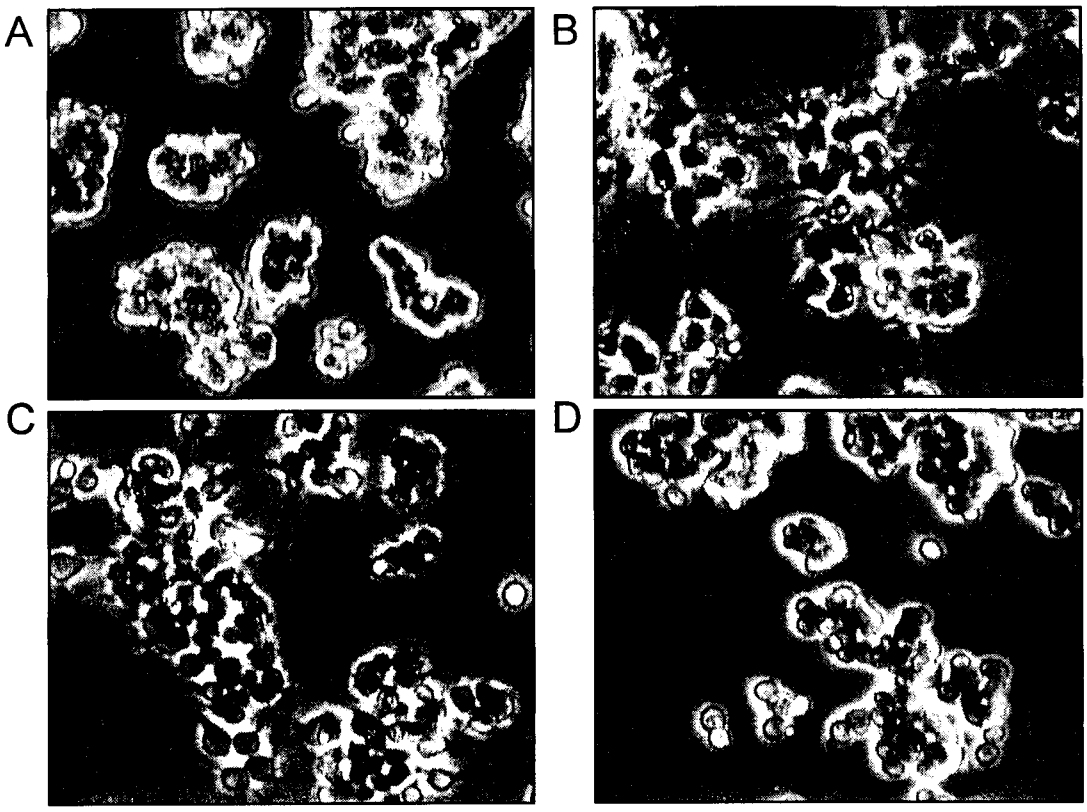

Figure 4: PD 98059 Dose Response

Primary bovine OL were observed under a phase contrast microscope 1 week after treatment.

A = untreated, control OL

B = OL treated with 100 nM PMA

C = OL treated with 25 μM PD 98059 for 30 min prior to treatment with 25 μM PD 98059 + 100 nM PMA

D = OL treated with 50 μM PD 98059 for 30 min prior to treatment with 50 μM PD 98059 + 100 nM PMA

Note that OL are still viable even one week after treatment. Also note that 25 μM PD 98059 was unable to completely abolish OL process extension. The ability of 50 μM PD 98059 to cause non-toxic inhibition of OL process extension was verified 5 times.

Bar = 20 μm

phosphotransferase kinase assays using either MBP or a synthetic peptide substrate. It was found that ERK1/2 phosphotransferase activity was increased by the phorbol ester treatment, and that this increase was reduced by pre-treatment of the cells with PD 98059 prior to addition of PD 98059 + PMA. By measuring the area under the peaks created by graphing the phosphotransferase activity values against the Mono Q fraction numbers, it was determined that PMA could increase ERK phosphotransferase up to 12-fold. Furthermore, PD 98059 was able to decrease this activity by approximately 70% (Fig. 5).

To ensure that the eluted and assayed fractions represented ERK1/2, samples of each Mono Q fraction were analyzed with both anti-ERK and anti-phosphotyrosine (4G10) Western blots. Prior to primary antibody incubation, the membranes were first subjected to a Ponceau S protein stain in an effort to account for the shift in peak phosphotransferase values between control or PMA-treated lysates and PD 98059 + PMA treated lysates. The stain indicated a total shift in protein elution between the two samples, rather than a specific shift in ERK1/2 elution. After destaining, the membranes were probed with either anti-ERK or anti-phosphotyrosine primary antibodies. The anti-ERK blots clearly showed the presence of both ERK1 and ERK2 in the eluted fractions (Fig. 6). Furthermore, ERK1 and 2 bandshifts to slower migrating forms were noted. These bandshifted fractions corresponded to fractions that showed increased activity in the phosphotransferase assays. The 4G10 Western blots also verified ERK1 and ERK2 activity in these same fractions (Fig. 7). As well, both blots confirmed that the ERK1/2 activity was not abolished, but only decreased, in PD

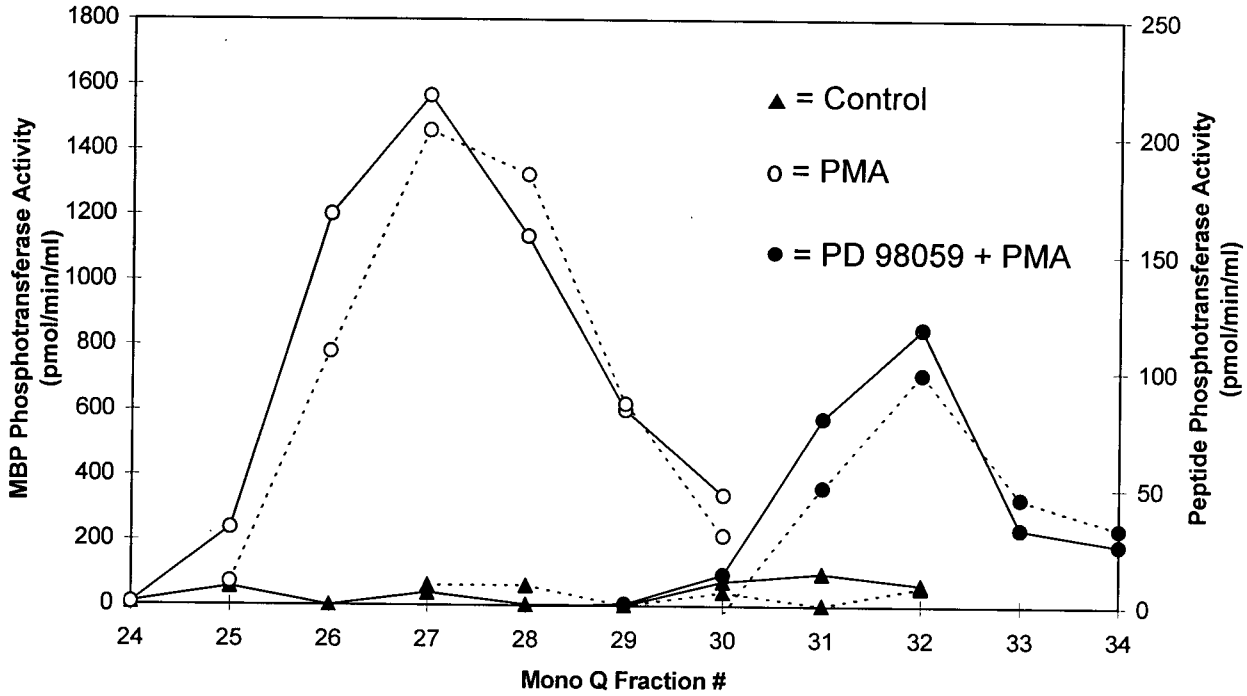

Figure 5: ERK1/2 Phosphotransferase Activity

Control = OL left untreated

PMA = OL treated with 100 nM PMA for 15 min

PD 98059 + PMA = OL treated with 50 μM PD 98059 for 15 min prior to treatment with 50 μM PD 98059 + 100 nM PMA for 15 min more.

After cell lysis, 1 mg of protein from each sample was subjected to Mono Q fractionation. Fractions containing 250 μl were collected and 10 μl of each fraction were assayed for ERK1/2 phosphotransferase activity. Assays were conducted using either an MBP —— or a peptide - - - substrate. Note the increase in ERK1/2 activity in PMA treated samples. Also note that the MEK1 inhibitor did not completely abolish PMA-induced ERK1/2 activity. This trend was observed in 5 experiments.

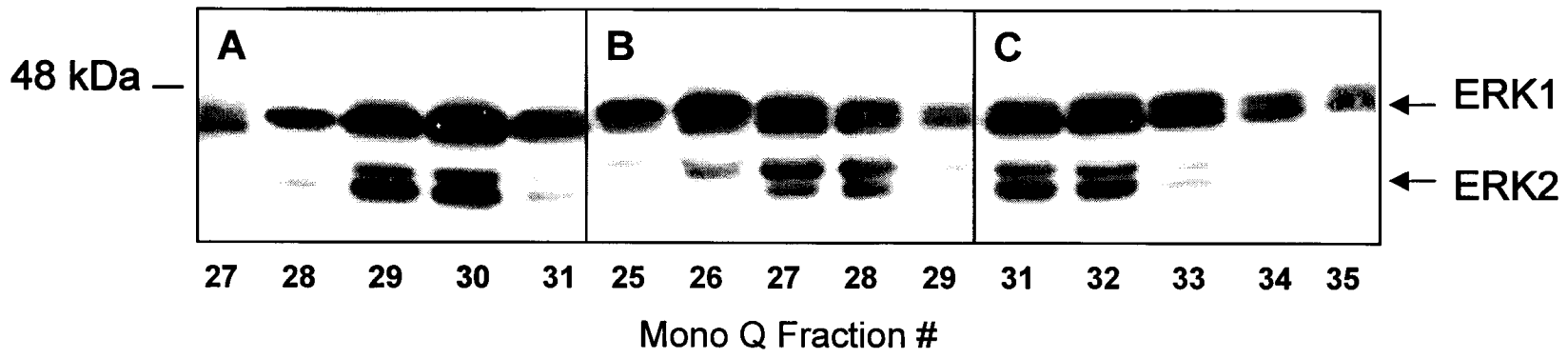

Figure 6: Anti-ERK Western Blot

A = Control OL

B = OL treated with 100 nM PMA for 15 min

C = OL treated with 50 μM PD 98059 for 15 min **prior to treatment with 50 μM PD 98059 + 100 nM PMA for** 15 min more

After cell lysis, 1 mg of protein from each sample was subjected to Mono Q fractionation prior to gel loading. Note the presence of both ERK1 and ERK2 in OL, as well as the shift to slower migrating ERK2 bands in the PMA-treated samples. This data is representative of 5 experiments.

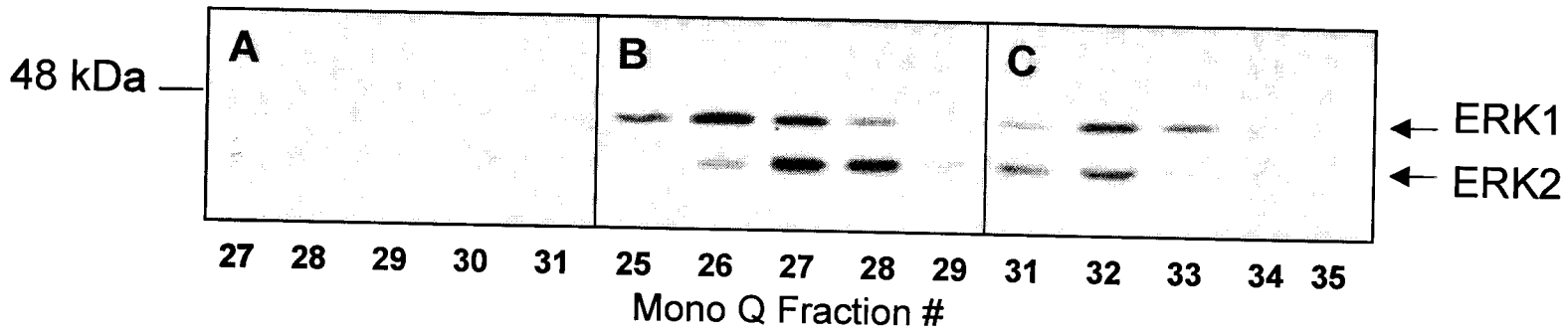

Figure 7: Anti-Phosphotyrosine Western Blot

A = Control OL

B = OL treated with 100 nM PMA for 15 min

C = OL treated with 50 μM PD 98059 for 15 min prior to treatment with 50 μM PD 98059 + 100 nM PMA for 15 min more

After cell lysis, 1 mg of protein from each sample was subjected to Mono Q fractionation prior to gel loading. Note that the majority of phosphorylated ERK1/2 is found in the PMA-treated samples. This experiment was duplicated.

98059 treated cells. A densitometry comparison of the two most intense 4G10 bands in PMA-treated lanes to the two most intense 4G10 bands in PD 98059 + PMA-treated lanes determined that the PMA-bands were approximately 30% more intense than the PD 98059 + PMA-bands.

Finally, immunoprecipitations and Western blots were undertaken to determine if Raf is potentially upstream of ERK1/2 in OL. All three known Raf isoforms, Raf-1, Raf A, and Raf B, were detected (Fig. 8).

3.3 Discussion

These experiments were conducted in order to confirm or deny a role for ERKs 1 and 2 in OL process extension. Through a series of morphological studies coupled with phosphotransferase assays and Western blots, ERK1/2 activation was shown to be a necessary component of phorbol ester-induced process extension. Other studies have demonstrated that ERK1/2 activation may also play a role in NGF-induced process extension, as NGF has been shown to induce both process extension and ERK1/2 activation in mature OL (Althaus et al., 1992; 1997). This indicates that ERK1/2 activation could be a general requirement of OL process extension, rather than simply a component of phorbol ester-induced OL process extension.

The potential substrates of ERK1/2 in OL also support a general role for this kinase in process extension. One such substrate, MBP, is a major myelin protein that comprises up to 25-30% of the total protein in myelin (Deber and

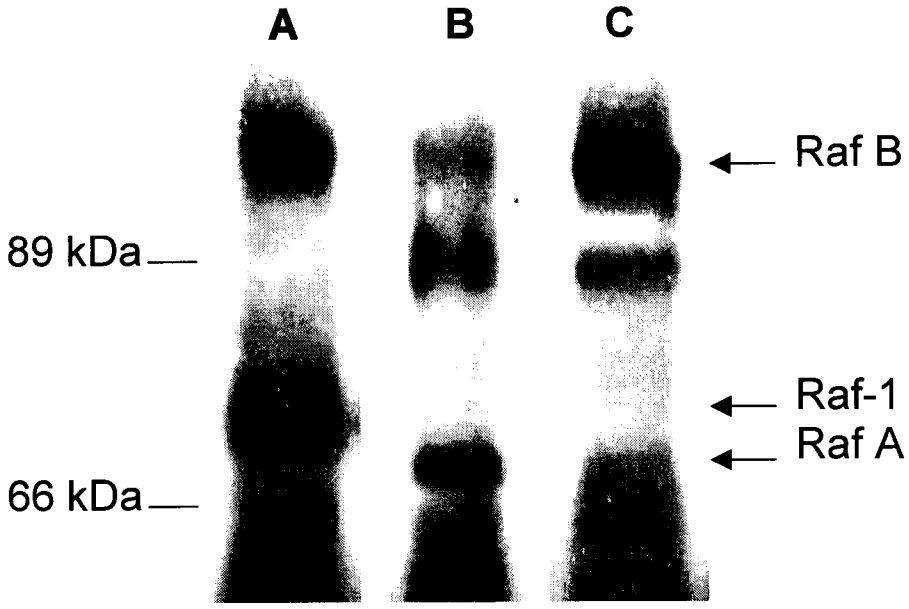

Figure 8: Raf Immunoprecipitations

A = Raf-1 immunoprecipitation

B = Raf A immunoprecipitation

C = Raf B immunoprecipitation

OL were lysed and 500 μg of cytosolic lysate were subjected to Raf immunoprecipitations. Note the evidence of all three Raf isoforms in OL. These blots are representative of 3 experiments.

Reynolds, 1991). MBP has been shown to associate with the OL cytoskeleton, specifically in the thick processes of mature cells, and radiolabelling studies have shown that phosphorylation of MBP occurs with the onset of myelin formation (Ulmer and Braun, 1983; Wilson and Brophy, 1989). Thus it is conceivable that the ERK1/2 activation observed in these studies could lead to phosphorylation of MBP in preparation for myelination following process extension.

ERK1/2 also has the potential to directly affect cytoskeletal dynamics via influencing the cytoskeletal protein, actin. Studies have linked ERK1/2 activation to actin reorganization in embryonic avian corneal cells, actin-mysoin assembly in immune cells, and redistribution of the actin cytoskeleton in progenitor OL (Baron et al., 1999; Chu et al., 2000; Stupack et al., 2000). Since OL processes themselves are not comprised simply of actin filaments, but also of microtubules, microtubule modulation is another possible target of activated ERK1/2. One possible scenario involves ERK1/2 phosphorylation of stathmin, a microtubule depolymerizing factor. Phosphorylation of stathmin by ERK1/2 has been shown to decrease its disruptive effects on microtubule polymerization (Moreno and Avila, 1998). This, in turn, has been shown to lead to microtubule rearrangement resulting in the formation of microtubule bundles that extend to the cell rim (Lovric et al., 1998).

Another potential role for ERK1/2 is induction of the c-fos gene, since activation of ERK1/2 can lead to c-fos gene induction via phosphorylation of the Elk-1 transcription factor. As well, c-fos induction in response to PKC activation has already been documented in progenitor OL, and is therefore also likely to

occur in mature OL (Bhat et al., 1992). Induction of c-fos has been linked to differentiation in osteoclasts, as well as to neurite extension in rat cerebellar granule cells and neuroblastoma cells (Rossino et al., 1995; Reddy and Roodman, 1998; Vaudry et al., 1998). Therefore, it is not unreasonable to hypothesize a similar role for c-fos induction in OL.

It is conceivable that the actions of ERK1/2 during process extension involve both the cytoplasmic/cytoskeletal and nuclear substrates mentioned above. This hypothesis is supported by immunocytochemical studies demonstrating the presence of ERK1/2 in the cytoplasm, processes, and nucleus of OL induced to extend processes (Stariha et al., 1997). Therefore, contact with MBP, actin and stathmin in the cytoplasm and processes is viable, as is induction of c-fos in the nucleus.

The ability of the MEK1 inhibitor PD 98059 to abolish phorbol ester-induced process extension provides tangible evidence of a role for ERK1/2 in process extension. However, MEK1 is upstream of ERK1/2, and there are no commercially available ERK inhibitors. Therefore, studies were undertaken to verify the inhibition of ERK1/2 by the MEK1 inhibitor.

The ERK1/2 activity profile was measured in four separate ways. The first two measurements involved assessment of ERK1/2 phosphotransferase activity, and the second two measurements involved Western blotting. The two phosphotransferase assays were conducted using either MBP or a synthetic peptide substrate (Fig. 5). The synthetic substrate was used to reduce the chances of artificially inflating the ERK1/2 activity levels via the presence of other

MBP-kinases in the eluted fractions. As might be expected, therefore, the activity counts were lower when using the synthetic peptide than when using MBP. However, both substrates showed the same trend of substantial ERK1/2 activation in lysates from PMA-treated OL, and a substantial reduction of ERK1/2 activation in lysates from OL pre-treated with PD 98059 prior to addition of PMA. Although it is possible that the level of ERK1/2 activity in the PMA-treated fractions could still be inflated by the presence of other MBP-kinases, MBP-kinases other than ERK1/2 would not likely be affected by PD 98059. Therefore, some of the activity seen in the MEK1-inhibited fractions could be due to other MBP-kinases, and the drop in ERK1/2 activity could actually be greater than 70%. Examples of such MBP-kinases are Raf-1 and protein kinases B and C.

Oddly, the activity elution profiles consistently showed that ERK1/2 in PD 98059 treated samples eluted later than ERK1/2 in control or PMA-treated samples. This anomaly was examined further via Western blotting. Western blots using an anti-ERK antibody confirmed the presence of ERK in the fractions that showed activity in the kinase assays (Fig. 6). They also confirmed that ERK1/2 in control and PMA-treated samples eluted earlier than those seen in PD 98059 + PMA-treated samples. To visualize the general protein elution profile for the various treatments, the membranes were subjected to a Ponceau S protein stain. It was observed that the entire protein elution profile appeared to shift in the PD 98059 + PMA-treated samples. Since the samples had always been applied to the column in the same order, namely control samples followed by PMA-treated samples followed by PD 98059 + PMA-treated samples, it was

speculated that the PD 98059 was affecting the elution profile. As well, it is possible that the column was becoming progressively clogged over time.

In the above-mentioned anti-ERK1/2 Western blots, eluted fractions were probed with an antibody that recognizes both ERK1 and ERK2. ERK1/2 bandshifts were used as an indication of ERK1/2 activity, and were noted predominantly in PMA-treated samples. While these bandshifts are difficult to distinguish due to the large amount of ERK1/2 present in the samples, parallel anti-phosphotyrosine blots clearly showed a predominance of phosphorylated ERK1/2 in PMA-treated samples (Fig. 7). Furthermore, both blots confirm that ERK1/2 activity is not abolished, but merely reduced, in PD 98059 + PMA-treated OL. This lack of complete abolition of ERK1/2 activity indicates that ERK1/2 is required to reach a certain level of activity before OL will commit to process extension. In OL treated with PD 98059 + PMA, therefore, the less robust ERK1/2 activation may not be enough to cross the necessary activity threshold. It is plausible that a major cell function, such as the differentiation and myelination associated with process extension, would not be turned on or off with only minor changes in kinase activity. Having a threshold of activity could be akin to having a safe-guard against premature differentiation of OL in response to small ERK1/2 activity spikes. Another factor that must be taken in consideration, but which cannot be answered by these experiments, is whether or not compartmentalization of ERK1/2 also affects process extension. In such a case, it would not simply be activation of ERK1/2 but also translocation of activated ERK1/2 to the nucleus and/or processes that would mediate process extension.

This possibility is supported but not verified by the noted translocation of ERK1/2 to the nucleus and processes of PMA-treated OL (Stariha et al., 1997).

Finally, the anti-ERK Western blot results indicate that ERK1, rather than ERK2, is the dominant isoform in mature bovine OL. While the 4G10 Western blots indicate similar phosphorylation levels of ERK1 and ERK2 in these studies, another set of studies using in gel assays have shown that NGF-induced OL process extension leads to phosphorylation of predominantly ERK1 over ERK2 (Althaus et al., 1997). Thus, it appears that ERK1 may play a larger role in process extension than ERK2. However, isoform specific immunoprecipitations and kinase assays would be required to make this distinction.

Preliminary studies were also undertaken to determine other potential components of the signalling cascade necessary for OL process extension. As expected, a phosphotransferase assay utilizing a c-Jun substrate for JNK and an ATF-2 substrate for p38 showed no significant phosphotransferase activity upon PMA-treatment of OL (data not shown). As a control, MBP-phosphotransferase activity was still observed. These experiments were done to verify that specifically ERKs, and not MAPKs in general, are responsible for process extension. Furthermore, the potential contribution of Raf to the process extension signalling cascade was assessed by Raf immunoprecipitations followed by Western blotting and MBP-phosphotransferase assays. Three Raf isoforms, Raf-1, Raf A, and Raf B were present in these cells, as seen via Western blotting (Fig. 8). However, Raf-1 was the only isoform that showed an approximately 2-fold increase in MBP phosphotransferase activity (data not

shown). Raf-1 activation has also been linked to phorbol ester stimulation in bovine luteal cells and NIH3T3 fibroblasts (Reuter et al., 1995; Chen et al., 1998).

Preliminary morphological studies were undertaken to determine if a variety of protein kinase inhibitors and/or activators could either induce process extension or inhibit phorbol ester-induced process extension. The PI 3-kinase inhibitor, wortmanin, was employed as there is evidence linking PI 3-kinase to the ERK pathway and to neurite extension (Kim et al., 1998; Pandey et al., 1999). The calcium-calmodulin dependent kinase II inhibitor, KN-62, was employed as studies have linked calcium modulation to process extension in OL (Yoo et al., 1999). As well, the protein kinase A (PKA) activator, forskolin, was employed, since PKA is another fairly ubiquitous kinase that has been implicated in OL differentiation and proliferation (Baron et al., 1998; Shi et al., 1998; Baron et al., 2000b). Wortmanin was shown to have no effect on control OL at concentrations up to 200 nM (Fig. 9). As well, pretreatment of cells for 24 hr with 200 nM wortmanin followed by 24 hr of wortmanin + PMA-treatment showed no change in the formation of PMA-induced process extensions (Fig. 10). Similar results were found with KN-62 at concentrations of 5 -10 μM, although 20 μM of KN-62 was cytotoxic (Fig. 9, 10). Finally, forskolin did not appear to induce process extensions at a dose of 100 nM, nor did it inhibit PMA-induced process extensions (Fig. 9, 10). Therefore, the only kinases definitively linked to OL process extension at this time are PKC and ERK1/2.

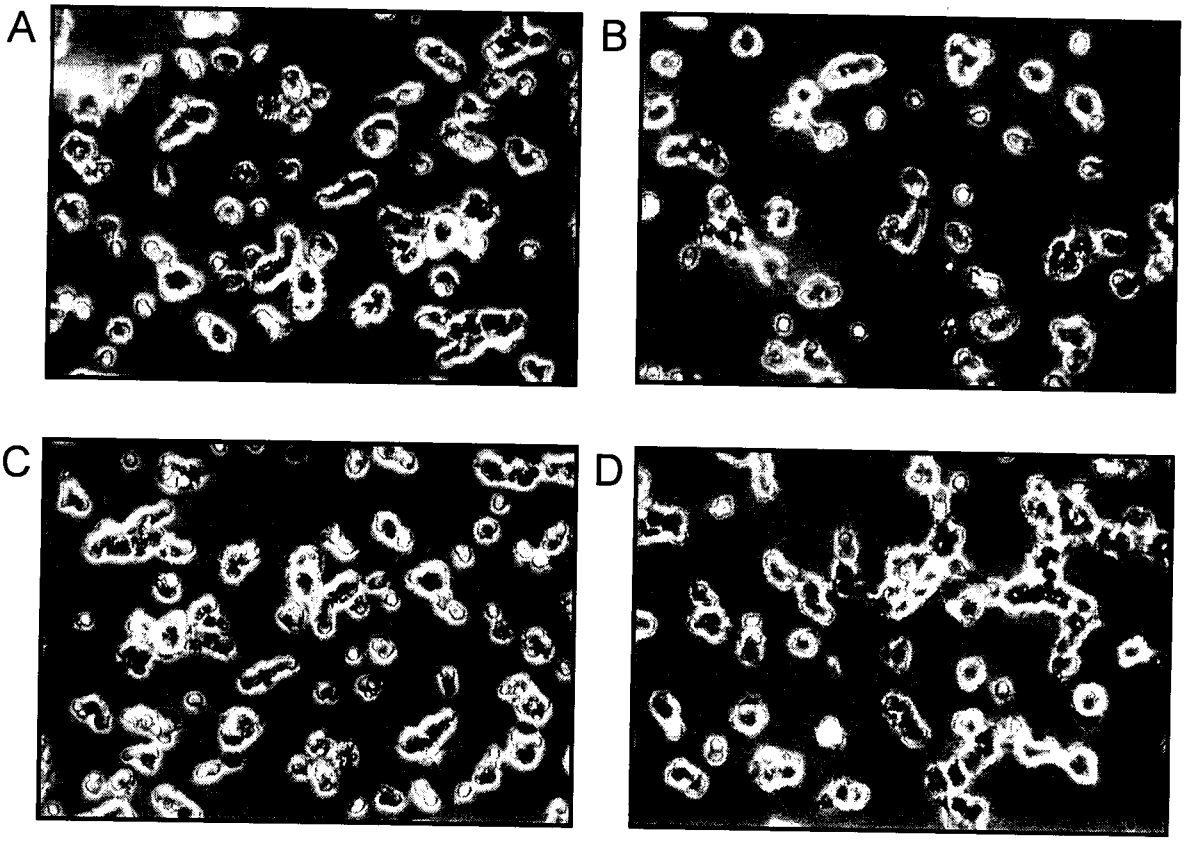

Figure 9: The Effects of Wortmanin, Forskolin, and KN-62 on Primary Bovine OL

Primary bovine OL were treated with various compounds for 24 hr. Photographs were taken using a phase contrast.

A - control OL

B - OL treated with 200 nM wortmanin

C - OL treated with 100 nM forskolin

D - OL treated with 5 μM KN-62

Note that none of the treatments induced noticeable process extensions from the OL. (For PMA-induced process extensions, see Fig. 10.) This indicates that PI 3-kinase (panel B), PKA (panel C), and calcium/calmodulin-dependent kinase II (panel D) do not play a role in OL process extension. This data is representative of 3 experiments.

Bar = 20 μm

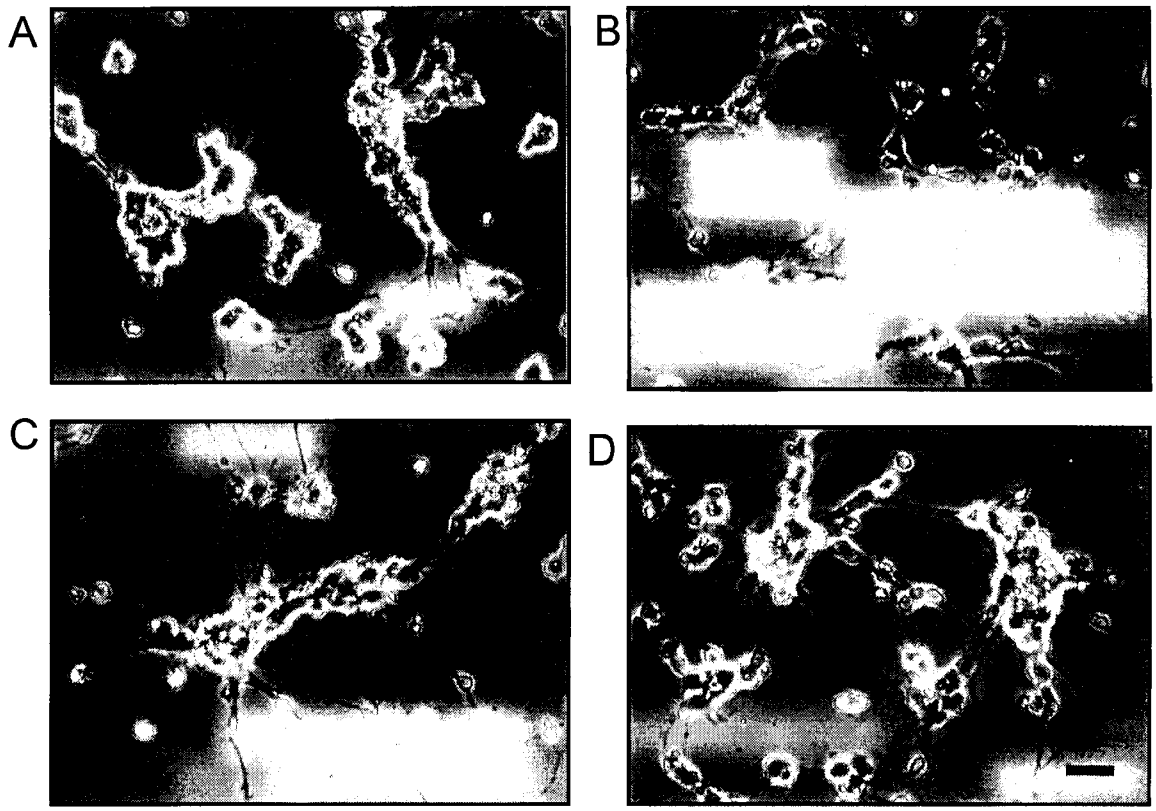

Figure 10: The Effects of Wortmanin, Forskolin, and KN-62 on PMA-induced Process Extension in Primary Bovine OL

Primary bovine OL were pretreated with various compounds for 24 hr prior addition of PMA. Photographs were taken using a phase contrast microscope.

A - OL treated with 100 nM PMA for 24 hr.

B - OL treated with 200 nM wortmanin for 24 hr followed by 200 nM wortmanin + 100 nM PMA for 24 hr more.

C - OL treated with 100 nM forskolin for 24 hr followed by 100 nM forskolin + 100 nM PMA for 24 hr more.

D - OL treated with 5 μM KN-62 for 24 hr followed by 5 μM KN-62 + 100 nM PMA for 24 hr more.

None of the treatments visibly affected PMA-induced process extension in three experimental trials. (For untreated control OL, see Fig. 9). Bar = 20 μm

CHAPTER 4: A COMPARISON OF THE KINASE EXPRESSION PROFILE BETWEEN PRIMARY RAT OL AND CG-4

4.1 Introduction

After conducting process extension studies on primary cultures of mature OL as described in Chapter 3, the quantity and variability problems associated with primary OL cultures led to a search for a better model. To generate sufficient quantities of mature primary OL for experimentation, bovine brains had been employed. However, although it was possible to produce a large quantity of cells from these brains, there was no control over the age or genetics of the animals. It was therefore logical to switch to a model that would allow for the generation of a large number of cells, while at the same time allowing for a certain amount of control over culture variability.

The search for an OL model brought to attention the CG-4 cell line. Based on immunocytochemical data, CG-4 cells are similar to primary OL. As well, CG-4 cells and primary OL share the expression of many proteins and share many similar actions, as described in the Introduction. However, since there are also reports of differences between CG-4 cells and primary OL, it was necessary to verify the suitability of the CG-4 model for signal transduction studies. An exhaustive analysis of the kinase expression patterns between CG-4 cells and primary OL was undertaken to determine this suitability. Since the CG-4 line

initially arose from a spontaneous mutation of rat O2A cells, the comparison was made using primary rat OL.

4.2 Results

In an initial comparison of primary rat OL to CG-4 OL, it was found that both cell types stained with an anti-CNP antibody (Fig. 11). Both cell types also developed a multipolar morphology, although the processes on primary OL had the potential to become slightly more intricate than the processes on CG-4 OL (Fig. 12).

For a further comparison of primary rat OL to CG-4-derived OL, cell lysates were sent to Kinexus Bioinformatics Corporation for a multi-kinase immunoblotting expression profile. CG-4 cells in bipolar and astrocytic differentiation states were also sent for analysis. Prior to lysis, CG-4 cells were allowed to differentiate over a one week period (Fig. 13).

The Kinetworks™ analysis is a proprietory method used to quantitate the level of expression of at least 75 known protein kinases. The screen employs the use of independently validated commercial antibodies for the protein kinases shown in Table 2. After the Kinetworks™ screen, selected protein kinase expression levels were confirmed at least twice on verification Western blots using fresh cell lysates. Any changes in kinase expression were quantitated by averaging the results of densitometric measurements taken from two verification Western blots.

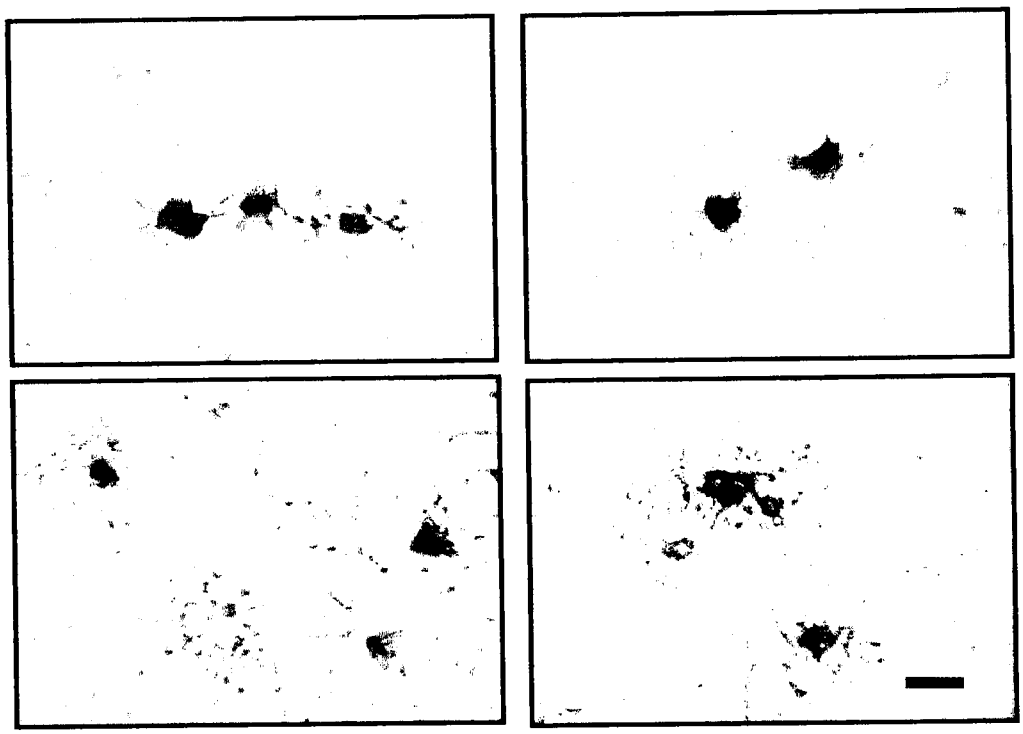

Figure 11: CNP Staining of CG-4 OL and Primary Rat OL

The panels on the left represent CG-4 cells differentiated into CG-4 OL by incubation in N1 medium for 48 hr. The panels on the right represent primary rat OL maintained in 5% HS medium.

Note that both cell types stain for the OL marker, CNP. Bar = 10 μm

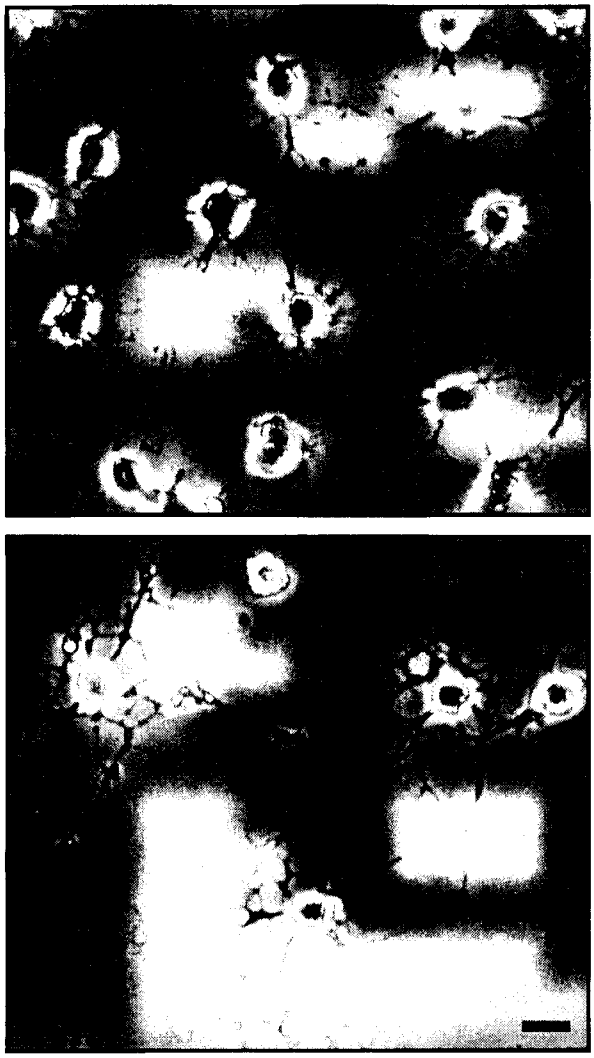

Figure 12: CG-4 OL and Primary Rat OL

The upper panel represents CG-4 cells differentiated into OL-like cells in N1 medium. The lower panel represents primary rat OL maintained in 5% HS medium. Photographs were taken using a phase contrast microscope.

Note that both cell types are multipolar, although the processes on the primary OL appear to be slightly more intricate than those on the CG-4 OL. Bar = 10 μm

Bipotential CG-4 Cells Astrocytic CG-4 Cells Oligodendrocytic CG-4 Cells

Figure 13: The Three Differentiation States of the CG-4 Cell Line

CG-4 cells were either maintained in their bipotential state or allowed to differentiate over a 1 week period. Bipotential CG-4 cells were maintained in 70/30 medium, astrocytic CG-4 cells were differentiated in N1 + 20% serum-containing medium, and oligodendrocytic CG-4 cells were differentiated in N1 medium. Photographs were taken using a phase contrast microscope.

Note the multipolar processes of the oligodendrocytic as compared to the bipotential CG-4 cells. All three differentiation states have distinct morphologies. Bar = 10 μm

Bmx	MEK6
Btk	Mek7
Calmodulin-dependent kinases kinase	Mnk2
Calmodulin-dependent kinase 1	Mos
Calmodulin-dependent kinase 4	Mst1
Cyclin-dependent kinases 1 (Cdc2)	Nek2
Cyclin-dependent kinase 2	p38 Hog MAPK
Cyclin-dependent kinase 4	Pak α (Pak 1)
Cyclin-dependent kinase 5	Pak β (Pak 3)
Cyclin-dependent kinase 6	PDK1 (PKB kinase)
Cyclin-dependent kinase 7	Pim1
Cyclin-dependent kinase 9	PKA (cAMP-dep. protein kinase)
Casein kinase 1 δ	PKB α (Akt1)
Casein kinase 1 ε	PKG1 (cGMP-dep. protein kinase)
Casein kinase 2 α/α'/α"	PKR
Cot (Tpl2)	Protein kinase C α
Csk	Protein kinase C β1
DAPK	Protein kinase C γ
DNAPK	Protein kinase C δ
Extracellular regulated kinase 1	Protein kinase C λ
Extracellular regulated kinase 2	Protein kinase C ε
Extracellular regulated kinase 3	Protein kinase C ζ
Extracellular regulated kinase 6	Protein kinase C θ
Focal adhesion kinase	Protein kinase C μ
Fyn	Pyk2
GCK	Raf1
GRK2 (BARK)	RafB
GSK3 α/β	ROK α
Hpk1	Rsk1
Inhibitor NF κB kinase α	Rsk2
JAK1	S6K p70
JAK2	SAPK (JNK2)
Ksr1	Src
Lck	Syk
Lyn	Yes
Mek1	ZAP70 kinase
Mek2	ZIP kinase
Mek4	

Table 2: Kinetworks™ Protein Kinase Screen

The expression levels of the 75 protein kinases listed above were assessed by the Kinetworks™ analysis performed by the Kinexus Bioinformatics Corporation.

4.2.1 Comparison of Primary OL to CG-4 OL

A number of kinases were not detected by the Kinetworks™ analysis in either primary OL or in the CG-4 line. These kinases include ERK6 from the MAPK family, as well as PKC's γ, λ, and θ. For a complete list of kinases not detected in primary OL or in CG-4 cells, see Table 3.

Among the kinases that were detected by the Kinetworks™ analysis were members of the MAPK family. In fact, most members of the MAPK family showed similar expression profiles in primary OL and in CG-4 OL. For a change in kinase expression to be considered relevant, the averaged densitometric values of two blots must have shown a minimum of 1.5-fold increase or decrease in expression. Furthermore, any trends of increased or decreased protein expression must have been supported by the original Kinetworks™ analysis. Based on these criteria, there were no major differences detected in ERK1, ERK2, p38, or SAPK expression (Fig 14). As well, upstream regulators of the MAPK family, such as MEK, Raf-1 and Raf B, and downstream substrates, such as Rsk-1, also showed similar expression levels (Fig. 15). There was, however, a noticeable change in Pak-α expression levels, and Pak-α has been shown to act upstream of the JNK and HOG members of the MAPK family. Pak-α expression was approximately 5-fold higher in primary OL than in CG-4 OL, and also at least 2-fold higher in primary OL than in astrocytic or bipotential CG-4 cells (Fig 16).

Btk	Mst1
Calmodulin-dependent kinase 4	Nek2
Casein kinase 1 ε	Pak β
CSK	Pim1
DAPK	PKG1 (cGMP-dep. protein kinase)
DNAPK	PKR
Extracellular regulated kinase 6	Protein kinase C γ
GCK	Protein kinase C λ
HPK1	Protein kinase C θ
Ksr1	Pyk2
Lck	ROK α
Lyn	Syk
Mnk2	Yes

Table 3: Protein Kinases Undetected by the Kinetworks™ Analysis

Antibodies that targeted the protein kinases listed above were present in the Kinetworks™ screen performed on extracts from primary rat OL, CG-4 OL, bipotential CG-4 cells and astrocytic CG-4 cells. However, the expression of these kinases was not detected in any of their cell lysates.

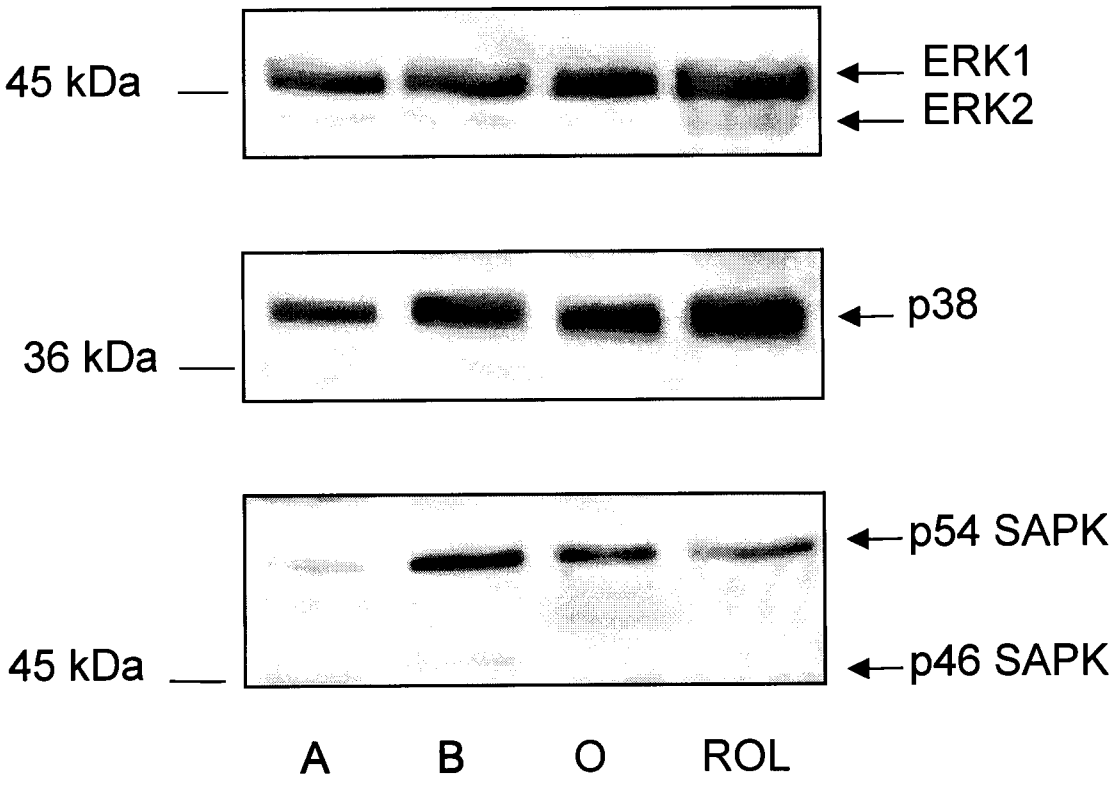

Figure 14: MAPK Expression Profile

Twenty μg of cytosolic protein lysate were loaded into each lane.

A = astrocytic CG-4 cells, cultured for 1 week in N1 medium + 20% FBS

B = bipotential CG-4 cells, cultured for 1 week in 70/30 medium

O = oligodendrocytic CG-4 cells, cultured for 1 week in N1 medium

ROL = mature rat oligodendrocytes, primary culture

Note that the ERK1/2 and p38 expression levels are similar across all lanes. Based on averaged densitometric values from two blots, there is a 2-fold upregulation of p54 SAPK in bipotential CG-4 cells in comparison to astrocytic CG-4 cells.

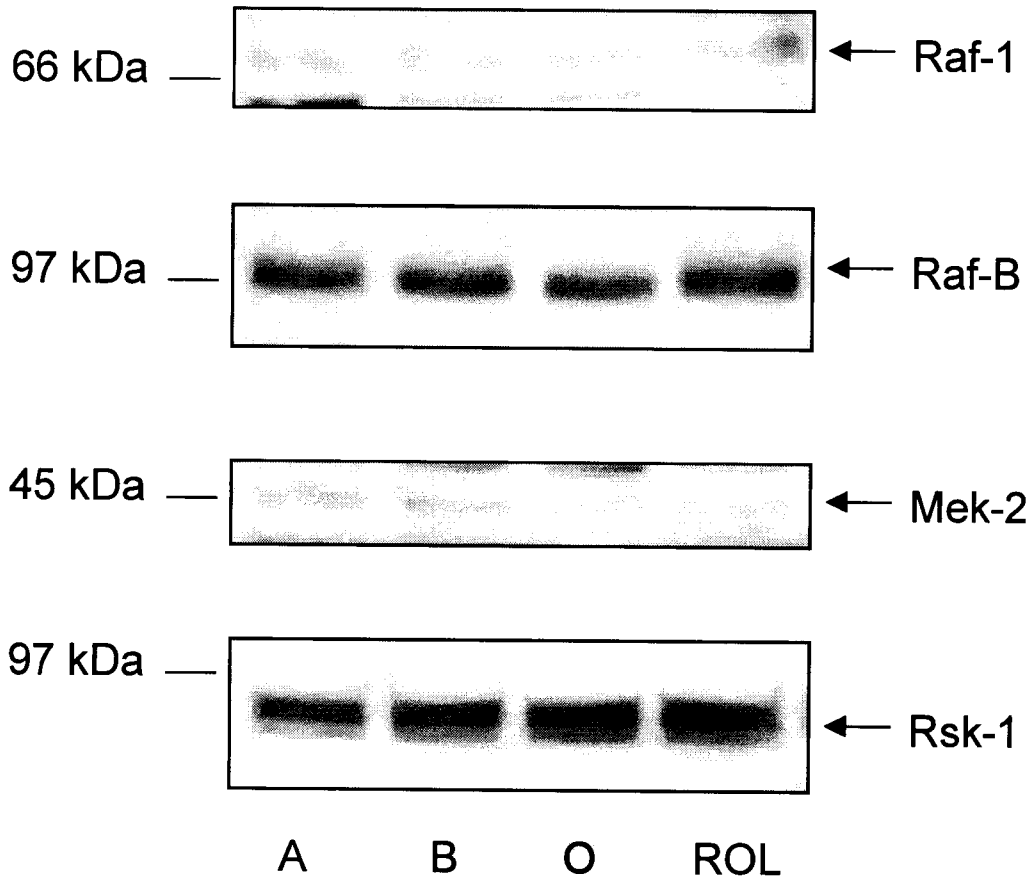

Figure 15: Western Blot Kinase Expression Profile

Twenty μg of cytosolic protein lysate were loaded into each lane.

A = astrocytic CG-4 cells, cultured for 1 week in N1 medium + 20%FBS

B = bipotential CG-4 cells, cultured for 1 week in 70/30 medium

O = oligodendrocytic CG-4 cells, cultured for 1 week in N1 medium

ROL = mature rat oligodendrocytes, primary culture

Note that the expression levels Raf-1, Raf-B, Mek-2 and Rsk-1 are similar across all lanes.

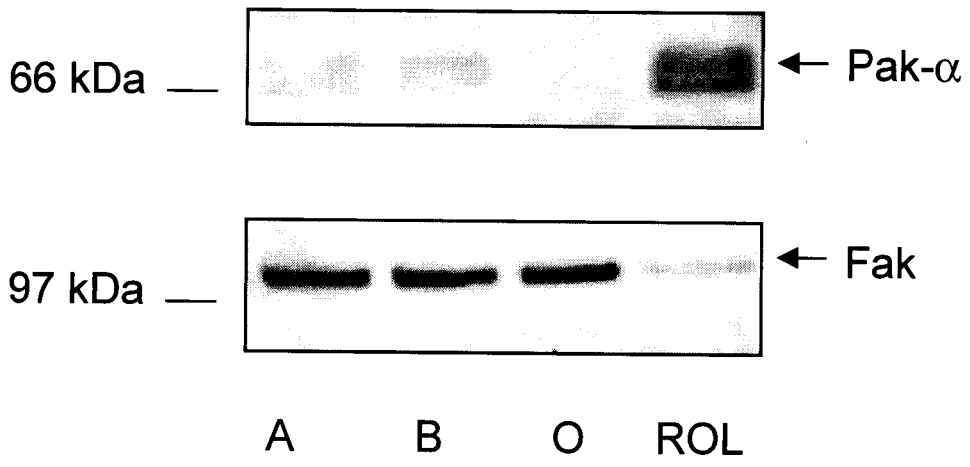

Figure 16: Western Blot Kinase Expression Profile

Twenty μg of cytosolic protein lysate were loaded into each lane.

A = astrocytic CG-4 cells, cultured for 1 week in N1 medium + 20%FBS

B = bipotential CG-4 cells, cultured for 1 week in 70/30 medium

O = oligodendrocytic CG-4 cells, cultured for 1 week in N1 medium

ROL = mature rat oligodendrocytes, primary culture

Based on averaged densitometric values from two blots, ROL has at least a 2-fold higher expression of Pak-α and a 2-fold lower expression of FAK as compared to A, B, or O.

Other kinases assessed included PKA, PKB, and PKC family members. While PKA and PKB expression was similar between primary OL and CG-4 OL, there were differences among some PKC isoforms (Fig. 17,18). In terms of PKC-α, the Kinetworks™ analysis detected kinase expression in CG-4 OL but not in primary OL. Verification blots, however, did detect PKC-α expression in both cell types. These verification blots showed a slight increase (1.3-fold) of PKC-α expression in CG-4 OL as compared to primary OL (Fig. 18).

The Kinetworks™ analysis and verification blots both indicated increased expression of PKC-β1 in CG-4 OL as compared to primary OL. Again, however, this increase was still only approximately 1.3-fold (Fig. 18). The expression of PKC-γ was undetectable in either cell type.

Two novel PKC isoforms, ε and δ, were also studied. It was found that primary OL expressed levels of PKC-ε that were at least 2-fold lower than in any of the CG-4 cells, and levels of PKC-δ that were at least 2-fold higher than in any of the CG-4 cells (Fig. 18). The expression of the atypical PKC-ζ isoform also appeared significantly higher in CG-4 OL than in primary OL in the Kinetworks™ analysis, but this difference was not confirmed on the verification blots (Fig. 18).

Outside of the MAPK and PKC pathways, interesting results were noted in two other kinases: cyclin-dependent kinase (CDK) and focal adhesion kinase (FAK). The expression of CDK5 was visible in primary OL, but not in CG-4 cells, on the Kinetworks™ analysis. Although verification blots did detect CDK5 in the CG-4 cell line, they also indicated that CDK5 expression was approximately 2-fold higher in primary OL than in any of the CG-4 cells (Fig. 19). In contrast to

CDK5, the expression of CDK7 was approximately 1.6-fold higher in CG-4 OL and bipotential CG-4 cells than in primary OL (Fig. 19). Finally, FAK expression was approximately 2-fold higher in all CG-4 cells as compared to primary OL (Fig. 16).

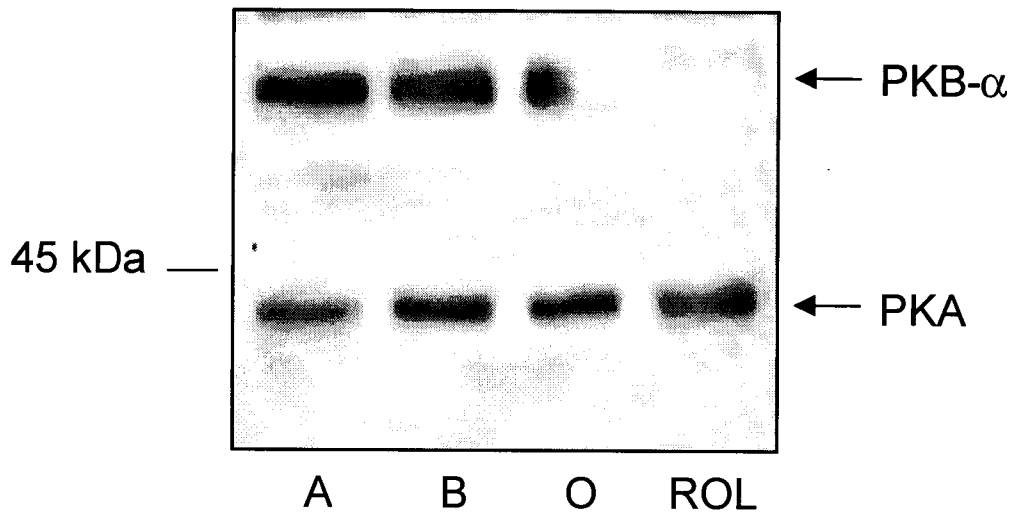

Figure 17: PKB-α and PKA Expression Profile

Twenty μg of cytosolic protein lysate were loaded into each lane.

A = astrocytic CG-4 cells, cultured for 1 week in N1 medium + 20% FBS

B = bipotential CG-4 cells, cultured for 1 week in 70/30 medium

O = oligodendrocytic CG-4 cells, cultured for 1 week in N1 medium

ROL = mature rat oligodendrocytes, primary culture

Note that the expression of PKB-α and PKA is similar in all lanes.

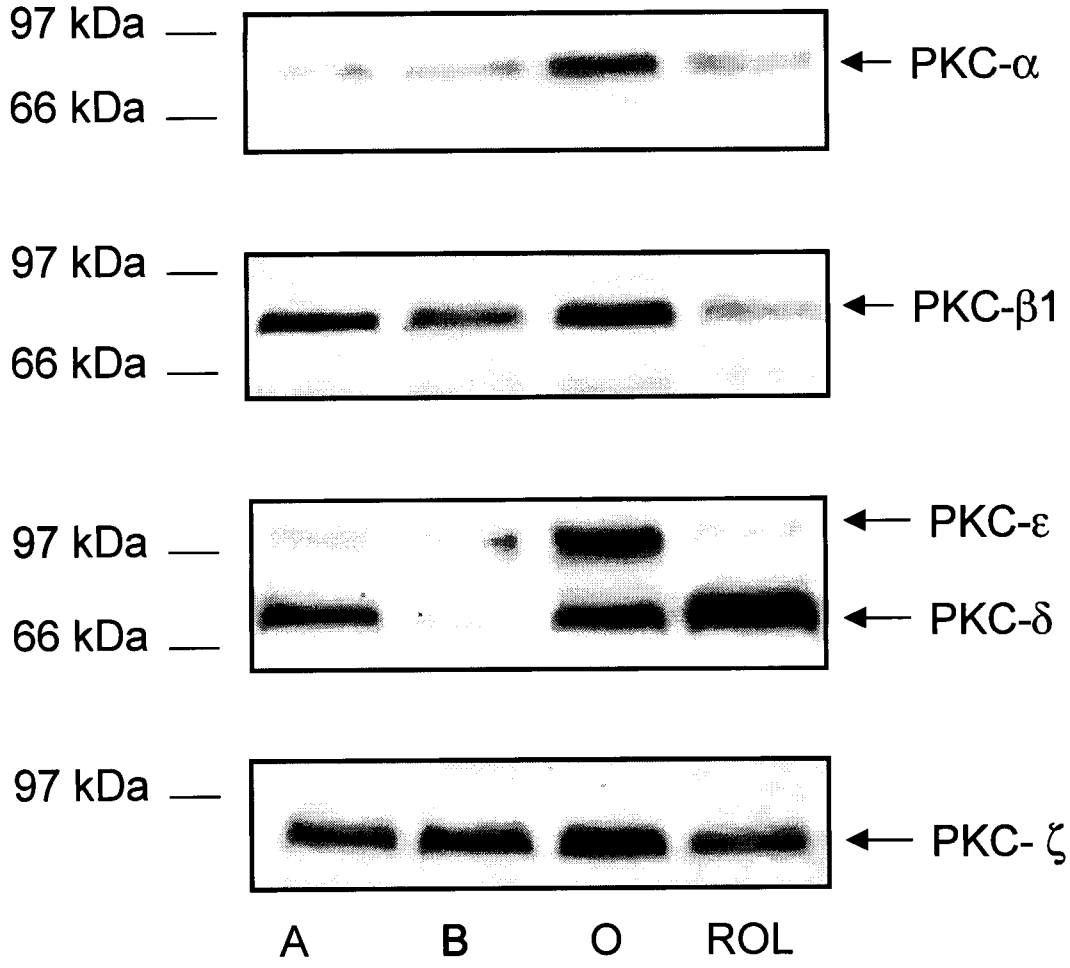

Figure 18: PKC Expression Profile

Twenty μg of cytosolic protein lysate were loaded into each lane.

A = astrocytic CG-4 cells, cultured for 1 week in N1 medium + 20%FBS

B = bipotential CG-4 cells, cultured for 1 week in 70/30 medium

O = oligodendrocytic CG-4 cells, cultured for 1 week in N1 medium

ROL = mature rat oligodendrocytes, primary culture

Based on averaged densitometric values from two blots, B has at least a 2-fold lower expression of PKC-α and PKC-β1 than O. As well, ROL has an approximately 1.3-fold lower expression of these isoforms than O. Finally, the expression of PKC-ε is at least 2-fold lower and the expression of PKC-δ is at least 2-fold higher in ROL as compared to A, B, or O.

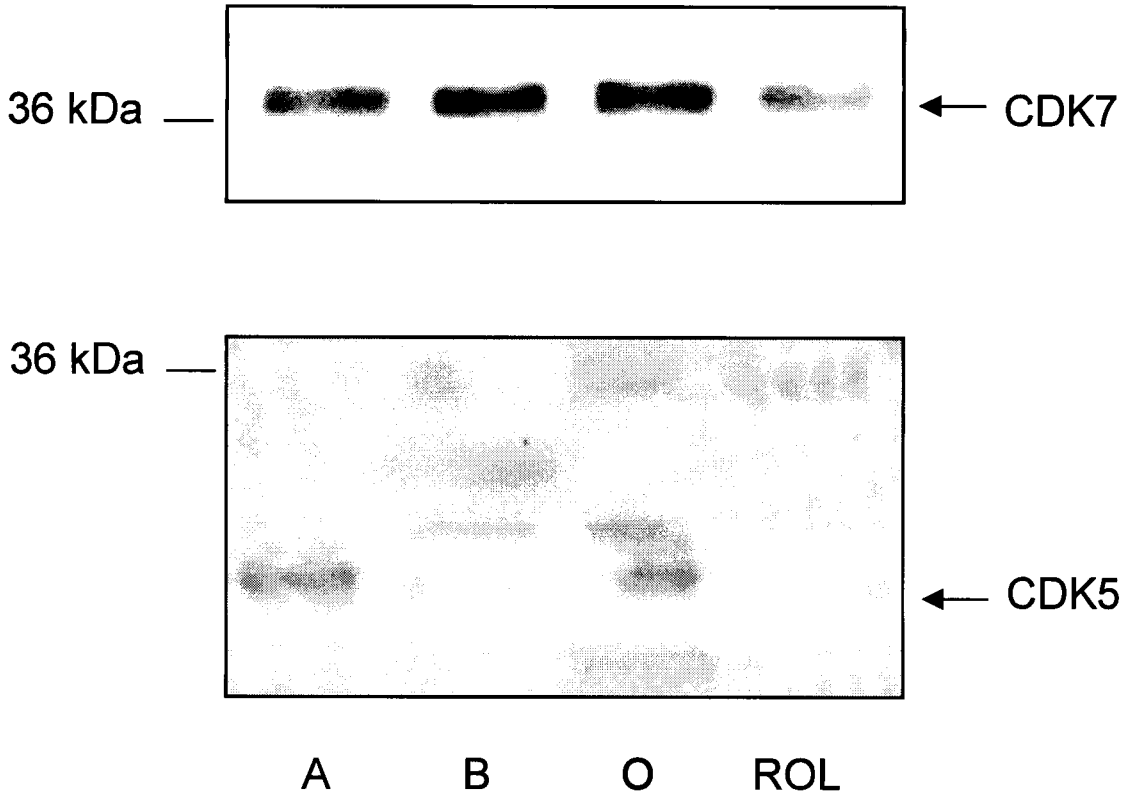

Figure 19: CDK7 and CDK5 Expression Profile

Twenty μg of cytosolic protein lysate were loaded into each lane.

A = astrocytic CG-4 cells, cultured for 1 week in N1 medium + 20% FBS

B = bipotential CG-4 cells, cultured for 1 week in 70/30 medium

O = oligodendrocytic CG-4 cells, cultured for 1 week in N1 medium

ROL = mature rat oligodendrocytes, primary culture

Based on averaged densitometric values from two blots, the expression of CDK7 is approximately 1.6-fold higher in B and O than in ROL. As well, the expression of CDK5 is approximately 2-fold higher in ROL than in A, B, or O. Also note the apparent bandshift in CDK5 in B and O.

4.2.2 A Comparison of the Three Differentiation States of CG-4

The Kinetworks™ multi-kinase immunoblot was also used to compare the bipotential, astrocytic, and oligodendrocytic forms of the CG-4 cell line. It was determined that most members of the MAPK family did not change expression drastically during the differentiation of bipotential CG-4 cells to astrocytic or oligodendrocytic CG-4 cells (Fig. 14). The largest MAPK change was a 2-fold increase of p54 SAPK expression in bipotential CG-4 cells as compared to astrocytic CG-4 cells. As well, Pak-α expression in oligodendrocytic CG-4 cells was at least 2.5-fold lower than in astrocytic or bipotential CG-4 cells. Furthermore, the Pak-α levels in all three CG-4 differentiation states were at least 2-fold lower than those seen in primary OL (Fig. 16).

The levels of PKA and PKB were also similar among all three CG-4 differentiation states (Fig. 17). However, there were some differences among PKC isoforms. For instance, PKC-α expression was at least 1.5-fold higher in oligodendrocytic and astrocytic CG-4 cells than in bipotential CG-4 cells (Fig. 18). There was also a 1.5-fold higher expression of PKC-β1 in oligodendrocytic CG-4 cells as compared to bipotential CG-4 cells, although an apparently similar increase of PKC-β1 expression in astrocytic CG-4 cells could not be confirmed (Fig. 18). While verification blots indicated an apparent increase of PKC-δ expression in astrocytic CG-4 cells as compared to bipotential CG-4 cells, this trend was not observed in the Kinetworks™ analysis (Fig. 18).

Finally, there was little difference in the expression levels of CDK7 and CDK5 among the three CG-4 differentiation states. The largest difference was an approximately 1.4-fold higher expression of CDK7 in bipotential and oligodendrocytic CG-4 cells as compared to astrocytic CG-4 cells (Fig 19).

4.3 Discussion

The main purpose of the Kinetworks™ analysis was to determine if the CG-4 OL model is suitable to use for signal transduction studies on OL process extension. Since process extension studies conducted on primary OL have already outlined a role for the MAPK and PKC families, the similarities in MAPK and PKC expression patterns between primary OL and CG-4 OL indicate that the CG-4 cell line may be a suitable OL model.

To begin with, all three branches of the MAPK cascade are present in both primary OL and CG-4 OL. Furthermore, no major changes in the expression levels of these MAPKs were noted between primary OL and CG-4 OL. Most upstream and downstream regulators of the MAPK cascades, such as various MEK, Raf, and Rsk isoforms, were also similarly expressed (Fig. 14,15). However, there was a large increase in the expression of one upstream regulator of the MAPK cascade, Pak-α, in comparison to all three CG-4 differentiation states (Fig. 16).

Pak-α is known to be enriched in the brain. The human homologue of Pak-α, Pak-1, has been implicated in the activation of various MAPKs (Knaus

and Bokoch, 1998). For instance, activation of JNK has been demonstrated by over-expression of Pak-1 in 293 cells, and has also been noted after addition of Pak-1 to cell free *Xenopus* oocyte extracts (Polverino et al., 1995; Frost et al., 1996). As well, other studies have demonstrated mediation of p38 activity by Pak-1 (Frost et al., 1996). Expression of dominant negative Pak-1 has been shown to suppress induction of p38 activity, while Pak-1 has been shown to be an upstream component of p38 activation in NK cells (Zhang et al., 1995; Mainiero et al., 2000). Although the consequences of JNK/p38 activation in many of these studies were not assessed, both JNK and p38 have been linked to apoptosis in OL (Casaccia-Bonnefil et al., 1996; Hida et al., 1999; Ladiwala et al., 1998). Therefore, the elevated expression of Pak-α in primary cultures might indicate that these cells are more susceptible to the induction of apoptotic cell death than the CG-4 cell line. However, since Pak-1 has also been shown to act in an anti-apoptotic manner via phosphorylation of the pro-apoptotic protein Bad, further studies would be needed to assess this hypothesis (Schurmann et al., 2000).

There is also evidence for Pak-α influence on the ERK members of the MAPK family. While studies have indicated that Pak isoforms do not directly lead to activation of ERK1/2, over-expression of the Pak-1 regulatory domain has been shown to inhibit ERK1/2 activation (Polverino et al., 1995; Frost et al., 1996; 1997). It is unclear from these ERK1/2 results why Pak-α should be upregulated in primary OL as opposed to CG-4 cells. However, the morphological consequences of Pak-α activation may provide some clues.

Pak-α has been shown to play a role in the morphological signalling pathways of various cell types. For instance, targeting of Pak-1 constructs to the plasma membrane in PC12 cells has been shown induce neurite extension independently of kinase activity (Daniels et al., 1998). Therefore, the upregulation of Pak-α expression in primary OL may explain why these cells are able to form an overall more intricate network of processes than CG-4 OL (Fig. 12). However, other reports have indicated that activation of Pak isoforms, rather than simply their expression, can cause retraction of the cell periphery (Manser et al., 1997). The role of Pak isoforms in cellular process extension, therefore, appears to depend on regulation of its activation state. Interestingly, CDK5 has been shown to stimulate neurite outgrowth by inactivation of Pak-1, and CDK5 appears to be highly expressed in primary OL (Nikolic et al., 1998) (Fig. 19).

As was mentioned above, the expression of PKA and PKB was also similar among primary OL and CG-4 cells (Fig. 17). The PKA pathway has been implicated in OL differentiation and proliferation, while PKB has been linked to PI 3-kinase signalling. PI-3 kinase, in turn, has been linked to OL survival (Vemuri and McMorris, 1996). Therefore, it seems that kinases from a variety of important signalling cascades are expressed in both the cell line and in primary cells. This again speaks to the suitability of the CG-4 model for OL signal transduction studies.

The expression of conventional PKC isoforms was also largely similar between primary OL and CG-4 OL, with a few significant differences. First, the noted lack of PKC-γ expression in primary OL and CG-4 OL is corroborated by

some studies and contradicted by other studies (Asotra and Macklin, 1993; Schmidt-Schultz and Althaus, 1994; Yong et al., 1994). This lack of consensus on the expression of PKC-γ in OL could be due to species variation. For instance, PKC-γ has been detected in cultured porcine OL, but not in human OL. Furthermore, PKC-γ has been found to be poorly expressed in rat OL, even though expression of this isozyme was detected in high levels in purified myelin. Since there is also evidence of developmental-dependent expression of various PKC isozymes in OL, the level of maturation of the OL used in the above-mentioned studies could also have affected the amount of PKC-γ detection. In contrast to PKC-γ, primary OL and CG-4 OL were both found to express PKC-α and βI (Fig. 18). Previous studies have also demonstrated the expression of these isoforms in OL, as well as an upregulation of PKC-α and βI in mature OL as compared to immature OL (Asotra and Macklin, 1993). This upregulation can again be seen when comparing bipotential CG-4 cells to CG-4 OL, indicating that upregulation of PKC-α and β1 may be required for OL cell differentiation (Fig. 18). The slight upregulation of PKC-α in astrocytic CG-4 cells as compared to bipotential CG-4 cells also speaks to a possible role for this isozyme in differentiation. In terms of the functions of PKC-α and PKC-β in OL, studies have indicated that PKC-α rather than PKC-β is the important mediator of OL process extension. In one such study, the PKC-α and β agonist thymeleatoxin was found to stimulate OL process extension. However, the PKC-β agonist, resiniferatoxin, was found to have no effect (Yong et al., 1994). In another study, MGDG was shown to increase both OL process extension and PKC-α activity (Schmidt-

Schultz and Althaus, 1994). Given that PKC-α may play a role in OL process extension, it is interesting that CG-4 OL appear to express slightly higher levels of PKC-α than primary OL. Since CG-4 OL extend processes within hours of attachment to a substratum, while primary OL can take days or weeks to send processes, the higher expression of PKC-α in CG-4 OL may partially explain the rapid ability of these cells to extend processes.

Perhaps the most significant differences in PKC isoform expression were in the δ and ε isoforms (Fig. 18). PKC-δ was expressed to a greater degree in primary OL than in any CG-4 differentiation state. A putative function of PKC-δ is inhibition of cell proliferation, as over-expression of PKC-δ has been shown to inhibit proliferation of NIH 3T3 cells (Mischak et al., 1993). Since the astrocytic and bipotential forms of the CG-4 cell line are highly proliferative, while primary OL are not, it seems reasonable that PKC-δ may play a role in the prevention of primary OL proliferation. This argument does not apply to CG-4 OL, however, as these cells also exhibit diminished proliferative capacity. Another putative function of PKC-δ is differentiation. Differentiation of murine erythroleukemia cells has shown to be blocked by decreases in PKC-δ expression, and inhibition of PKC-δ has been shown to block neurite outgrowth in PC-12 and H19-7 cells (Leng et al., 1993; Corbit et al., 1999). In this case, the lower level of PKC-δ expression in CG-4 OL could account for the generally lower level of differentiation attained by these cells in comparison to primary OL (Yim et al., 1995).

PKC-ε, by contrast, shows lower expression in primary OL than in any of the CG-4 differentiation states. This isoform could play on oncogenic role, as overexpression of PKC-ε in rat colonic epithelial cells has been shown to cause cell transformation (Perletti et al., 1996). Thus, the higher expression of PKC-ε in CG-4 cell as compared to primary OL indicates that this isoform may contribute to the mutation of primary O2A cells into the CG-4 cell line. However, conflicting studies also indicate that PKC-ε may play a role in differentiation, rather than proliferation, as over-expression of this isoform has been shown to increase EGF-induced neurite outgrowth (Brodie et al., 1999).

As well as the MAPK and PKC families, the cyclin-dependent kinase (CDK) family demonstrated interesting kinase expression patterns. On the one hand, the expression of CDK5 was higher in primary OL than in any of the CG-4 differentiation states. On the other hand, expression of CDK7, especially in the bipotential and oligodendrocytic CG-4 cells, was higher in the CG-4 cell line than in primary OL (Fig. 19).

The CDK family of kinases is involved in cell cycle dynamics by modulation of cell cycle regulatory protein known as cyclins. Specifically, CDK7 has been implicated in the activation of cyclin A and cyclin B complexes, leading to cell division (Larochelle et al., 1998). With respect to its proliferative function, inhibitors of CDK7 have been shown to inhibit tumour cell proliferation (Hajduch et al., 1999). CDK7 has also been found to be moderately upregulated in tumour cells as compared to normal control cells (Bartkova et al., 1996). Although the CG-4 cell line is purported to be non-tumorigenic, the moderate upregulation of

CDK7 in CG-4 cells as compared to primary OL could be a function of the self-renewal capacity of this cell line. CDK5, on the other hand, although it has been shown to associate with cyclins D1 and E, has not yet been shown to function as a kinase in these complexes (Xiong et al., 1992; Miyajima et al., 1995; Lee et al., 1997). It has, however, been shown to phosphorylate neurofilaments and to colocalize with actin and tubulin (Veeranna et al., 2000). These results imply that CDK5 plays a role in cytoskeletal dynamics. As primary OL are eventually able to produce more elaborate and intricate process extensions than CG-4, perhaps the upregulation of CDK5 in these cells speaks to its role as a cytoskeletal modulator. There also appears to be a CDK5 bandshift in the bipotential and oligodendrocytic CG-4 cells (Fig. 19). This could represent phosphorylation/ activation of this kinase, and activation of CDK5 has been shown to be involved in the outgrowth of neurites from neuronal cells (Nikolic et al., 1998; Li et al., 2000; Zukerberg et al., 2000). Therefore, the seemingly endogenous activation of the CDK5 in bipotential and oligodendrocytic CG-4 could be one reason why these cells are able to extend processes within hours of attachment to a substratum.

Finally, there was higher expression of FAK in all three CG-4 differentiation states than in primary OL (Fig. 16). Functions associated with FAK include cell adhesion and motility, as well as cell survival (Schlaepfer et al., 1999). In terms of cell adhesion, plating of BALB/c 3T3 fibroblasts onto a fibronectin substrate has been shown to activate FAK (Hanks et al., 1992). Since both CG-4 and primary OL can adhere to a poly-L-lysine/fibronectin substratum,

this perhaps explains why FAK expression is noted in both cell types. Another well-documented role for FAK is cell migration. Over-expression of FAK in Chinese hamster ovary cells has been shown to stimulate migration, and FAK-deficient fibroblasts have been shown to demonstrate reduced migration (Ilic et al., 1995; Cary et al., 1996). Since *in vivo* studies have shown that bipotential CG-4 cells have the ability to migrate, while mature primary OL do not, this could explain why FAK expression is higher in bipotential CG-4 than in primary OL (Duncan, 1996). It does not, however, explain why the expression of this kinase should also be higher in astrocytic and oligodendrocytic CG-4 cells. A possible explanation for the noted upregulation of FAK in all three CG-4 differentiation states could be its tumorigenic effects. Studies have shown that blocking FAK expression in tumour cells can induce apoptosis, and elevated FAK levels have been found in a variety of tumours (Weiner et al., 1993; Owens et al., 1995; Xu et al., 1996; Kornberg, 1998). It is possible that the general increase in FAK expression seen in the CG-4 cell line is characteristic of its immortalization potential rather than its cell adhesion/motility functions.

CHAPTER 5: PROCESS EXTENSION IN CG-4

5.1 Introduction

After surveying the kinase expression profile of the CG-4 cell line as described in Chapter 4, experiments using CG-4 cells were undertaken to further the process extension experiments begun on primary OL. CG-4 cells begin to extend processes almost immediately after adherence to a substrate in serum-free medium, and thus do not require a phorbol ester stimulus. As well, bipotential CG-4 cells maintained in 70/30 medium already demonstrate long, bipolar processes. However, since switching CG-4 cells to an N1 medium induces the formation of multipolar processes, the endpoint of these experiments was the expression of a multipolar phenotype. A multipolar phenotype is defined in this thesis as one in which the CG-4 cells exhibit upwards of four extensions that form a network of branches encircling the cell body.

Since inhibition of PKC can lead to an inhibition of phorbol ester-induced process extension in primary OL, the Ro-32 PKC inhibitor was initially applied to CG-4 cells in an attempt to block multipolar process extensions. Subsequent experiments were undertaken to determine the effects of MEK1/2 inhibition and PKC activation on the formation of multipolar processes in the CG-4 cell line.

5.2 Results

5.2.1 PKC Inhibitor Studies

CG-4 cells were maintained in 70/30 medium, then switched to N1 medium containing the PKC inhibitor Ro-32. Concentrations of Ro-32 ranging from 1 to 20 μM were applied, and concentrations of 5-10 μM were able to prevent the formation of multipolar processes (Fig. 20). A concentration of 20 μM Ro-32 was found to be cytotoxic.

Western blots were performed to determine if CG-4 cells cultured in N1 medium express an endogenous level of phosphorylated ERK1/2. Phosphorylation of ERK1/2 was used as an indication of increased ERK1/2 activation. Western blots were also used to determine if inhibition of any endogenous ERK1/2 activity by Ro-32 is responsible for the inhibition of process formation observed in Figure 20. The Western blots indicated that CG-4 cells in N1 medium with or without Ro-32 did not express the phosphorylated and more active forms of ERK1/2. CG-4 cells in 70/30 medium, however, did express marked levels of the phosphorylated and bandshifted forms of ERK1/2 (Fig 21). The bandshift in ERK1 was particularily evident, whereas the phosphorylated ERK2 co-migrated very close to the dephosphorylated ERK1. These blots also indicated that the phosphorylation of ERK1/2 seen in bipotential CG-4 cells is transient, as the phosphorylated ERK1/2 isoforms were nearly undetectable 1-2 hr after the application of fresh 70/30 medium. To assess if the transient ERK1/2

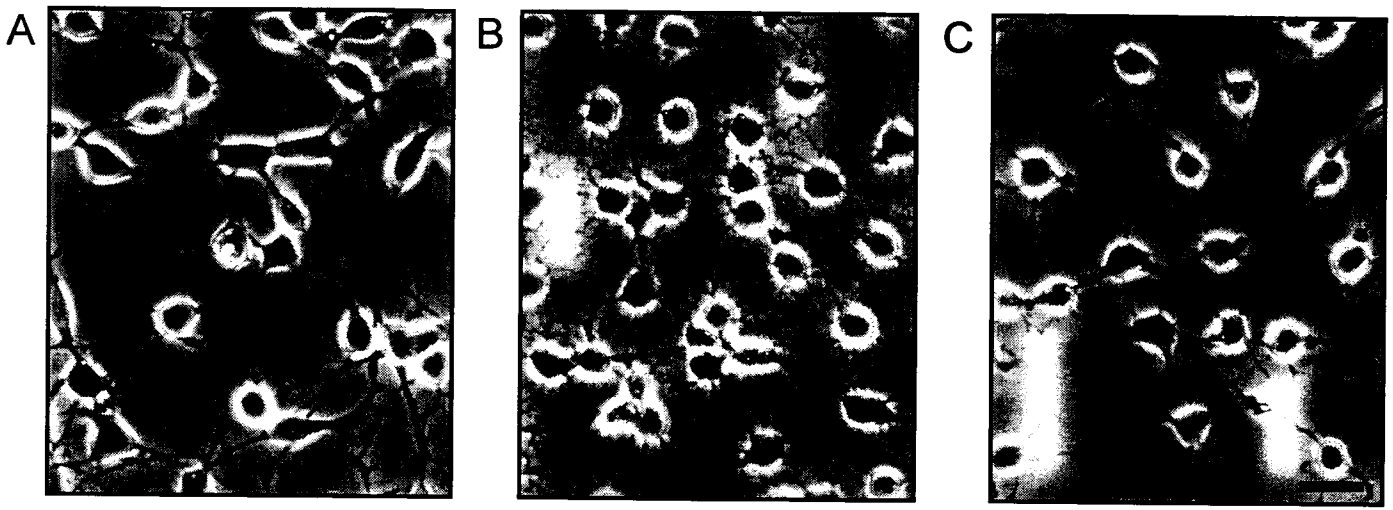

Figure 20: The Inhibitory Effects of Ro-32 on the Development of a Multipolar Phenotype in CG-4 OL

CG-4 cells were maintained in 70/30 medium for at least 48 hr prior to treatment. Photographs were taken using a phase contract microscope 24 hr after treatment.

A = 70/30 medium

B = N1 medium

C = N1 medium + 5 μM Ro-32

Note that the addition of Ro-32 to the N1 medium prevented the CG-4 cells from acquiring the multipolar phenotype seen in panel B. Results are typical of 5 experiments. Bar = 20 μm

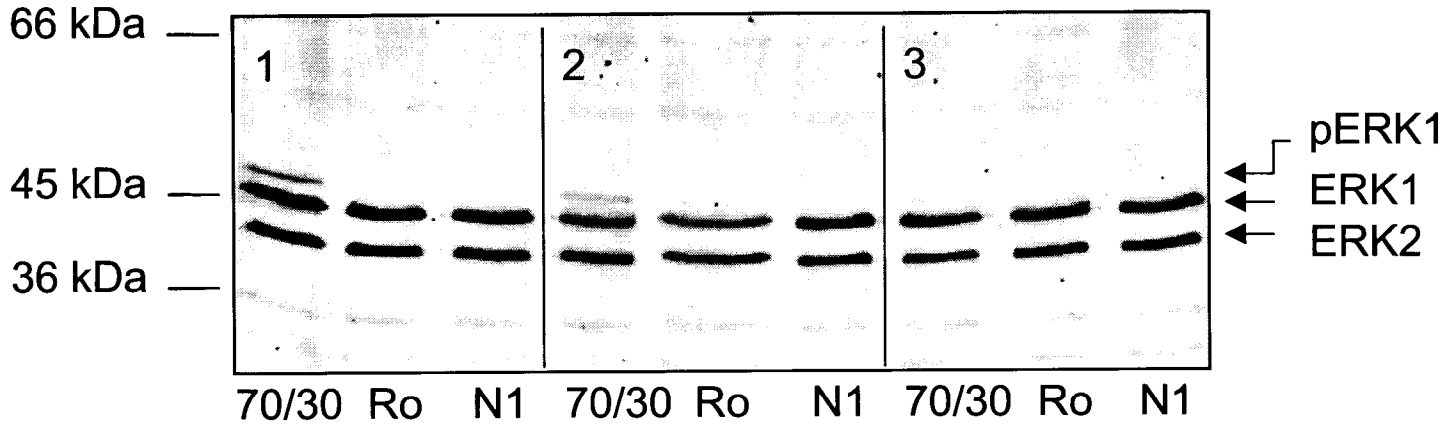

Figure 21: The Effects of 70/30, N1, and Ro-32 on ERK1/2 Activation in CG-4 Cells

CG-4 cells were lysed at 30 min (panel 1), 1 hr (panel 2), or 2 hr (panel 3) time points after exposure to the conditions described below:

70/30 = CG-4 cells fed with 70/30 medium

Ro = CG-4 cells fed with N1 medium + 5 μM Ro-32

N1 = CG-4 cells fed with N1 medium

Note the appearance of phosphorylated ERK1 at 30 min in CG-4 cells fed with 70/30 medium, and the comigration of phosphorylated ERK2 near the dephosphorylated ERK1. This trend was noted in 8 experiments. The disappearance of the ERK1 bandshift at later time pints was confirmed in 3 experiments.

activation provided by the addition of fresh 70/30 medium every 48 hr prevents CG-4 cells from developing a multipolar morphology, CG-4 cells were cultured in 70/30 medium for 4 days with no medium change. With a lack of fresh 70/30 medium, these cells did indeed begin to form multipolar process extensions (Fig. 22).

The next experiments were undertaken in order to clarify the effects of Ro-32 and ERK1/2 activation in CG-4 cells. First, bipotential CG-4 cells were switched to fresh 70/30 medium containing 5 μM Ro-32 in an attempt to block ERK1/2 activation. Second, the cells were lysed after 30 min of Ro-32 treatment and subjected to Western blotting. It was found that Ro-32 had no effect on the transient ERK1/2 activation noted in bipotential CG-4 cells, even when these cells were pre-incubated with the inhibitor prior to the medium change (Fig. 23). Not surprisingly, therefore, a parallel experiment showed that Ro-32 had no morphological effects on CG-4 cells in 70/30 medium.

Since Ro-32 is a PKC inhibitor, CG-4 cells in both 70/30 and N1 media were next assessed for any endogenous PKC-α activity. CG-4 cells were switched to either fresh 70/30 or N1 medium for 15 min, then lysed. The lysates were separated into cytosolic and membrane fractions and subjected to Western blotting. The activity of PKC-α was assessed by observing any translocation from cytosolic to membrane fractions. Although it is possible that some of the PKC-α in the cytosolic fraction could be active, translocation of PKC-α to the membrane fraction commonly signifies activation of this kinase. PKC-α was not detected in the membrane fractions of CG-4 cells in either 70/30 or N1 medium.

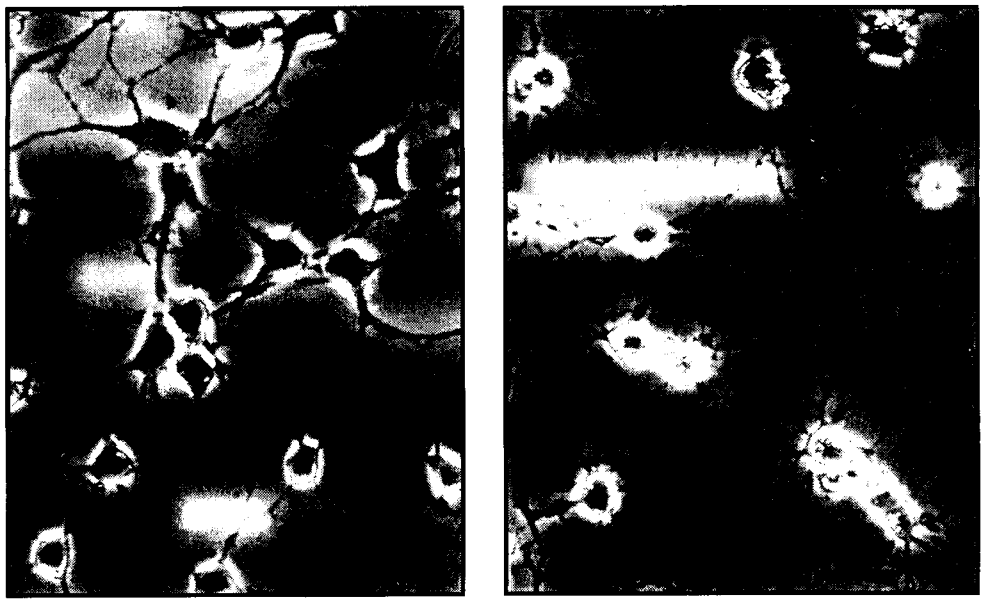

Figure 22: Morphological Changes of CG-4 Cells

CG-4 cells in the left panel had 70/30 medium replaced every 48 hr for 4 days. CG-4 cells in the right panel remained in the same 70/30 medium for 4 days. Note that, in CG-4 cells which did not receive fresh 70/30 medium every 48 hr, multipolar processes began to form. Photographs were taken using a phase contrast microscope and are representative of 3 experiments. Bar = 20 μm

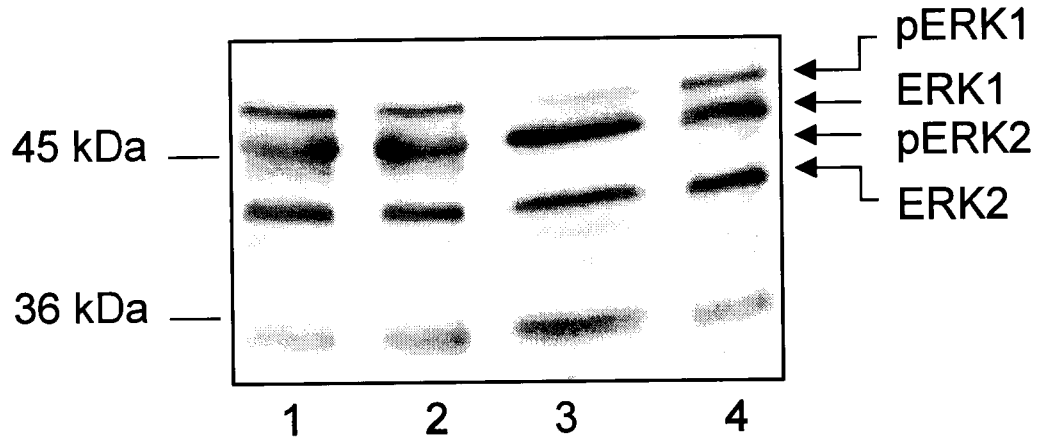

Figure 23: The Effects of Ro-32 on ERK1/2 Activation in CG-4 Cells

CG-4 cells were lysed 30 min after various treatments.

1 = CG-4 cells exposed to 70/30 medium

2 = CG-4 cells exposed to 70/30 medium + 5 μM Ro-32

3 = CG-4 cells exposed to N1 medium

4 = CG-4 cells pretreated with 5 μM Ro-32 for 20 min prior to exposure to 70/30 medium + 5 μM Ro-32

Note the ERK1/2 bandshift in all 70/30-treated samples, regardless of the addition of Ro-32. The inability of Ro-32 to inhibit ERK1/2 activation in duplicate experiments indicates that the ERK1/2 activation seen in 70/30 treated cells is not necessarily linked to PKC activation.

In confirmation of the multi-kinase blots conducted in Chapter 4, however, PKC-α was detected in the cytosolic fractions (Fig. 24). A parallel blot also confirmed that phosphorylation of ERK1/2 can occur independently of PKC-α activity in these cells (Fig. 24).

To determine if kinases other than ERK1/2 are differentially phosphorylated between CG-4 cells in either 70/30 or N1 medium, a Kinetworks™ phosphokinase profile was performed 20 min after medium change. Since it is unclear why Ro-32 prevented the expression of a multipolar phenotype in CG-4 fed with N1 medium + 5 μM Ro-32, these Ro-32 treated cells were also profiled. The multi-kinase blot did not reveal significant differences between CG-4 cells in either N1 or 70/30 medium, other than the increased ERK1/2 phosphorylation previously noted in Figure 21 (Table 4). In terms of Ro-32 treatment, preliminary results from the Kinetworks™ phosphoprotein screen indicated a decrease of STAT3 phosphorylation in CG-4 cells treated with N1 medium + 5 μM Ro-32 as compared to CG-4 cells in either N1 or 70/30 medium alone (Table 4).

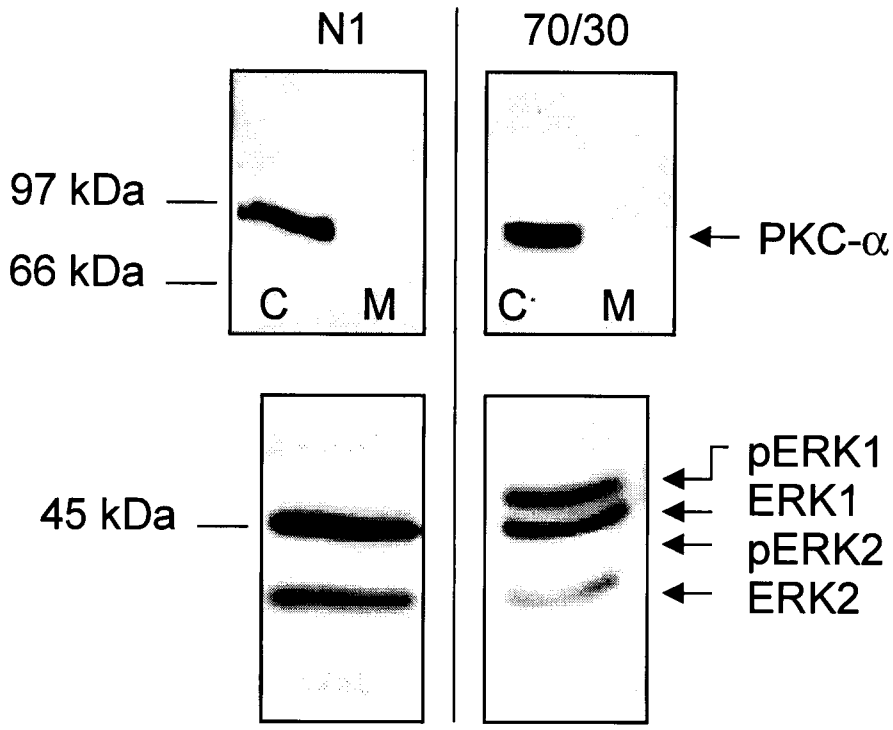

Figure 24: The Effects of 70/30 and N1 on PKC-α Activity in CG-4 Cells

CG-4 cells were maintained in 70/30 medium for at least 48 hr prior to medium change. Cells were lysed 15 min after medium change. The blots on the left correspond to cells fed with N1 medium, while the blots on the right correspond to cells fed with 70/30 medium. PKC-α activation was assessed by separating the lysates into cytosolic (C) and membrane (M) fractions. ERK1/2 activation was assessed by observing bandshifts.

Note that PKC-α did not appear to be activated by either N1 or 70/30 medium, as the kinase is not detected in the membrane fractions. Furthermore, the ERK1/2 bandshift noted in cells fed with 70/30 medium occurred independently of PKC-α translocation. This data is representative of three experiments.

Phosphoprotein	N1 + Ro-32	N1	70/30
NR1	8	9	11
Adducin	14	13	16
ERK1	24	19	57
PKB/Akt	6	6	9
GSK3 α/β	18	14	14
Protein kinase C ε	10	8	11
Protein kinase C α	21	20	18
Protein kinase C α/β	17	21	22
Src	12	13	10
STAT3	14	24	22

Table 4: Kinetworks™ Phosphoprotein Analysis

The phosphoproteins listed above are part of the phosphoprotein screen conducted by Kinexus. The numerical values represent the relative quantity (%) of the ECL signals from individual bands within each lane. CG-4 cells were maintained in 70/30 medium for at least 48 hr prior to treatment, and then lysed for analysis 20 min after treatment.

N1 + Ro-32 = CG-4 treated with N1 medium containing 5 μM Ro-32

N1 = CG-4 treated with N1 medium

70/30 = CG-4 treated with fresh 70/30 medium

Note the approximately 3-fold increase in the relative quantity of phosphorylated ERK1 in 70/30 samples as compared to N1 samples. These was also an approximately 1.7-fold increase in the relative quantity of phosphorylated STAT3 in N1 samples as compared to N1 + Ro-32 samples.

5.2.2. MEK Inhibitor Studies

Since all the results described above implicate ERK1/2 activation in the bipolar nature of CG-4, these cells were next treated with MEK1/2 inhibitors. PD 98059 and UO-126 were employed in an attempt to inhibit ERK1/2 and to induce the expression of a multipolar phenotype. However, morphological monitoring showed that inhibitor concentrations ranging from 12.5 to 50 μM did not affect the bipolar CG-4 cell phenotype, while 100 μM was cytotoxic (Fig. 25). Even preincubation of the cells in either inhibitor for 20 min prior to treatment had no morphological effect. Next, Western blots were performed to determine the level of phosphorylated and active ERK1/2 in these cells. The blots showed that, while both 50 μM PD 98059 and 50 μM UO-126 were able to reduce ERK1/2 phosphorylation, neither inhibitor could completely abolish their phosphorylation (Fig. 26). To assess if a higher MEK inhibitor concentration could abolish ERK1/2 phosphorylation in CG-4, Western blots using 100 μM PD 98059 were conducted. These blots confirmed that ERK1/2 inhibition via MEK1 inhibition is possible in these cells (Fig. 27).

5.2.3. Phorbol Ester Studies

To test the hypothesis that activation of ERK1/2 appears to prevent the formation of multipolar processes in CG-4 cells, the phorbol ester PMA was employed in an attempt to phosphorylate ERK1/2 via activation of PKC-α. CG-4

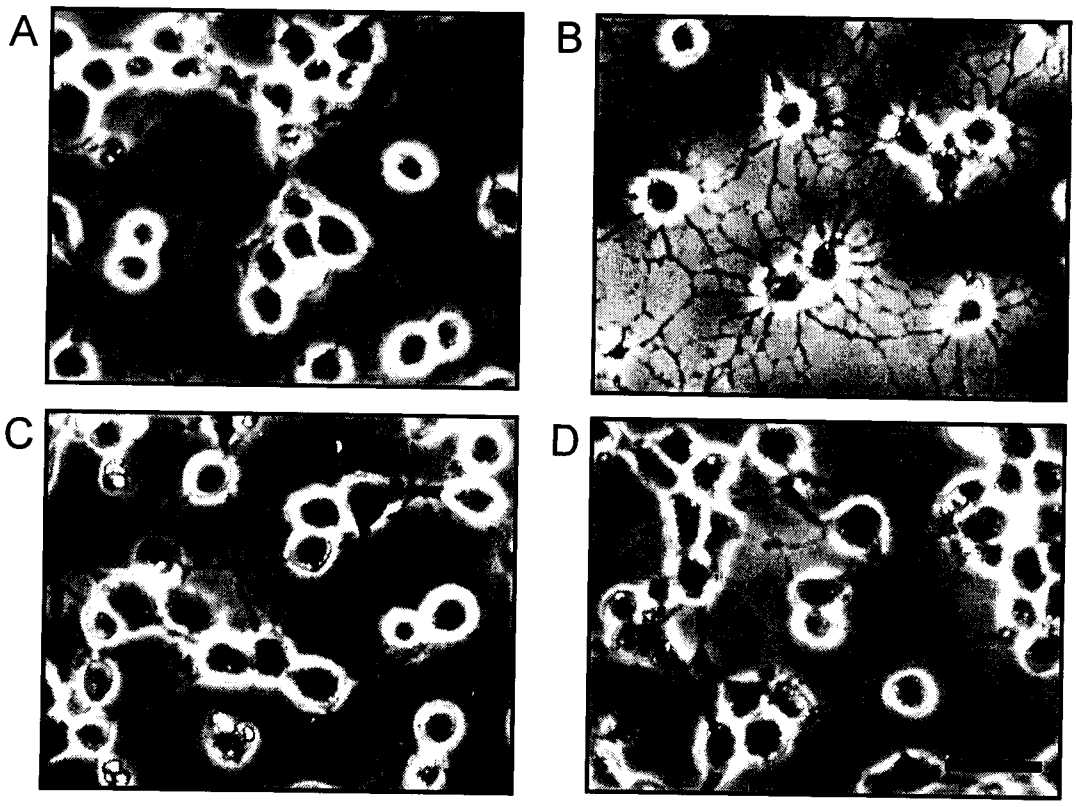

Figure 25: The Effects of PD 98059 and UO-126 on CG-4 Cell Morphology

CG-4 cells were maintained in 70/30 medium for at least 48 hr prior to treatment. Photographs were taken using a phase contrast microscope 24 hr after treatment.

A = CG-4 in 70/30 medium

B = CG-4 in N1 medium

C = CG-4 in 70/30 medium + 50 μM PD 98059

D = CG-4 in 70/30 medium + 50 μM UO-126

Note that neither MEK1/2 inhibitor induced CG-4 cells to adopt the oligodendrocytic, multipolar phenotype observed in panel B. Results are representative of 4 experiments. Bar = 20 μm

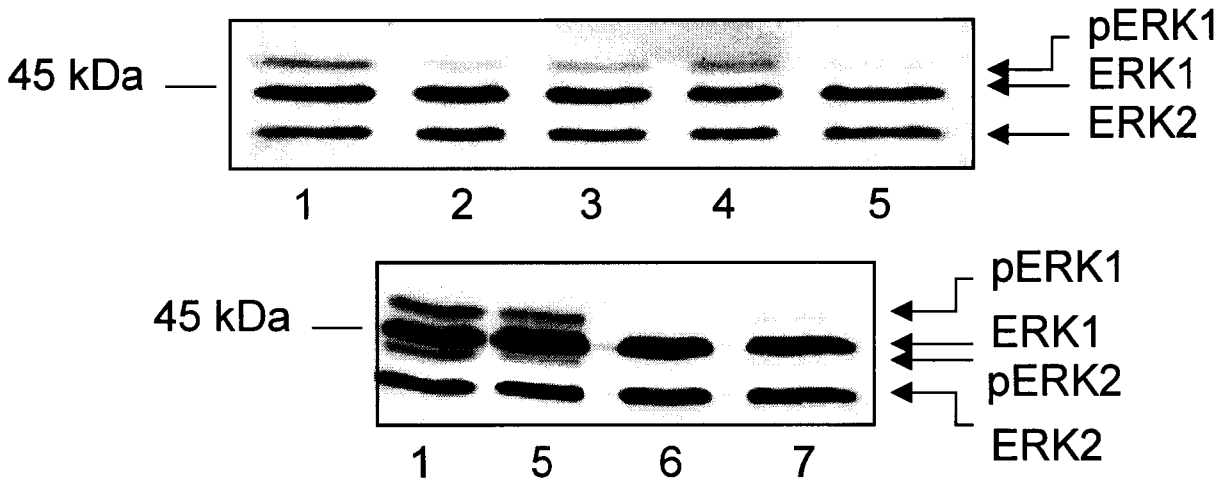

Figure 26: The Effects of PD 98059 and UO-126 on ERK1/2 Activation in CG-4 Cells

CG-4 cells were exposed to the MEK1/2 inhibitors PD 98059 and U0-126 during the change to fresh 70/30 medium. ERK1/2 bandshifts were observed in order to assess if the inhibitors could block the ERK1/2 activation associated with the addition of fresh 70/30 medium. Treatments were 30 min in length.

1 = 70/30 medium

2 = 70/30 medium + 25 μM UO-126

3 = 70/30 medium + 50 μM UO-126

4 = 70/30 medium + 25 μM PD 98059

5 = 70/30 medium + 50 μM PD 98059

6 = N1 medium

7 = CG-4 pretreated with 50 μM PD 98059 for 20 min prior to treatment with 70/30 medium + 50 μM PD 98059

In the upper panel, note that there was no marked reduction of phosphorylated ERK1 in MEK1/2 inhibitor-treated samples. Phosphorylated ERK2 comigrated near the dephosphorylated ERK1. In the lower panel, note that even pretreatment of the cells with PD 98059 did not decrease ERK1/2 phosphorylation to the level seen in N1 treated cells. This data is representative of duplicate experiments.

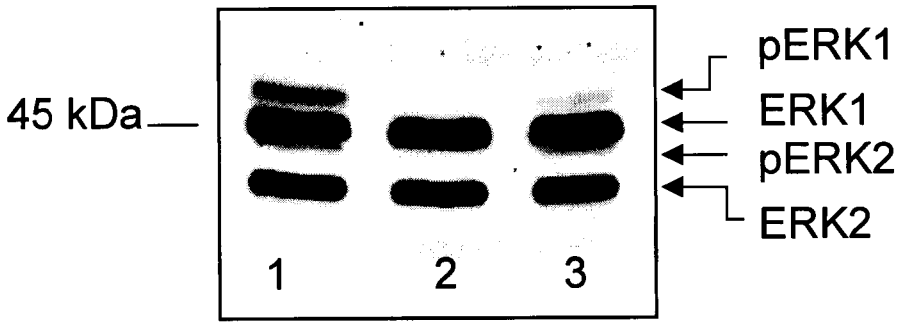

Figure 27: The Effects of PD 98059 on ERK1/2 Activation in CG-4 Cells

CG-4 cells were treated with a high dose of the MEK1 inhibitor PD 98059 to determine if the inhibitor has the ability to inhibit ERK1/2 phosphorylation.

1 = CG-4 cells in fresh 70/30 medium for 30 min

2 = CG-4 cells pretreated with 100 μM PD 98059 for 20 min prior to treatment with 100 μM PD 98059 in fresh 70/30 medium for 30 min more.

3 = CG-4 cells in N1 medium for 30 min

This high dose of the PD 98059 abolished the ERK1/2 phosphorylation associated with the addition of fresh 70/30 medium in duplicate experiments.

cells in N1 medium were exposed to PMA concentrations ranging from 1 to 40 nM PMA, and cell analysis was carried out 24-48 hr later. A PMA concentration of 20 nM was the lowest concentration sufficient to completely abolish the formation of a multipolar phenotype over a 24 hr period. After 48 hr, multipolar processes began to form even in PMA-treated cells (Fig. 28). Next, cells were treated with 20 nM PMA in N1 medium for 15 min prior to lysis and Western blotting. The lysates were separated into cytosolic and membrane fractions. The Western blots indicated a significant translocation of PKC-α, a very small translocation of PKC-β, and the appearance of activated ERK1/2 (Fig. 29). To further monitor the relationship between PKC-α translocation and ERK activation, a dose response using a concentration range of 1 nM to 20 nM PMA was conducted. After exposure to PMA for 15 min, CG-4 cells were lysed, fractionated, and Western blotted. The Western blots indicated that PKC translocation is dose dependent, and that noticeable ERK1/2 phosphorylation only occurs with the PKC-α translocation seen at the 20 nM PMA dose (Fig. 30). Therefore, 20 nM PMA was the lowest dose able to both completely block the formation of multipolar processes and to markedly increase ERK1/2 phosphorylation.

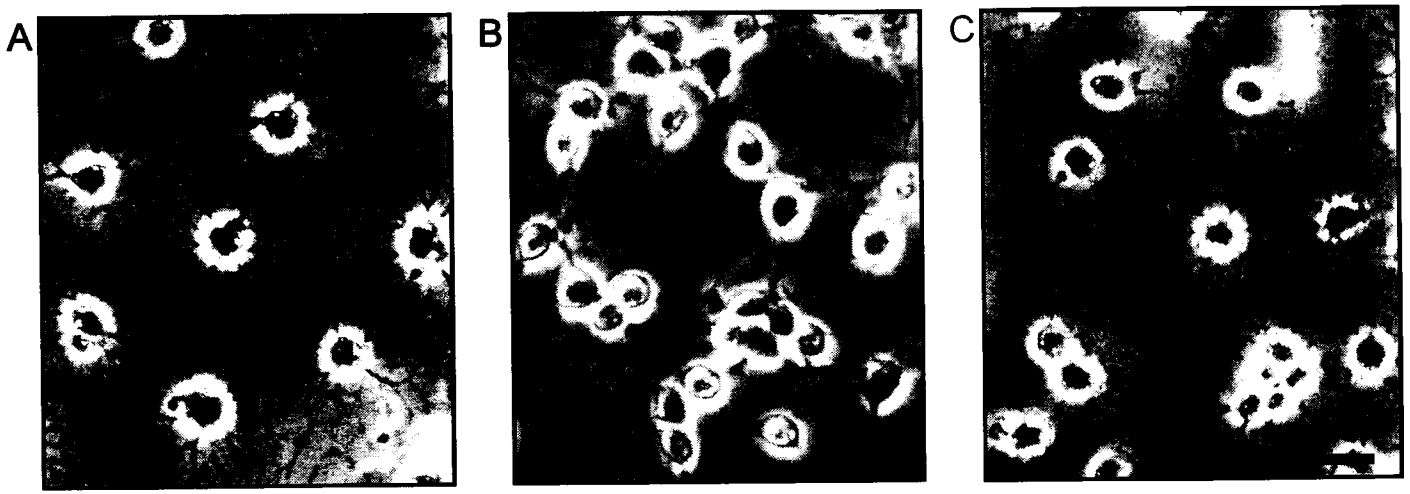

Figure 28: Transient Inhibition of a Multipolar Phenotype in CG-4 Cells by PMA Treatment

A = CG-4 cells cultured for 24 hr in N1 medium

B = CG-4 cells cultured in for 24 hr N1 medium + 20 nM PMA.

C = CG-4 cells cultured for 48 hr in N1 medium + 20 nM PMA.

Note that the addition of PMA to the culture medium prevented the CG-4 cells from acquiring a multipolar phenotype over a 24 hr period. After 48 hr, however, the cells were able to develop multipolar processes. Photographs were taken using a phase contrast microscope and are representative of 3 experiments. Bar = 20 μm

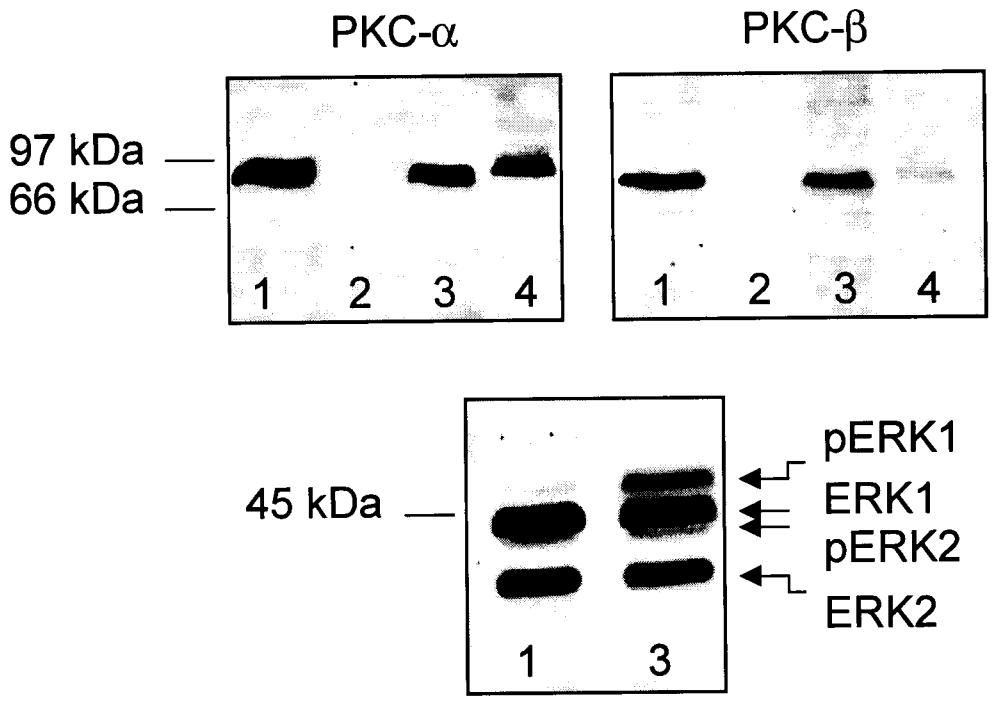

Figure 29: The Activation of PKC and ERK1/2 in CG-4 Cells Treated with PMA

CG-4 cells were maintained in 70/30 medium for at least 48 hr prior to feeding with N1 medium alone or N1 medium containing 20 nM PMA. Cells were lysed 15 min after treatment.

1 = Cytosolic fraction, N1 treatment

2 = Membrane fraction, N1 treatment

3 = Cytosolic fraction, N1 + PMA treatment

4 = Membrane fraction, N1 + PMA treatment

Note that the translocation of PKC-α was much greater than the translocation of PKC-β after PMA treatment. Furthermore, this translocation corresponded to an activation of ERK1/2. These results are representative of 3 experiments.

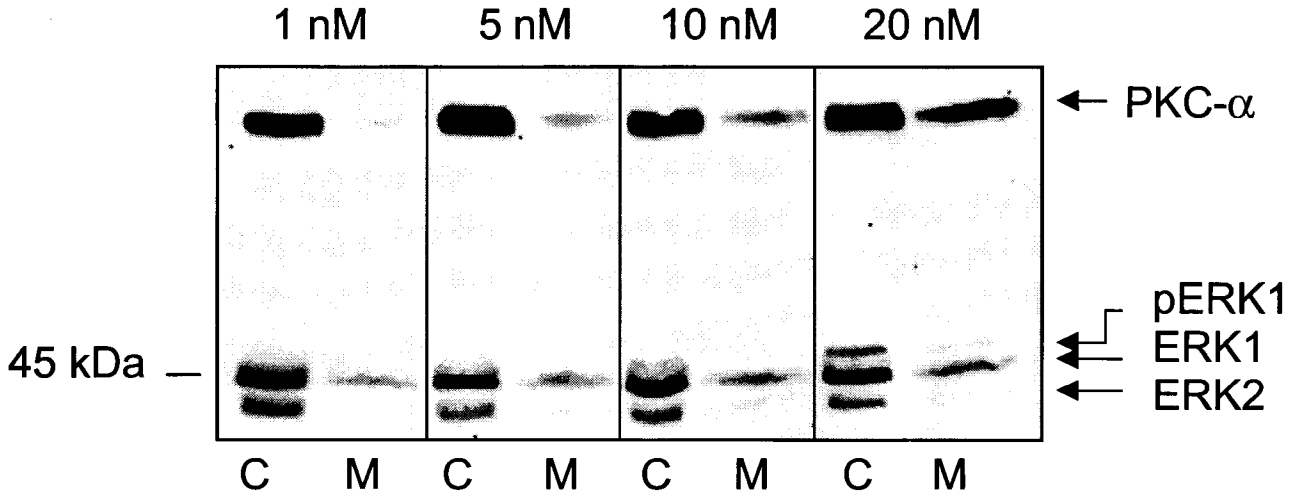

Figure 30: PMA Dose Response

CG-4 cells were maintained in 70/30 medium for at least 48 hr prior to treatment with N1 + PMA (1nM, 5 nM, 10 nM, or 20 nM). Cells were lysed 15 min after treatment, and were loaded as either cytosolic (C) or membrane (M) fractions.

Note that ERK1 activation appears to correlate to PKC-α activation, as both marked PKC-α translocation and ERK1 phosphorylation occurred after 20 nM PMA treatment. Phosphorylated ERK2 comigrated near dephosphorylated ERK1. Activation of ERK1 at the 20 nm PMA dose was representative of 3 experiments. The dose response was duplicated.

5.3 Discussion

5.3.1 PKC Inhibitor Studies

These CG-4 experiments were undertaken to first confirm the role of ERK1/2 in OL process extension, and to then expand the knowledge base on signal transduction events involved in OL process extension. Since PKC inhibitors have been shown to inhibit phorbol ester-induced process extension in primary OL, the PKC inhibitor Ro-32 was applied to CG-4 cells in an attempt to inhibit the formation of multipolar processes. Switching CG-4 cells from 70/30 medium, which contains B-104 mitogens, to N1 medium, which does not contain these mitogens, induces the formation of multipolar processes in CG-4 (Fig. 13). Therefore, studies were undertaken to determine whether including Ro-32 in the N1 medium could inhibit this process formation. As can be seen in Figure 20, CG-4 treated with Ro-32 in N1 medium did indeed demonstrate inhibition of multipolar process formation.

Working with the hypothesis that ERK1/2 activation is a crucial component in the induction of OL process extension, it was hypothesized that CG-4 cells in N1 medium may express an endogenous level of ERK1/2 activation. In such a scenario, the treatment of these cells with Ro-32 might have inhibited this activation and thereby inhibited process formation. However, the Western blot depicted in Figure 21 shows that CG-4 cells in N1 medium do not contain endogenously active ERK1/2, and therefore Ro-32 does not affect the level of

ERK1/2 activity in these cells. Instead, CG-4 cells in 70/30 medium were found to show a transient activation of ERK1/2 up to 1 hr after the application of fresh medium. ERK1/2 activation was subsequently hypothesized to inhibit multipolar process formation rather than to induce process formation in CG-4 cells.

To test this hypothesis, CG-4 cells were left in 70/30 medium for 4 days with no medium change. The medium is normally replaced every 48 hr in order to maintain CG-4 cells in their bipotential state, and thus bipotential CG-4 cells are subject to transient ERK1/2 activation every 48 hr. As can be seen in Figure 22, without the application of fresh 70/30 medium, CG-4 cells began to develop multipolar processes. This result implies that transient but repetitive ERK1/2 activation is required in order to prevent multipolar process formation in the CG-4 cell line.

The lack of ERK1/2 activation in CG-4 cells switched to N1 medium negates the possibility of ERK1/2 inhibition by Ro-32 in these cells, but it does not rule out the possibility of PKC inhibition. Therefore, CG-4 cells were assessed for PKC-α translocation 15 min after change to either N1 or 70/30 medium. PKC-α was the isoform chosen for assessment as it is one of the conventional PKC isoforms, and Ro-32 is a conventional PKC inhibitor (Wilkinson et al., 1993). Furthermore, PKC-α rather than PKC-β is the isoform implicated in primary OL process extension, while PKC -γ is virtually undetectable in these cells. The blots indicate, however, that PKC-α does not translocate to the membrane fraction in these cells, and is therefore not likely a potential candidate for Ro-32 inhibition.

The Western blots in Figure 23 also show that ERK1/2 phosphorylation is independent of PKC-α activation in CG-4 cells fed with 70/30 medium. Since 70/30 medium, by definition, contains 30% B-104 mitogens, and since N1 medium does not contain these mitogens, the B-104 conditioning must be responsible for this observed ERK1/2 phosphorylation. The most credible scenario involves phosphorylation and activation of ERK1/2 via activation of protein tyrosine kinase receptors, leading to the sequential activation of Ras, Raf-1, MEK1/2, and ERK1/2. It has been previously determined that the active components of B-104 conditioned medium are bFGF and PDGF (Louis et al., 1992). Therefore, these two growth factors are the best candidates for activation of protein tyrosine kinase receptors leading to ERK1/2 activation in bipotential CG-4 cells.

Speculating that the ERK1/2 activation cascade in CG-4 cells involves bFGF and PDGF activation of tyrosine kinase receptors, as opposed to activation of PKC-α, it becomes clear why Ro-32 had no effect on ERK1/2 phosphorylation in these cells. However, the fact remains that Ro-32 was able to inhibit the formation of processes of CG-4 cells in N1 medium. Therefore, a multi-phosphoprotein immunoblot conducted by Kinexus Bioinformatics Corporation was used to survey potential candidates for Ro-32 inhibition in these cells. Since the IC_{50} of Ro-32 is 9 nM for PKC-α and 28 nM for PKC-β, while the minimum concentration required for abolition of process formation in CG-4 cells is 5 μM, it is possible that this inhibitor was demonstrating non-specific effects in the CG-4 cell line. The phosphorylated proteins surveyed included PKC-α, β, and ε, but

these kinases showed no decrease in phosphorylation with the inhibitor. However, the survey did indicate that STAT3 may be non-specifically inhibited by Ro-32. Since STAT3 has been shown to play a role in the differentiation of PC-12 cells, inhibition of this kinase by Ro-32 could explain why CG-4 cells in N1 medium were prevented from differentiating into a multipolar phenotype (Wu and Bradshaw, 2000). Further experiments are necessary to confirm any significant inhibition of STAT3 by Ro-32.

5.3.2 MEK inhibitor studies

Once it became evident that the transient ERK1/2 activation associated with the application of fresh 70/30 medium could be involved in the inhibition of process formation in CG-4 cells, MEK inhibitors were applied in an attempt to block this activation and to induce process formation. Surprisingly, neither PD 98059 nor UO-126 was able to induce process formation at non-toxic doses. Since Western blots indicate, however, that these inhibitors were unable to abolish the phosphorylation of ERK1/2 at these doses, it is still possible that ERK1/2 activation inhibits process formation in CG-4 cells. The concept of a threshold of activation has already been seen with respect to ERK1/2 in primary OL, and may again play a role in the CG-4 cell line. In such a scenario, it is possible that the MEK1/2 inhibitors were unable to drop the level of activated ERK1/2 below the threshold necessary to remove its inhibitory effects on process formation. It is unclear why the MEK1/2 inhibitors were unable to abolish ERK1/2

activity in these cells. One possibility is that the inhibitor dose was simply not high enough, while another possibility is that ERK1/2 is downstream of a kinase other than MEK1/2 in CG-4 cells. To address these possibilities, a higher dose of PD 98059 was applied to the cells. Since 100 μM PD 98059 was able to abolish ERK1/2 phosphorylation, this indicates that ERK1/2 is indeed downstream of MEK1/2 in the CG-4 cell line. However, it does not rule out the possibility that other pathways could also lead to ERK1/2 activation in these cells.

5.3.3 Phorbol Ester Studies

After attempting to induce process formation by blocking ERK1/2 activation in CG-4 cells fed with 70/30 medium, PMA was used in an attempt to block process formation by inducing ERK activation in CG-4 cells fed with N1 medium. Since 20 nM PMA in N1 medium could inhibit the formation of a multipolar phenotype over a 24 hr period, while at the same time leading to ERK1/2 phosphorylation, these results support a role for ERK1/2 activation in the inhibition of process formation. Furthermore, the ability of CG-4 cells in N1 medium + 20 nM PMA to form multipolar processes after 48 hr strengthens the argument that repetitive transient activation of ERK1/2 is required to inhibit multipolar extensions.

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

6.1 The Role of ERKs 1 and 2 in Primary OL Process Extension

The first objective of this thesis was to use a primary OL model to determine whether or not ERKs 1 and 2 play a role in process extension. In these experiments, bovine brains were used as a source of OL. Bovine brains were chosen as they are able to provide a large quantity of cells, and the basal rate of bovine OL process extension is very slow.

From previous studies it has already been determined that phorbol esters can induce primary OL process extension. Experiments were therefore undertaken to assess if the MEK1 inhibitor PD 98059 could prevent this phorbol ester-induced response. It was found that PD 98059 could inhibit process extension, and could also inhibit the increase in ERK1/2 activity observed after PMA treatment. The inhibitor did not, however, completely abolish ERK1/2 activity, leading to speculation that a threshold of ERK1/2 activity is required for OL to commit to process extension.

There are other instances where the concept of a threshold of ERK1/2 activity may affect the functional biology of OL. First, in studies using NT-3 and NGF to induce progenitor OL proliferation, it was found that both compounds could lead to ERK1/2 phosphorylation. However, only NT-3 could stimulate progenitor OL proliferation (Cohen et al., 1996). Upon closer examination, it was shown that ERK1/2 was phosphorylated to a greater extent upon treatment of

cells with NT-3 than with NGF. It is therefore possible that NGF did not cause ERK1/2 activation to cross the threshold necessary to induce proliferation. Second, in the CG-4 studies described in Chapter 5, inhibition of ERK1/2 phosphorylation was unable to induce morphological changes. Once again, however, this inhibition was shown to be minimal, allowing for the possibility that ERK1/2 activation was not dropped below the threshold required for morphological change. From these results it may be concluded that OL do not commit to major proliferative or morphological responses unless significant kinase activity changes are affected.

As well as causing activation of ERK1/2, stimulation of primary OL with PMA has been shown to cause movement of ERK1/2 into the nucleus and processes of OL (Stariha et al., 1997). The potential substrates of ERK1/2 activation during OL process extension are numerous; in the cytoplasm and processes, the most likely candidates are MBP and stathmin. In the nucleus, activated ERK1/2 most likely leads to induction of the c-fos gene. Perhaps the most probable ERK1/2 substrate is MBP, as there is already circumstantial evidence linking ERK1/2 activation to MBP phosphorylation. First, our studies have shown that PMA-stimulation of OL causes ERK1/2 activation and process extension (Stariha et al., 1997). Second, other studies have demonstrated that PMA-stimulation of OL causes MBP phosphorylation (Vartanian et al., 1986). Since MBP is a commonly used *in vitro* substrate of ERK, there is a potential *in vivo* link between ERK1/2 phosphorylation, MBP phosphorylation, and process extension in OL. Phosphorylation of MBP during OL process extension could be

a preparatory step for myelination. However, it is possible that one or more other ERK1/2 substrates are also phosphorylated during OL process extension. Other such substrates, including stathmin, may play a more integral role than MBP in the formation of the processes themselves.

There is also evidence that the ERK1 and ERK2 isoforms are developmentally regulated in OL. For instance, ERK2 has been shown to play a role in the proliferation of progenitor OL (Kumar, 1998). Conversely, ERK1 has been shown to play a role in the proliferation of a subset of mature OL (Althaus, 1997). While our studies indicate that ERK1 is the dominant isoform in primary OL, the separate contributions of ERK1 and ERK2 to process extension were not determined. To examine this question, studies could be undertaken to individually immunoprecipitate ERK1 and ERK2 from OL and compare their activities before and after stimulation and inhibition of process extension.

Finally, our preliminary results indicate that p38, JNK, PKA, PI-3-kinase and calcium-calmodulin-dependent kinase II do not play a role in OL process extension. However, future studies are needed to verify and expand these results, as it is likely that kinases other than ERK1/2 and PKC-α are involved in the process extension phenomenon.

6.2 A Comparison of the Kinase Expression Profile Between Primary Rat OL and CG-4

The second objective of this thesis was to evaluate the usefulness of the CG-4 cell line as a model for OL signal transduction studies. A self-renewing OL model would greatly advance the knowledge of OL signal transduction, since primary mature OL are post-mitotic and therefore of limited quantity. Also, a cell line could overcome the inherent variability associated with primary cultures.

To evaluate the CG-4 cell line for use in OL signal transduction studies, a multi-kinase expression profile was conducted by Kinexus Bioinformatics Corporation. Any interesting results were then further confirmed via Western blotting. In general, there were few differences between the kinase expression profiles of primary OL and CG-4 OL. The main areas of difference were in the expression of Pak-α, which was higher in primary OL than in CG-4 cells, and the expression of FAK, which was lower in primary OL than in CG-4 cells. There were also differences in the expression of PKC-δ and -ε, as well as in the expression of CDKs 5 and 7.

The putative functions of Pak-α vary widely. For instance, this kinase has been shown to activate JNK and p38 pathways, potentially leading to apoptosis. However, it has also been shown to act in an anti-apoptotic manner via phosphorylation of Bad (Schurmann et al., 2000). Pak isoforms have also been shown to both induce neurite extension and to cause retraction of the cell periphery (Manser et al., 1997; Daniels et al., 1998). Therefore, the significance

of increased Pak-α expression in primary OL is unclear. One possible scenario evolves after examining the expression of Pak-α separately from the activation of Pak-α. Expression of Pak1 has been implicated in the above-mentioned induction of neurite extension, while activation of Pak-α has been implicated in the retraction of the cell periphery. It is therefore possible that the expression of Pak-α in primary OL, as opposed to the activation of Pak-α in these cells, may explain why primary OL can form more intricate networks of processes than CG-4 OL.

In contrast to Pak-α, FAK expression is higher in CG-4 cells than in primary OL. The most likely explanation for this increased expression is that FAK is involved in the self-renewal capacity of the CG-4 cell line. FAK expression has been found to upregulated in a variety of tumours, and blocking FAK expression in tumour cells can induce apoptosis (Owens et al., 1995; Xu et al., 1996). While the CG-4 cell line is not considered to be a tumour cell line, it is possible that this kinase contributes to the spontaneous mutation of primary progenitor OL into immortalized self-renewing CG-4 cells. The increased expression of PKC-ε in CG-cells as compared to primary OL may also play a similar role, since overexpression of PKC-ε has been shown to be involved in cell immortalization (Perletti et al., 1996).

As opposed to PKC-ε, PKC-δ expression is higher in primary OL than in CG-4 cells. PKC-δ has been implicated in both proliferation and differentiation, and it is unclear which of these roles it assumes in OL. A role for PKC-δ in OL differentiation could explain why this kinase is highly expressed in primary cells,

as primary OL are able to achieve a higher degree of differentiation than CG-4 OL (Yim et al., 1995).

Finally, the expression of CDK5 was higher in primary OL than in CG-4 cells. Conversely, the expression of CDK7 was lower in primary OL than in CG-4 cells. Since CDK7 has been found to be upregulated in tumour cells, it could share a role with FAK and PKC-ε in the mutation of primary OL into the self-renewing CG-4 cell line. As opposed to CDK7, CDK5 has been shown to play a role in cytoskeletal dynamics. Along with Pak-α, increased expression of this kinase in primary OL could explain why these cells form more intricate processes than CG-4 OL.

Overall, the differences in kinase expression between primary OL and CG-4 OL were few. Many major kinases, such as conventional PKCs, members of the MAPK family, PKA, and PKB, were all similarly expressed. Therefore, the CG-4 cell line appears to make a suitable model for OL signal transduction studies.

6.3 Process Extension in CG-4

After noting the similarities in the kinase expression patterns of CG-4 cells and primary OL, particularly with respect to the MAPK pathway, it was surprising to discover that ERKs 1 and 2 do not appear to play a role in the induction of mulitpolar process extensions from CG-4 cells. Rather, in contrast to their role in

primary OL, ERKs 1 and 2 appear to inhibit multipolar process formation in CG-4 cells.

First, transient ERK1/2 activation was noted in CG-4 cells fed with 70/30 medium, but not in CG-4 cells fed with N1 medium. However, CG-4 cells fed with 70/30 medium maintain a relatively bipolar phenotype, while CG-4 cells fed with N1 medium acquire a multipolar phenotype. Therefore, the ERK1/2 activation associated with 70/30 medium appears to prevent the expression of a multipolar phenotype. Further evidence of this was provided when CG-4 cells in 70/30 medium did not have their medium renewed for 4 days. These cells were able to develop a multipolar phenotype, presumably due a lack of fresh 70/30 medium leading to a lack of transient ERK1/2 activation. It is thought that the active components in 70/30 are the bFGF and PDGF mitogens contained in the 30% B-104 conditioning. These two growth factors, therefore, are likely leading to ERK1/2 phosphorylation in CG-4 cells fed with 70/30 medium.

Second, CG-4 cells fed with 70/30 medium were exposed to two different MEK1/2 inhibitors in an attempt to inhibit transient ERK1/2 activation and induce the formation of a multipolar phenotype. However, although the inhibitors were able to reduce ERK1/2 activation at non-toxic doses, they were unable to induce a multipolar phenotype. These results do not necessarily negate a role for ERK1/2 activation in the inhibition of multipolar process extensions, since the inhibitors were able to reduce but not to abolish ERK1/2 activity. As mentioned above, the ERK1/2 activity after inhibitor treatment could still be above the threshold required to keep the CG-4 cells in their bipolar state.

Finally, since N1 medium does not contain bFGF and PDGF, N1 medium alone does not cause ERK1/2 activation. Therefore, CG-4 cells in N1 medium are normally able to form a multipolar phenotype. PMA-induced activation of ERK1/2 in CG-4 fed with N1, however, was able to prevent the expression of a multipolar phenotype. This result supports a role for ERK1/2 activation in the inhibition of multipolar process extensions in CG-4 cells.

6.4 Conclusions

The results presented in Chapter 3 of this thesis provide evidence for a role for ERK1/2 activation in the induction of process extensions from primary OL. The results presented in Chapter 4 of this thesis then illustrate that the kinase expression profiles of primary OL and CG-4 OL are similar, implying that the CG-4 cell line is a useful model for OL signal transduction studies. Then, in Chapter 5, the signal transduction results on the role of ERK1/2 in CG-4 OL process extension were found to potentially contradict the results obtained from primary OL. While at first glance this seems to negate the usefulness of the CG-4 model for OL signal transduction studies, this is not necessarily the case. Due to the nature of primary OL as compared to CG-4 OL, there were differences in the experimental protocols used for these two cell types. Therefore, direct comparisons should not be drawn without careful consideration.

For instance, the primary bovine OL used in the experiments shown in Chapter 3 were mature cells that had lost their processes during the primary

culture procedure. These cells take weeks to re-grow their processes endogenously, and therefore phorbol esters were used to stimulate process extension and ERK1/2 activation. The endpoint was an observed extension of processes from phorbol ester-stimulated mature OL, as compared to a complete lack of processes in control OL. Such an endpoint is not possible with CG-4, as these cells endogenously extend processes within hours of differentiation towards mature OL-like cells. By the time CG-4 cells have fully differentiated into mature CG-4 OL, they already demonstrate a multipolar phenotype. Therefore, a distinction has to be made between "process extension" in primary OL and a "multipolar phenotype" in CG-4 OL. "Process extension" in primary OL is defined as the appearance of processes extending from the cell body, as opposed to a complete lack of processes. A "multipolar phenotype" in CG-4 cells is defined as a network of branched processes surrounding the cell body, as opposed to 2-4 unbranched processes that do not completely encircle the cell body.

One way to mimic the primary OL experiments as closely as possible would be to initially differentiate the CG-4 cells into CG-4 OL by the application of N1 medium. These CG-4 OL could then be replated, as most processes are lost during the replating procedure, and the effects of various kinase stimulators and inhibitors on the re-growth of processes could be monitored over the next few hours. This type of experiment was attempted; however, the survival and general well-being of the CG-4 OL were adversely affected by the replating procedure. Since bipotential CG-4 cells can be easily replated, a second type of experiment was devised.

In this second type of experiment, CG-4 cells were maintained as bipotential cells in 70/30 medium prior to any treatments. This is a significant departure from primary OL, which were maintained as mature cells prior to any treatments. However, the advantage of such an experimental set-up is that the change from the bipolar phenotype of CG-4 cells in 70/30 medium to the multipolar phenotype of CG-4 cells in N1 medium can be easily monitored. Various kinase stimulators and inhibitors were therefore employed in an attempt to either stimulate or inhibit the induction of a multipolar phenotype.

In this thesis, results obtained from the CG-4 cell line provide strong evidence to support a role for ERK1/2 activation in the inhibition of a multipolar phenotype in CG-4 cells. In addition, results obtained from primary OL experiments evidence a role for ERK1/2 activation in the formation of process extensions from mature OL.

Two potential conclusions can be drawn from the combined results of these CG-4 cell line and primary OL culture experiments:

1 – The first conclusion is that the CG-4 cell line is not a useful model for OL signal transduction studies. This conclusion is supported by the induction of process formation via ERK1/2 activation in primary OL, and the inhibition of process formation via ERK1/2 activation in CG-4 cells.

2 – The second conclusion is that ERKs 1 and 2 play a role in the induction of OL process extension, but only if the OL have fully matured. This conclusion is supported by studies of PKC in OL, since PKC activation has been shown to both prevent differentiation in immature OL and to induce process extensions in

mature OL. These PKC results demonstrate that the effects of kinase activation in OL can be dependent on the differentiation level of the cell. In this second conclusion, therefore, the CG-4 cell line is not necessarily negated as an appropriate model for OL signal transduction studies.

Future experiments using primary O2A cultures to outline the role of ERK1/2 in process extension during differentiation would help to elucidate any differentiation-dependent effects of ERK1/2 in OL. For instance, the effects of ERK1/2 activation on the differentiation of primary O2A cells into mature OL, as well as the effects of ERK1/2 activation on the differentiation of bipotential CG-4 cells into CG-4 OL, could be assessed using developmental marker expression. If ERK1/2 activation is shown to prevent the appearance of mature OL markers in both the primary model and CG-4 model, then the CG-4 cell line may still be a useful model for OL signal transduction studies. In such a scenario, the previously noted inhibition of a multipolar phenotype in CG-4 cells via ERK1/2 activation could be attributed to an inhibition of differentiation rather than a direct inhibition of process formation.

It is of paramount importance that the usefulness of the CG-4 cell line as an OL model be addressed. Due to the large number of OL-like cells that can be obtained from this cell line, it has rapidly become the model of choice for studies on OL functional biology. Since the results of this thesis raise the possibility that some protein kinase signalling cascades may function differently in CG-4 OL than in primary OL, care should be taken before applying any results obtained from the CG-4 cell line directly to primary OL. In terms of signal transduction

studies, the functional effects of any signalling pathways should first be determined in both primary OL and CG-4 OL cultures. If the functional results are the same in both models, then the unlimited supply of cells afforded by the CG-4 model could be employed to further define such signalling pathways. Once the pathways have been well-defined in CG-4 OL, the results should again be verified in primary OL.

In conclusion, therefore, this thesis underlines the importance of exercising caution when using the CG-4 cell line for OL studies. To truly eludicate the signal transduction pathways involved in OL functional biology, an approach that combines both the CG-4 and the primary OL culture models is the best option.

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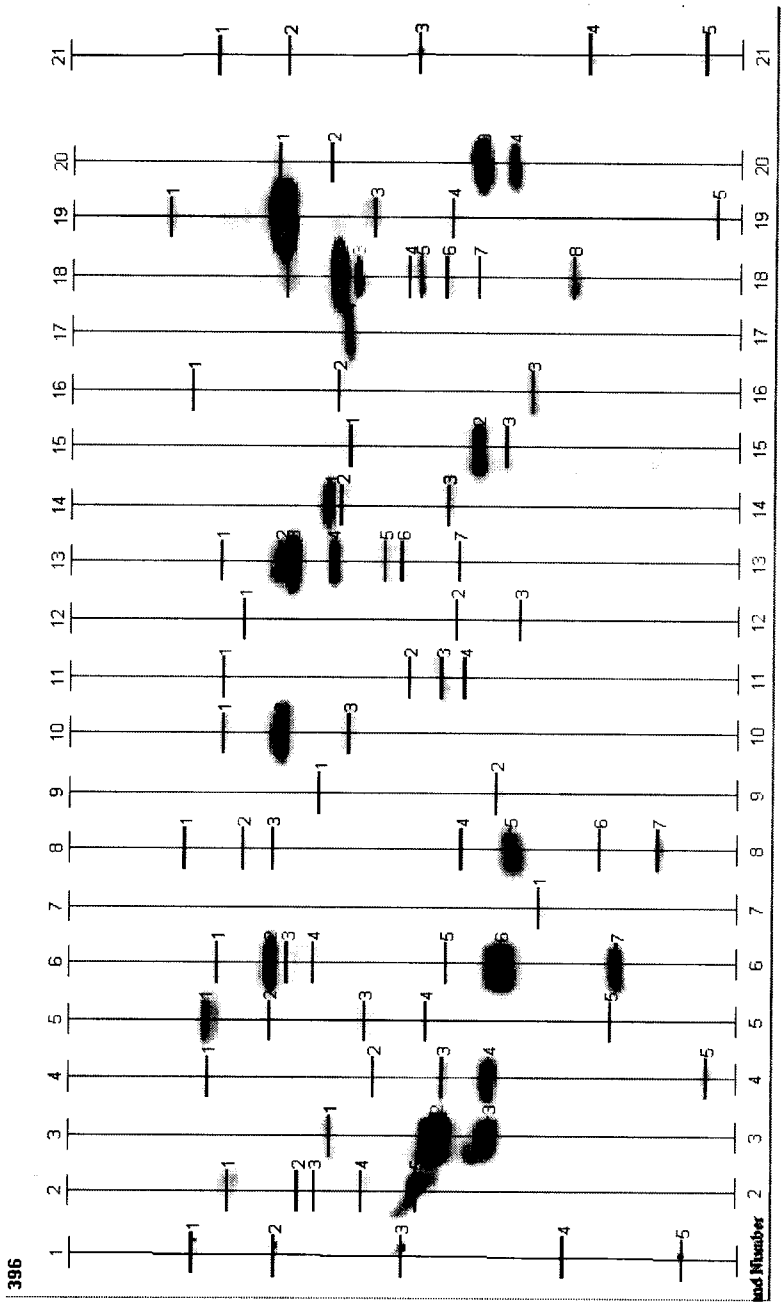
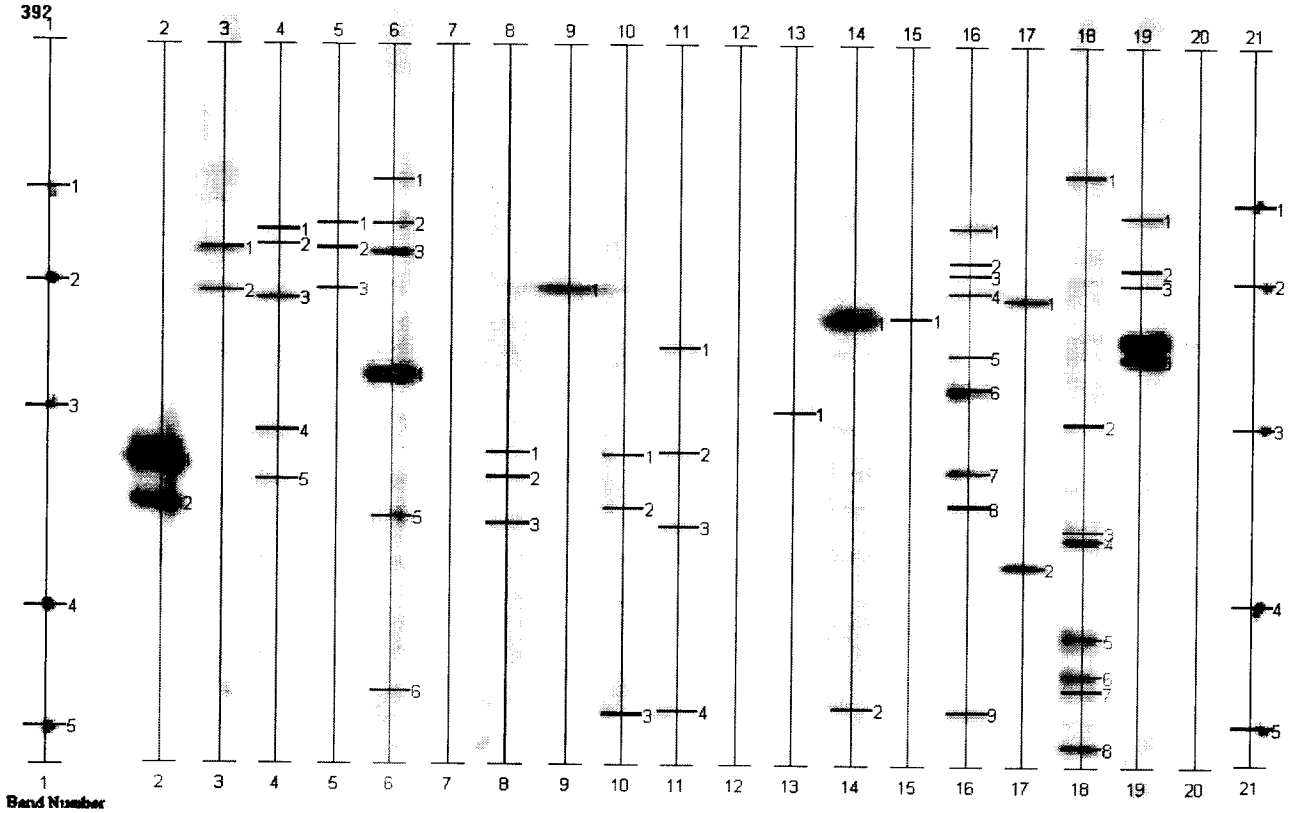


Appendix A: Kinetworks™ Analysis of Rat OL, Part 1

Appendix A: Densitometric Analysis of Rat OL Blot, Part 1

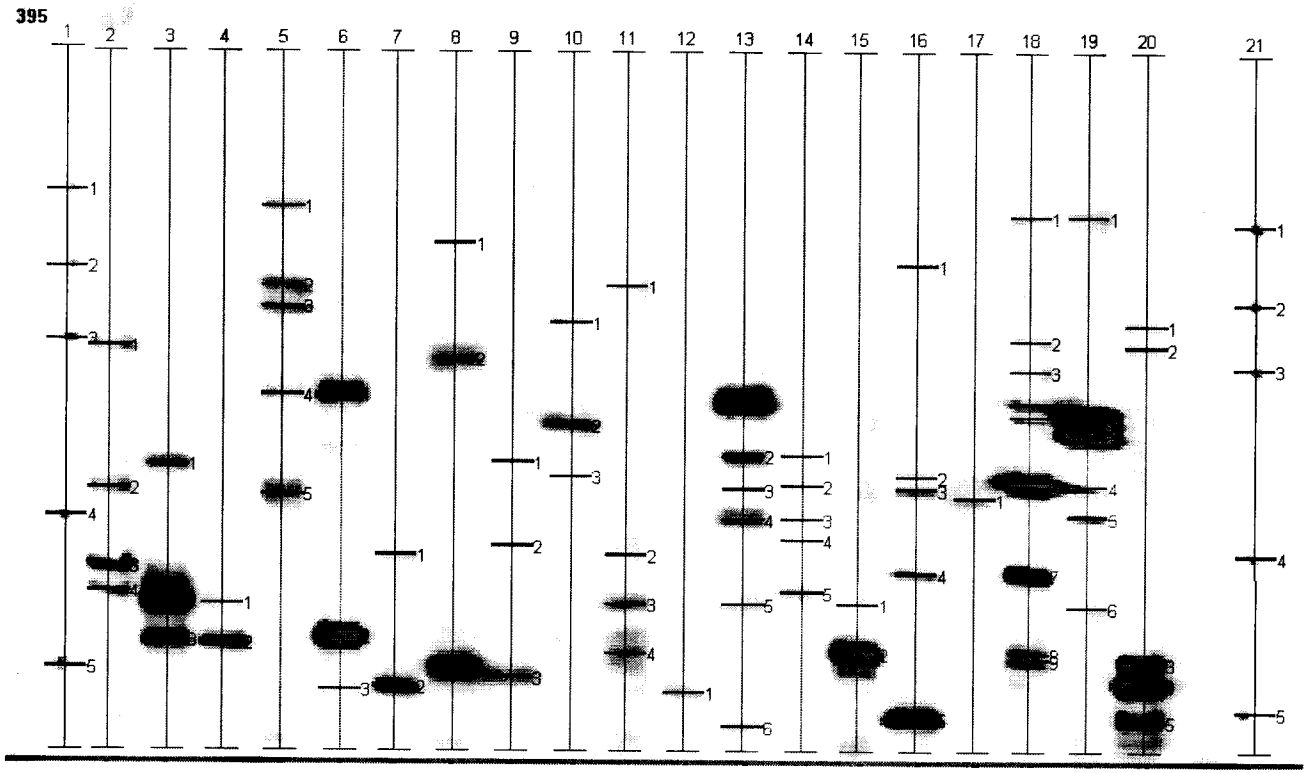
Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.183	112	standard	3462	1140.636	1254.7	14.566
1	2	0.304	81	standard	4970	1381.182	1519.3	17.638
1	3	0.496	49.9	standard	5063	1611.818	1773	20.584
1	4	0.739	36	standard	2781	1010.857	707.6	8.215
1	5	0.917	29.9	standard	3667	1019.9	1019.9	11.841
2	1	0.239	96.792	Raf B	7002	4374.765	7437.1	12.62
2	5	0.522	48.276	ERK1-III	19583	7593.708	18224.9	30.926
3	1	0.391	65.361	PKB-CT	7682	3875.929	5426.3	5.154
3	2	0.552	46.426	ERK1-CT	60765	24627.727	54181	51.458
3	3	0.63	41.822	ERK1-CT	24960	10813.429	22708.2	21.567
4	4	0.63	41.904	ERK 2	23097	7719.143	16210.2	33.387
5	1	0.204	107.718	PKC-mu	16268	6667.75	10668.4	21.648
6	2	0.3	83.116	PKC-beta 1	47782	19599.455	21559.4	19.407
6	6	0.648	41.105	p38	28906	14316.2	28632.4	25.774
6	7	0.822	33.317	CDK 5	22616	9118.4	13677.6	12.312
8	2	0.261	93.282	nPKC-epsilon	5940	3232.714	4525.8	9.137
8	5	0.661	40.56	CDK 7	15687	7107.708	17058.5	34.439
9	2	0.639	41.817	CDK 9	5719	2475.368	4703.2	23.544
10	2	0.313	81.033	PKC-delta	30463	9844.55	19689.1	38.613
11	2	0.509	49.796	SAPK-beta	4012	3057.909	3363.7	5.964
11	3	0.557	46.772	SAPK-beta	8126	5184.167	6221	11.031
13	3	0.33	78.142	PKC-zeta	37856	17068.077	22188.5	32.221
13	7	0.583	45.358	MEK 1	5325	2625	3412.5	4.955
14	1	0.387	67.992	PAK-alpha	23379	8073.875	12918.2	25.633
14	3	0.565	46.472	MEK2	6427	3364.714	4710.6	9.347
15	2	0.609	43.995	MEK4	30579	10854.533	16281.8	21.29
16	3	0.691	39.597	MEK 6	10707	3732.688	5972.3	17.132
18	2	0.4	66.489	S6K	38065	15949.375	25519	25.611
18	5	0.522	49.47	GSK 3 a/b	13895	7252.111	6526.9	6.55
19	2	0.313	83.207	RSK 1	63986	27906.947	53023.2	46.482
19	3	0.452	58.534	COT	11567	6057.933	9086.9	7.966
20	3	0.613	44.127	CK2 alpha-III	54360	15539.056	27970.3	39.679



Appendix A: Kinetworks™ Analysis of Rat OL, Part 2

Appendix A: Densitometric Analysis of Rat OL Blot, Part 2

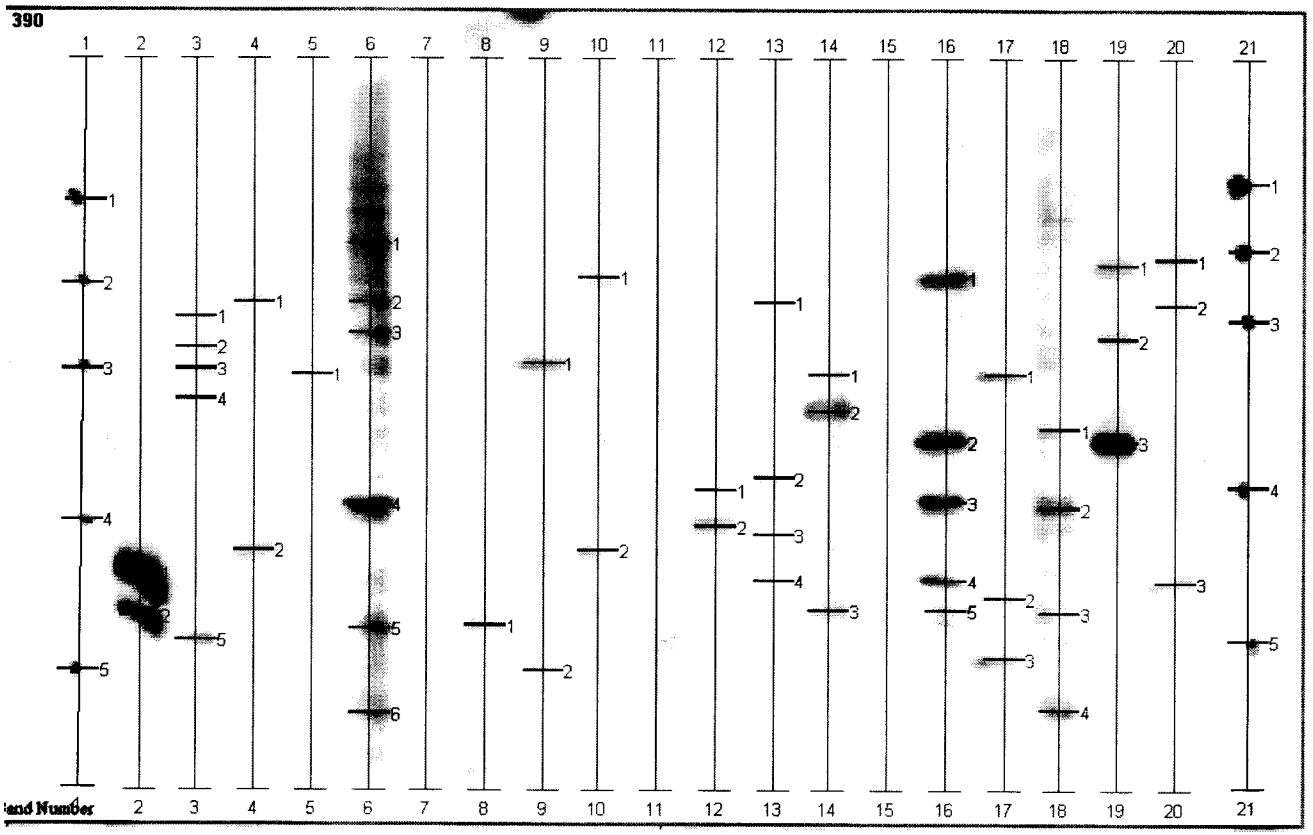
Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.204	112	standard	2899	1254.625	1003.7	8.996
1	2	0.332	81	standard	8974	2218.636	2440.5	21.874
1	3	0.506	49.9	standard	4808	1324.727	1457.2	13.061
1	4	0.783	36	standard	7336	1939.692	2521.6	22.601
1	5	0.949	29.9	standard	6772	1952.818	2148.1	19.253
2	1	0.579	45.867	ERK1-CT	50678	18220.81	38263.701	56.697
2	2	0.639	42.698	ERK1-CT	22571	8801.188	14081.9	20.866
3	2	0.343	78.393	IKK alpha	5350	2680.923	3485.2	10.077
4	3	0.352	76.578	BMX	6877	2942.182	3236.4	9.415
6	4	0.459	57.584	Fyn	20521	9489.688	15183.5	13.448
8	3	0.665	41.533	CK1 delta	3889	2068	3308.8	10.652
9	1	0.339	79.128	Zap70	8473	2619.529	4453.2	24.994
14	1	0.382	70.931	Raf-1	12589	4934.095	10361.6	21.338
16	1	0.253	100.651	FAK	7441	3714.067	5571.1	10.234
17	1	0.352	76.453	GRK2	8866	3013.875	4822.2	17.412
18	2	0.524	50.29	Zipk	7435	3811.6	3811.6	3.938
19	1	0.236	106.29	JAK 1	7014	3438.571	4814	7.36



Appendix A: Kinetworks™ Analysis of CG-4 OL, Part 1

Appendix A: Densitometric Analysis of CG-4 OL Blot, Part 1

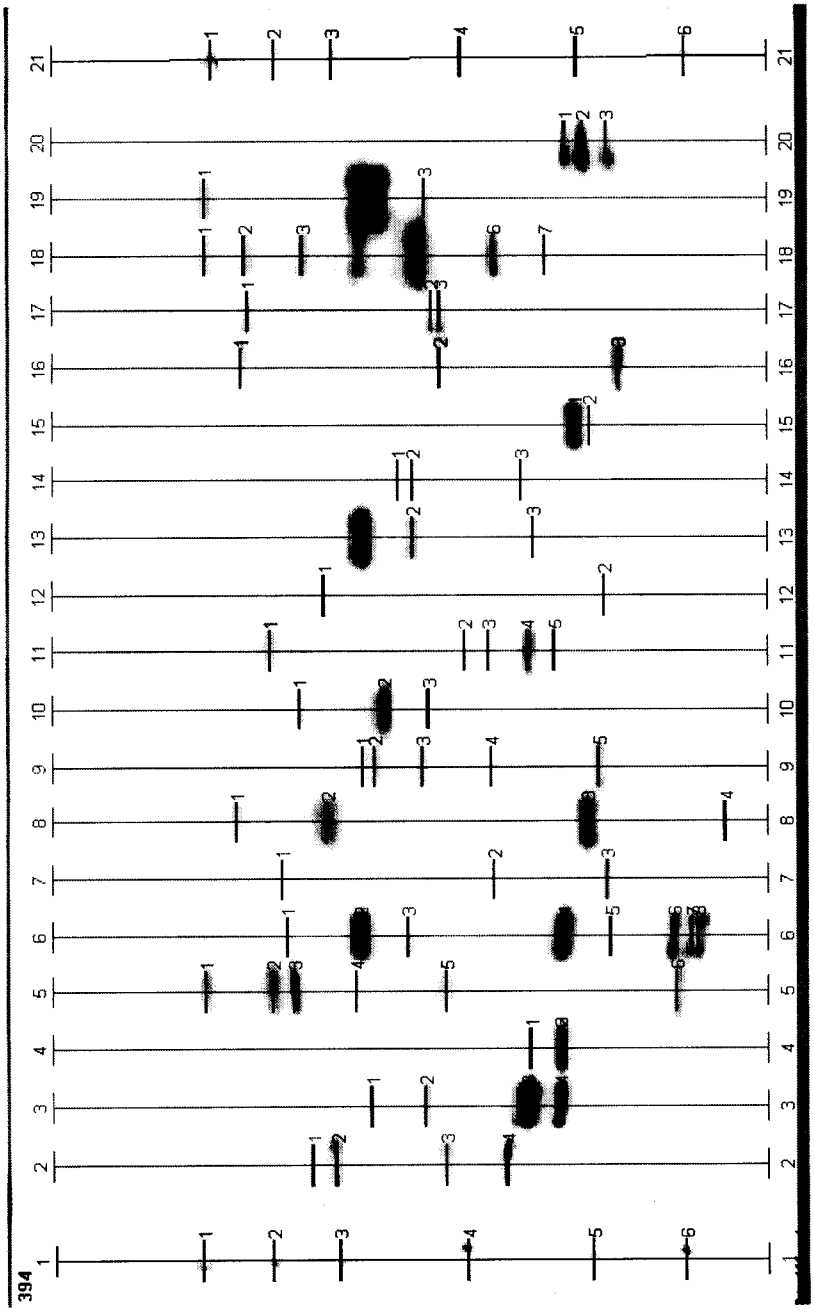
Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.203	201	standard	3637	1127.364	1240.1	7.224
1	2	0.312	130	standard	3660	1333.444	1200.1	6.991
1	3	0.416	94	standard	7544	2228.182	2451	14.277
1	4	0.667	48.6	standard	5742	2419	1935.2	11.273
1	5	0.883	36.4	standard	6256	1710.357	2394.5	13.948
2	1	0.422	92.797	Raf B	11091	5097.357	7136.3	8.232
2	2	0.626	54.328	ERK1-III	13687	6318.857	8846.4	10.205
2	4	0.774	42.231	ERK1-III	16778	7054.538	9170.9	10.579
3	1	0.591	59.877	PKB-CT	20110	6296.611	11333.9	8.528
3	2	0.787	41.658	ERK1-CT	64441	29718.348	68352.202	51.429
3	3	0.843	38.637	ERK1-CT	52405	16264.056	29275.301	22.027
4	2	0.848	38.554	ERK 2	40526	11461.813	18338.901	34.631
5	3	0.365	112.574	PKC-u	14667	8248.667	7423.8	10.07
6	2	0.839	39.301	p38	64012	25781.111	46406.001	32.892
6	3	0.913	35.623	Cdk 5	7335	5367.111	4830.4	3.424
7	2	0.909	35.965	Cdk 6	43643	9761.905	20500.001	47.512
8	2	0.439	91.058	nPKC-epsilon	15160	6635.4	13270.8	15.785
8	3	0.878	37.596	Cdk 7	60626	23132.895	43952.501	52.279
9	3	0.896	36.884	Cdk 9	15920	6016.611	10829.9	39.668
10	2	0.535	72.026	PKC-delta	20535	8752.929	12254.1	27.318
12	1	0.917	36.251	Mos III	8885	3803.571	5325	14.848
13	1	0.5	79.827	PKC-zeta	64441	29415.588	50006.502	44.065
13	5	0.791	42.952	MEK 1	8657	4572.765	7773.7	6.85
14	5	0.774	44.104	MEK 2	9117	4617.176	7849.2	13.718
15	2	0.861	39.489	MEK 4	61707	25570.25	40912.401	36.638
18	6	0.617	61.13	S6K	58299	23130.235	39321.401	23.12
18	7	0.748	46.273	GSK 3 a/b	61298	20055.938	32089.501	18.868
18	8	0.861	39.927	GSK 3 a/b	18538	12996.444	11696.8	6.878
19	2	0.53	76.155	RSK 1	64441	33880.611	60985.102	36.636
19	3	0.548	72.963	RSK 1	64441	30276.833	36332.201	21.826
19	5	0.665	54.65	COT	18630	9194.091	10113.5	6.076
20	3	0.874	39.547	CK2 alpha III	60274	19534.824	33209.201	24.667
20	4	0.904	38.012	CK2 alpha III	63338	35589.154	46265.901	34.365



Appendix A: Kinetworks™ Analysis of CG-4 OL, Part 2

Appendix A: Densitometric Analysis of CG-4 OL Blot, Part 2

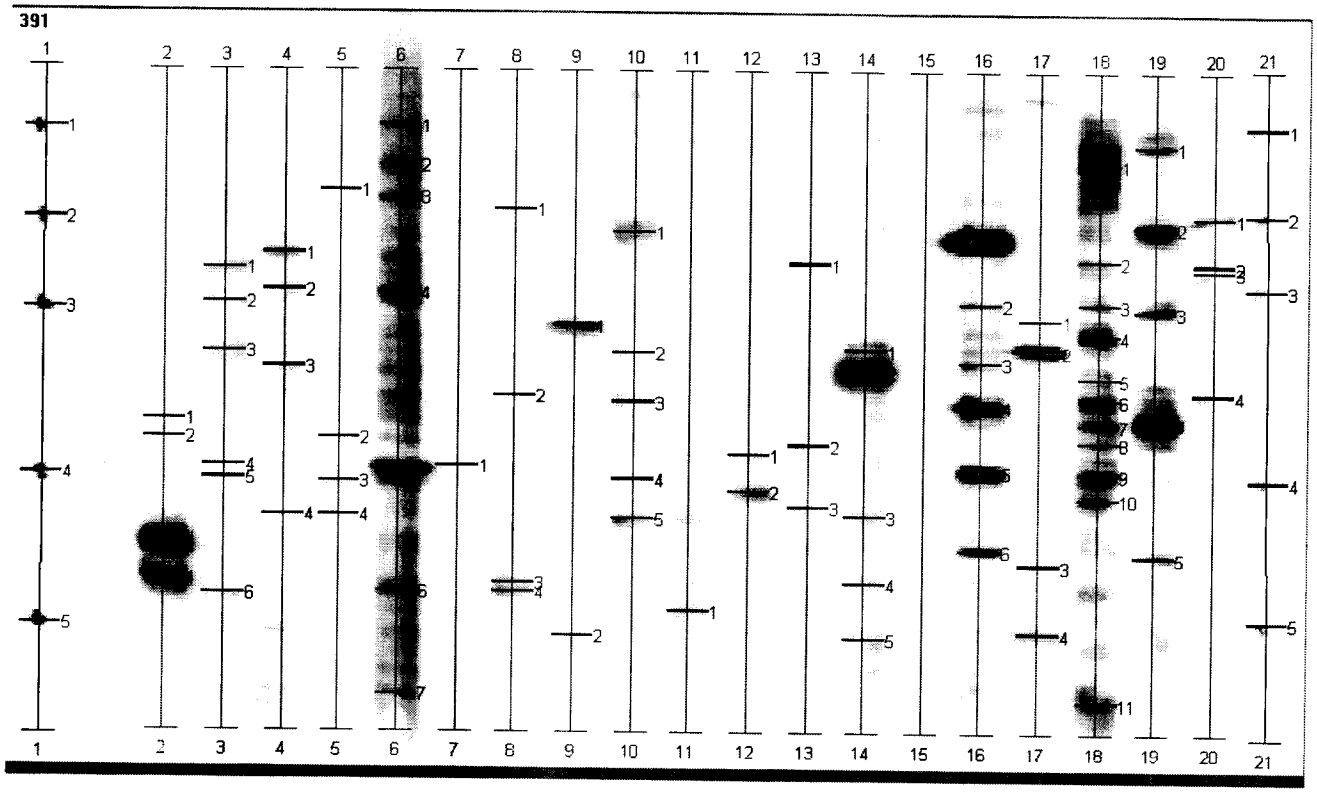
Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.196	201	standard	8268	2792	3071.2	15.115
1	2	0.308	130	standard	5859	1860.182	2046.2	10.07
1	3	0.425	94	standard	6701	2025.091	2227.6	10.963
1	4	0.633	48.6	standard	6267	2234.778	2011.3	9.899
1	5	0.842	36.4	standard	6222	1995.923	2594.7	12.77
2	1	0.705	43.834	ERK1-CT	53403	24098.667	57836.802	61.782
2	2	0.763	40.443	ERK1-CT	21009	10846.588	18439.201	19.697
8	1	0.776	39.066	Zap70	3486	2323.6	3485.4	8.393
14	2	0.481	70.009	Raf1	9758	5868.333	8802.5	15.661
16	1	0.299	120.249	FAK	14424	5619.722	10115.5	12.988
17	1	0.432	79.009	GRK2	8355	3279.133	4918.7	15.047
19	1	0.282	123.928	JAK1	9524	4779.188	7646.7	9.925
20	1	0.274	126.376	JAK2	4398	1991.75	2390.1	11.882



Appendix A: Kinetworks™ Analysis of Bipotential CG-4, Part 1

Appendix A: Densitometric Analysis of Bipotential CG-4 Blot, Part 1

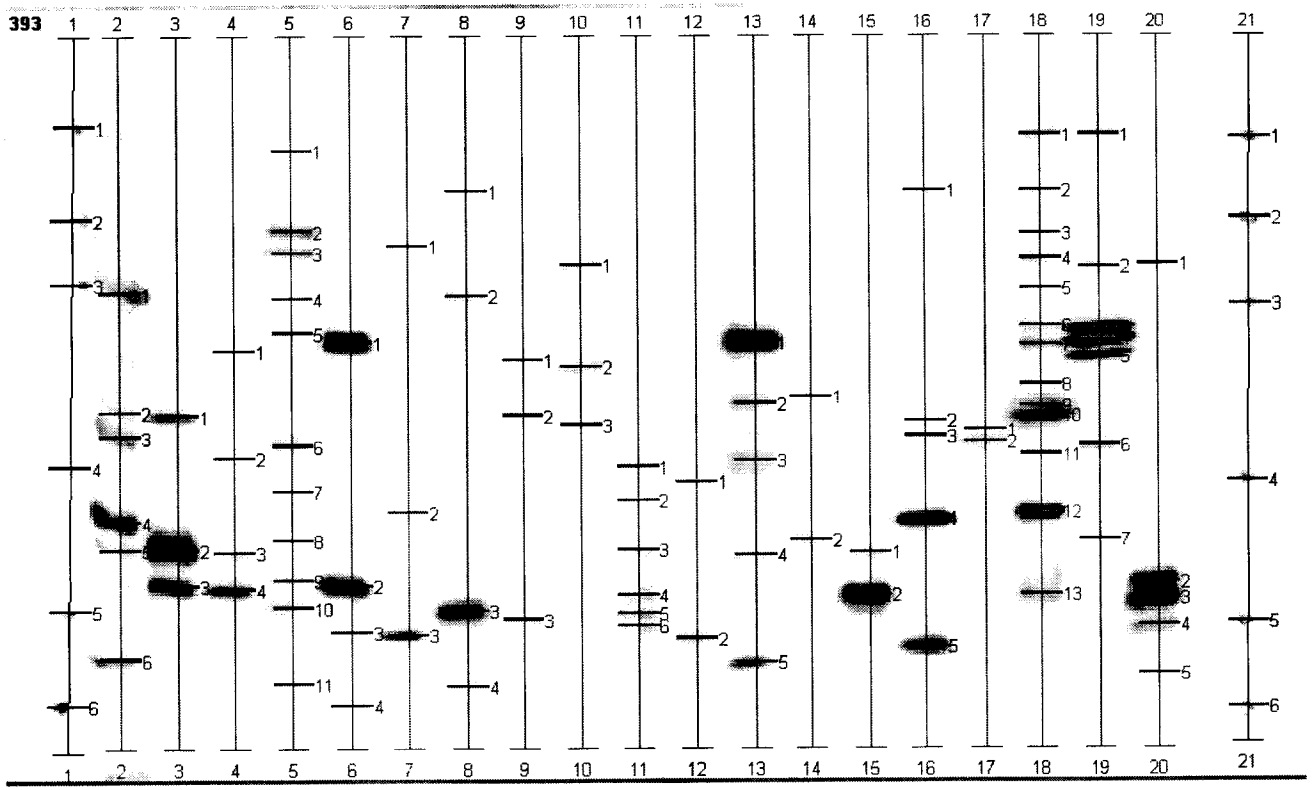
Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.207	201	standard	3038	1746.778	1572.1	13.922
1	2	0.304	130	standard	3890	1209.818	1330.8	11.785
1	3	0.397	94	standard	4088	1170.364	1287.4	11.4
1	4	0.578	48.6	standard	5655	1472.231	1913.9	16.948
1	5	0.755	36.4	standard	2863	1006.778	906.1	8.024
1	6	0.886	29.8	standard	7224	1652.083	1982.5	17.556
2	2	0.395	94.402	Raf B	13931	6839.143	9574.8	10.918
2	4	0.634	44.267	ERK1 III	20940	7194.923	9353.4	10.666
3	2	0.521	59.627	PKB-CT	13060	4363.526	8290.7	8.469
3	3	0.664	42.138	ERK1-CT	46000	17297.9	34595.801	35.341
3	4	0.71	39.05	ERK1-CT	43073	13222.412	22478.101	22.962
4	2	0.71	38.998	ERK2	42702	13684.385	17789.701	31.116
5	3	0.336	116.357	PKC-mu	22874	12594.556	11335.1	13.744
5	4	0.424	84.57	PKC-alpha	6370	4318.071	6045.3	7.33
6	2	0.429	83.204	PKC-beta	43558	14359.864	31591.701	21.653
6	4	0.714	38.625	p38	39635	13462	26924.001	18.454
6	5	0.782	34.751	CDK 5	5030	3836	4603.2	3.155
6	3	0.777	34.927	CDK 6	13073	4442.733	6664.1	18.124
6	2	0.382	98.294	nPKC-epsilon	17536	8303.143	17436.601	22.379
7	3	0.748	36.407	CDK 7	38011	11451.6	22903.201	29.395
9	2	0.462	73.31	PKC-delta	21977	8581.636	18879.601	36.925
11	1	0.433	81.348	PKC-zeta	50161	17111.95	34223.901	49.092
17	5	0.513	60.478	S6K	40349	14553.391	33472.801	27.668
18	6	0.618	44.93	GSK 3 a/b	23144	7514.813	12023.7	9.939
18	7	0.689	39.678	GSK 3 a/b	6210	4426.7	4426.7	3.659
18	2	0.437	79.612	RSK 1	60411	24692.621	71608.602	52.252
18	3	0.521	58.586	COT	10460	6537.231	8498.4	6.201
18	1	0.718	37.582	CK2 alpha-III	20059	7774.462	10106.8	17.653
18	2	0.744	36.062	CK2 alpha-III	30380	16440.889	14796.8	25.844



Appendix A: Kinetworks™ Analysis of Bipotential CG-4, Part 2

Appendix A: Densitometric Analysis of Bipotential CG-4 Blot, Part 2

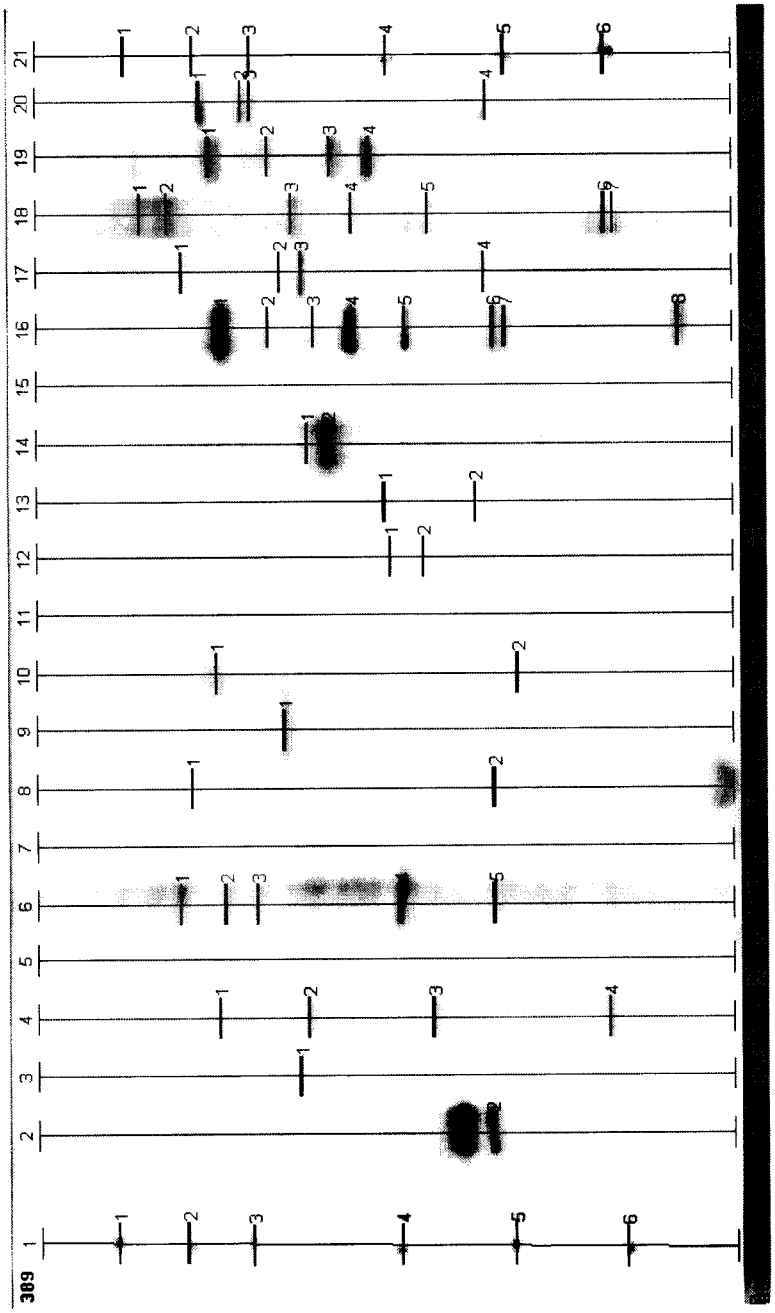
Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.093	201	standard	2993	1268.636	1395.5	12.866
1	2	0.227	130	standard	3164	1173.5	1408.2	12.983
1	3	0.361	94	standard	6306	2038	2038	18.789
1	4	0.611	48.6	standard	3234	1631.375	1305.1	12.032
1	5	0.838	36.4	standard	6741	1993.1	1993.1	18.376
2	3	0.72	42.313	ERK1	39665	15261.158	28996.2	49.543
2	4	0.771	39.615	ERK1	32867	10866	18472.2	31.562
6	5	0.603	49.801	Fyn	20811	8490.706	14434.2	13.038
9	1	0.383	85.954	Zap70	7314	2547.889	4586.2	27.873
11	1	0.818	37.142	CaMK1	3183	2042.636	2246.9	8.287
12	1	0.579	52.832	CaMKK-CT	1871	894.5	1073.4	9.331
14	2	0.453	71.521	Raf1	16743	9231.471	15693.5	29.55
16	1	0.252	118.748	Fak	46527	14469.65	28939.3	30.096
17	2	0.421	76.808	GRK2	21498	5773.056	10391.5	31.578
18	9	0.607	49.474	Zipk	8404	6351.4	6351.4	5.72
19	2	0.234	124.473	JAK1	15005	5966.389	10739.5	13.329
20	1	0.22	129.397	JAK2	5285	2115.333	2538.4	18.378



Appendix A: Kinetworks™ Analysis of Astrocytic CG-4, Part 1

Appendix A: Densitometric Analysis of Astrocytic CG-4 Blot, Part 1

Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.124	201	standard	3249	1435.625	1148.5	7.246
1	2	0.253	130	standard	2157	928.273	1021.1	6.442
1	3	0.343	94	standard	5246	1737	1389.6	8.767
1	4	0.601	48.6	standard	2785	1058.222	952.4	6.009
1	5	0.803	36.4	standard	4542	1455.222	1309.7	8.263
1	6	0.936	29.8	standard	9337	2900.6	2900.6	18.3
2	1	0.358	90.953	Raf B	7174	4493.059	7638.2	8.594
2	3	0.56	54.103	ERK3	9090	5772.688	9236.3	10.393
2	4	0.681	43.405	ERK1-III	23449	9670.105	18373.201	20.673
2	5	0.72	41.057	ERK1-III	8047	4873.455	5360.8	6.032
3	1	0.53	58.688	PKB-CT	13647	3895.526	7401.5	10.055
3	2	0.72	41.141	ERK1-CT	35857	15613.81	32789.001	44.544
3	3	0.772	38.2	ERK1-CT	25798	9310.235	15827.4	21.502
4	4	0.776	38.037	ERK2	17641	5686.235	9666.6	30.478
5	3	0.302	110.626	PKC-mu	11733	5468.2	5468.2	10.899
5	10	0.802	36.725	CDK 4	7661	4362.1	4362.1	8.694
6	1	0.427	77.505	PKC-beta 1	23500	10562.471	17956.201	23.783
6	2	0.772	38.428	p38	29626	11150.25	17840.401	23.63
8	2	0.362	92.341	nPKC-epsilon	4164	2008.385	2610.9	7.109
8	3	0.806	36.714	CDK 7	27429	8429.826	19388.601	52.794
9	3	0.819	36.082	CDK 9	4192	1943.556	3498.4	28.666
10	2	0.461	72.092	PKC-delta	7067	2718.737	5165.6	20.735
13	1	0.427	79.826	PKC-zeta	33653	13245.389	23841.701	39.345
13	4	0.728	41.459	MEK 1	4206	2144.909	2359.4	3.894
14	1	0.504	65.535	PAK alpha	4054	2530.6	3795.9	9.775
14	2	0.707	42.85	MEK 2	5608	2738.579	5203.3	13.399
15	2	0.784	38.396	MEK 4	34739	13398.474	25457.101	36.285
18	10	0.53	62.264	S6K	29765	13728.538	17847.101	16.959
18	12	0.668	45.684	GSK 3 a/b	38478	12669.5	20271.201	19.262
18	13	0.784	38.622	GSK 3 a/b	11700	8039.222	7235.3	6.875
19	3	0.409	85.658	RSK1	34795	14273.308	18555.301	20.155
19	4	0.422	82.816	RSK1	40187	29553	20687.101	22.47
19	6	0.573	55.87	COT	11201	5935.7	5935.7	6.447
20	2	0.767	39.757	CK2 alpha-III	34181	14242.4	21363.601	29.778
20	3	0.789	38.538	CK2 alpha-III	35899	22899.909	25189.901	35.111



Appendix A: Kinetworks™ Analysis of Astrocytic OL, Part 2

Appendix A: Densitometric Analysis of Astrocytic CG-4 Blot, Part 2

Lane	Band	RF	MOLWT	BandName	PeakQty	AverageQty	TraceQty	RelativeQty
1	1	0.111	201	standard	6243	1669.5	2337.3	13.126
1	2	0.209	130	standard	3571	1374.636	1512.1	8.492
1	3	0.302	94	standard	4359	1382.818	1521.1	8.542
1	4	0.515	48.6	standard	3923	1593.636	1753	9.844
1	5	0.681	36.4	standard	6475	1840.727	2024.8	11.371
1	6	0.843	29.8	standard	6889	1786	2143.2	12.036
2	1	0.609	41.217	ERK1-CT	58849	17063.6	34127.201	51.375
2	2	0.651	38.28	ERK1-CT	32975	8639.5	13823.2	20.809
3	1	0.374	75.062	IKK-alpha	4034	2015.6	3023.4	9.694
4	2	0.387	72.087	BMX	7371	3018.5	4829.6	10.321
6	4	0.519	47.938	Fyn	22279	9230.25	14768.4	12.968
9	1	0.353	80.255	Zap70	7200	2835.067	4252.6	14.532
14	2	0.417	65.116	Raf-1	21891	11262.5	15767.5	23.426
16	1	0.264	110.413	FAK	49911	15459.056	27826.301	26.616
17	3	0.379	73.754	GRK2	15877	5010.083	6012.1	16.57
19	1	0.247	118.821	JAK 1	16917	7242.588	12312.4	16.147
20	1	0.234	125.336	JAK2	13131	4102.533	6153.8	21.636