UNIVERSITY OF
CALGARY

UNIVERSITY OF CALGARY

Regulation of Glioma Phenotypes by Specific Protein Kinase C Isoforms

By

Arnaud Besson

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

April 2001

Abstract

Astrocytomas are the most common primary brain tumors. The prognosis for patients with glioblastoma multiforme, the most common and the more malignant type of astrocytomas, is very poor and has not significantly improved in the past 30 years. Hence, better therapeutic strategies are needed to improve the outcome of the disease. There is increasing evidence to suggest an important role for the PKC family in the regulation of glioma cell phenotypes, including proliferation, migration, and invasion; however the identity of the PKC isoforms involved and the mechanisms by which they accomplish their functions remain elusive.

We found that PKC a was both necessary and sufficient to promote cell cycle progression of human glioma cells. PKC a activation induced expression of the cyclin-CDK inhibitor p21/Wafl/Cipl, which was incorporated into various cyclin-CDK complexes; the kinase activity of these complexes was increased, resulting in cell cycle progression. Thus PKC a activity controls glioma cell cycle progression through the upregulation of p21/Wafl/Cipl, which facilitates active cyclin/CDK complex formation.

PKC e was found to positively regulate integrin-dependent adhesion and motility of glioma cells. PKC e activation was associated with increased focal adhesion formation and integrin clustering, and was required for PMA-induced adhesion and motility. The scaffolding protein RACK1 mediated the interaction between integrin (3 chain and activated PKC e; this was accompanied by the association of FAK and vinculin with integrin. Depletion of RACK1 by an antisense strategy prevented PKC e-induced adhesion and migration. These results provide a mechanistic link between PKC activation and integrin-mediated adhesion and motility. Activation of the ERK pathway downstream of PKC was required for PKC-induced integrin-mediated adhesion and migration. Moreover, PKC e was able to target activated ERK to focal adhesions to mediate glioma adhesion and motility.

In conclusion, we have found that specific isoforms of PKC regulate distinct phenotypes of glioma cells. PKC a activation stimulates cell cycle progression and proliferation, while PKC e activation induces integrin-mediated adhesion and motility.

These findings may prove useful in designing new therapeutic strategies targeting PKC isoforms in astrocytomas.

Acknowledgements

I wish to thank my supervisor Dr. Wee Yong for the opportunity to do my PhD in his laboratory, for his support, his patience, and for giving me the freedom to work on the topics that were most interesting to me. I've learned a lot in your lab, thanks Wee.

For their helpful suggestions and comments throughout the years, I wish to thank the members of my supervisory committee Drs. Steve Robbins, Mike Walsh, and Peter Forsyth. Special thanks to Dr. Steve Robbins, who kept his door always opened to me and for the countless advices he gave me. I also wish to thank Drs. Phyllis Luvalle, Karl Riabowol, and Michael Berens, for accepting to serve as external examiners in my examination committees.

Special thanks to Lori Robertson, from the Flow Cytometry Laboratory, for her technical expertise.

I would like to thank all the members of the "Yong Lab", and more particularly my friends, Dr. Sophie Chabot, Dr. Luke Oh, Shannon Corley, Luigi Riscaldino, Dr. Jack Vecil, and Dr. Uma Ladiwala. Special thanks to Tammy Wilson for putting up with me at the bench for the last four years.

I would like to thank Drs Charline Piettre, Ginette Soret, Christophe Merlin, and Yves Marckowicz, for giving me my first taste of biological research.

I wish to extend my thanks to all our friends, who contributed in making our stay in Calgary most enjoyable.

Many thanks to my parents Monique and Gerard, my brother Fabrice, and all my family, for their continuous support and their love.

Finally, I thank Dr. Alice Davy for her unconditional support, her suggestions and comments, and for sharing my life during the past five years.

Dedication

A mon grand-pere, Gabriel (1917-1996)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A488	Alexa 488
AMV-RT	avian myeloblastosis virus reverse transcriptase
ANOVA	analysis of variance
BBS	BES-buffered solution
BES	***N, N-bis*** (2-hydroxyethyl)-2-aminoethanesulfonic acid
BIS	Bisindolylmaleimide I
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
CalpC	Calphostin C
CCD	charge-coupled device
CDK	cyclin-dependent kinase
CIP1	CDK-interacting protein 1
Cy3	Cyanine 3
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced-chemiluminescence
EGFR	epidermal growth factor receptor
EDTA	ethylenediamine tetraacetic acid
ERK	extracellular-signal regulated kinase
FCS	fetal calf serum
FN	fibronectin
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
h	hour
HEPES	Af-2-hydroxyethylpiperazine-Af-2-ethanesulfonic acid
HGF	hepatocyte growth factor
HRP	horseradish peroxidase

IP	immunoprecipitation
kDa	kilodalton
KIP1	kinase-inhibitory protein 1
LN	laminin
pM	micromolar
pi	microliter
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase/extracellular-signal regulated kinase kinase
ml	milliliter
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue
ng	nanogram
nM	nanomolar
NP-40	Nonidet P-40
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	log **10** proton concentration
PH domain	pleckstrin homology domain
PI 34cinase	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4, 5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylfluoride
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
RACK1	Receptor for activated C-kinase 1
RPA	RNase protection assay

rpm	rotation per minute
PvNA	ribonucleic acid
RTK	receptor tyrosine kinase
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
THY	thymeleatoxin
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
V	volt
VN	vitronectin
WAF1	Wild-type p53 activated fragment 1

CHAPTER ONE

Introduction

1.1. Protein Kinase C.

1.1.1. Overview.

Protein kinase C (PKC) is a family of serine/threonine kinases comprising 12 members in mammalian cells (reviewed in Newton 1995, Jaken 1996, Mellor and Parker 1998, Ron and Kazanietz 1999). Each PKC isozyme is the product of an individual gene, with the exception of PKC 3i and **p2,** which are spliced variants of the same gene. PKCs have been divided into three subgroups, based on the cofactor requirement of each isoform for activation: Ca^{2+}, diacylglycerol (DAG), and phosphatidylserine (PS) are required for full activation of the conventional PKCs (a, pi, (3$_2$, and ***y),*** while Ca^{2+} is not required for activation of the novel PKCs (5, e, ***r\,*** 6, v, and p/PKD). Both Ca^{2+} and DAG are dispensable for atypical PKC activation (i/X, and Q (Fig. 1). The substrate specificity of PKC is thought to be mediated by a set of anchoring proteins and by a tight regulation of their subcellular localization. PKC is activated downstream of many transmembrane receptors, including receptor tyrosine kinases (RTKs) and integrins, and is involved in many cellular processes such as proliferation, cell cycle progression, apoptosis, migration/motility, invasion, and angiogenesis. Phospholipase C (PLC) is one of the main enzymes involved in PKC activation. PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and inositol 1,4,5-trisphosphate (IP_3) (Fig. 2). Four classes of PLCs have been characterized so far: PLC p_**4,** PLC **81-4,** PLC **Y1-2,** and PLC e. PLC p is activated by heterotrimeric G-proteins. PLC **8** is regulated by changes in intracellular calcium. PLC ***y*** is activated by binding to phosphotyrosine residues on receptor tyrosine kinases (RTKs) through its SH2 domain; and by phosphoinositide (PI) 3-kinase, and focal adhesion kinase (FAK). The recently cloned PLC e is directly activated by Ras, thus placing PKC downstream of Ras activation (Kelley et al. 2001). PKC may participate in mitogenic signaling by activating the Raf/MEK/MAPK pathway. Indeed, PKC can directly phosphorylate and activate Raf-1 (Kolch et al. 1993), although Ras appears to be required to recruit Raf to the membrane (Marais et al. 1998). PKC appears to be hyper-activated in gliomas (Couldwell et al. 1991, 1992), possibly due to amplification and

constitutive activation of several growth factor receptors. Another event that could possibly account for PKC hyper-activation in gliomas is PTEN inactivation, which could lead to activation of PLC y and phosphoinositide-dependent protein kinase-1 (PDK-1), two enzymes that play critical roles in PKC activation.

1.1.2. Structure and regulation of PKC.

PKC contains an N-terminal regulatory region and a C-terminal catalytic region (Fig. 1). The pseudosubstrate domain (auto-inhibitory domain) binds to the substrate-binding site in the catalytic domain, keeping the enzyme in an inactive conformation in the absence of activators. Upon cofactor binding to the enzyme, the pseudosubstrate domain is removed from the catalytic site and becomes proteolytically labile.

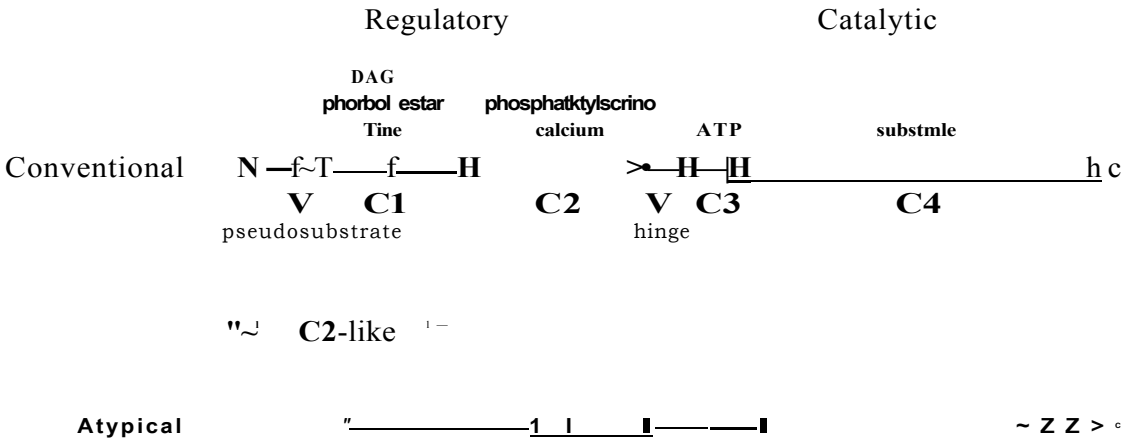


Figure 1: Domain structure of the PKC family.
The pseudosubstrate domain interacts with the kinase domain (C4) when PKC is inactive, preventing the interaction with substrates. When activated, the pseudosubstrate domain becomes highly sensitive to proteolysis. The CI domain mediates the interaction with diacylglycerol (DAG), and also with the artificial activators phorbol esters. Co-factors binding to the CI domain serve as hydrophobic anchors to recruit PKC to the membrane, stabilize the active conformation of the enzyme, and facilitate phosphatidylserine (PS) binding. The C2 domain mediates the binding to PS, which anchors the protein to the membrane, and induces a conformational change that displaces the pseudosubstrate domain from the catalytic site. The C2 domain of novel PKCs lacks several amino acids that mediate binding to calcium but is readily able to bind phospholipids; hence novel PKCs are calcium independent. The hinge region becomes sensitive to proteolysis when the enzyme is in its active conformation, allowing the degradation of active PKC (downregulation). The C3 domain is the ATP-binding site, and the C4 domain is the kinase domain, which requires several phosphorylations on key residues to render the enzyme catalytically competent. (Adapted from Newton 1995).

The CI domain is present in all PKC isoforms and contains a cysteine-rich region, involved in the binding of DAG in cPKCs and nPKCs. This domain is duplicated in all PKCs except aPKCs. Each cysteine-rich region binds two Zn^{2+} ions. DAG binding to the CI domain serves as a hydrophobic anchor that facilitates the recruitment of PKC to the membrane (Fig. 2). DAG binding increases the affinity of PKC for PS and increases the catalytic activity of the enzyme that is already bound to PS. The CI domain of aPKCs lacks critical residues for DAG binding, hence the absence of requirement for DAG for aPKC activation.

The tumor promoting phorbol esters are a class of chemicals that can bind to the CI domain of conventional and novel PKCs (using the same binding site as DAG) and induce the membrane translocation and activation of PKC. The compounds are therefore widely used as PKC activators. However, one has to keep in mind when using these agonists that PKCs are not the only proteins that can bind to phorbol esters, since other proteins containing a CI domain bind and are activated by phorbol esters (reviewed in Ron and Kazanietz 1999, Kazanietz 2000). For example, RAS-GRP, a guanine nucleotide exchange factor (GEF) for Ras, binds and is activated by phorbol 12,13-dibutyrate (PDBu), and enhances Ras activity (Ebinu et al. 1998, Tognon et al. 1998, Lorenzo et al. 2001). The chimaerins are a family of GTPase activating proteins (GAP) for Rac; therefore chimaerin activation leads to Rac inactivation (Ahmed et al. 1993). Chimaerins comprise 4 members (oti, 02, Pi, and P2), which are activated by phorbol esters. However, the DAG analog thymeleatoxin is a poor activator of chimaerins (Kazanietz 2000). Finally, CalDAG-GEFl, a GEF for the small GTPase Rapl, is also activated by phorbol esters.

The C2 domain of PKC mediates binding to Ca^{2+} and phosphatidylserine (or other negatively charged phospholipids) (Fig. 2). This domain is also present in over 20 other proteins, including PLCs, phospholipase A2, and several GAPs (Ponting and Parker 1996). The affinity and selectivity of the C2 domain for PS is greatly increased by binding of DAG to the CI domain. Binding of Ca^{2+} and PS to the C2 domain induces a conformational change in the enzyme, removing the pseudosubstrate domain from the catalytic site, and activating the enzyme. For cPKCs, the binding of Ca^{2+} to the C2 domain increases its affinity for negatively charged phospholipids. In nPKCs, critical Asp

residues of the C2 domain involved in Ca^{+} binding are replaced by Arg residues, thus changing the conformation of the Ca^{2+} binding pocket, rendering it readily adapted for the binding of negatively charged phospholipids in the absence of calcium; hence it is termed C2-like domain. The C2 domain is absent from aPKCs, hence their lack of requirement for phospholipids for activation. The C2 domain of PKC is also involved in mediating protein-protein interactions and contains the binding site for the receptor for activated C-kinase (RACK) (Ron et al.1994, 1995).

C-terminal to the C2 domain is the hinge region, which becomes exposed and sensitive to proteolytic cleavage when PKC switches to its active conformation. This process is thought to participate in the downregulation of the protein. Downregulation of PKC is also mediated by ubiquitination and targeting of the enzyme for degradation by the 26S proteasome (Lu et al. 1998).

The catalytic region of PKC is formed of the C3 domain, which contains the ATP-binding site; and the C4 domain, which contains the substrate binding/catalytic site. In its inactive conformation, the pseudosubstrate domain occupies the substrate-binding site. Newly synthesized PKC undergoes a maturation process. The catalytic region of PKC requires phosphorylation events to be catalytically competent (Fig. 2). Phosphorylation of the activation loop of PKC by PDK-1, followed by autophosphorylation on two additional sites, is necessary for the release of newly synthesized PKC into the cytosol and to obtain a catalytically competent conformation. PDK-1 can phosphorylate all PKC isoforms at the activation loop of the kinase domain; this step is required for PKC to be catalytically competent (Le Good et al. 1998, Dutil et al. 1998, Garcia-Paramio et al. 1998). The two subsequent autophosphorylation events (Thr-638 and Ser-657 in PKC a) are important to lock and stabilize the enzyme in its catalytically competent conformation and to render it resistant to dephosphorylation by protein phosphatases (Bornancin and Parker 1996, 1997). PDK-1 has a PH domain that mediates, at least in part, by binding to **PIP2** or **PIP3,** its activation and localization to the plasma membrane. In view of the critical role of PDK-1 in PKC activation, one can hypothesize that inactivation of PTEN, which decreases the availability of phosphoinositides phosphorylated at position 3, could lead to PDK-1 overactivation in gliomas, and subsequent accumulation of catalytically competent PKC.

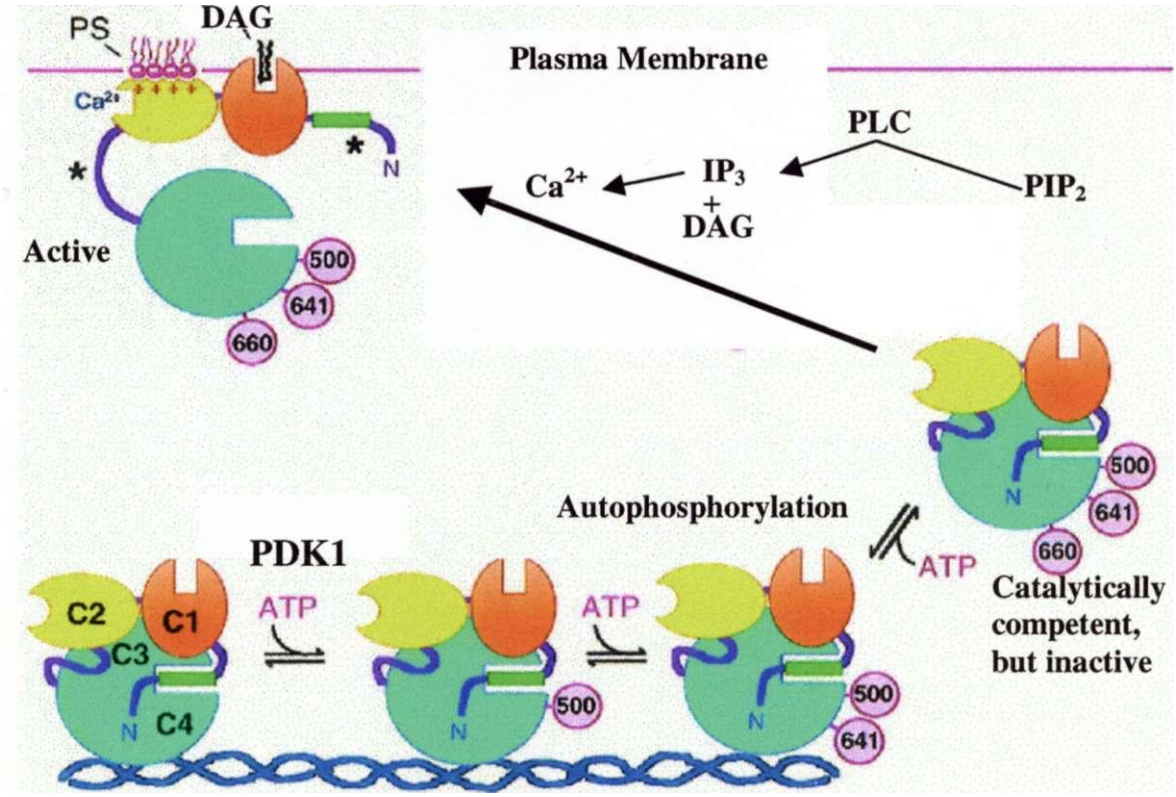


Figure 2: Regulation of PKC by phosphorylation and cofactors.
Maturation: After synthesis, PKC is found in the detergent insoluble fraction of the cells. Its maturation to a catalytically competent form requires a first phosphorylation event mediated by PDK1 at the activation loop of the kinase domain. Then 2 autophosphorylation steps take place, which stabilize the catalytically competent conformation, and trigger the release of the enzyme into the cytosol. The numbers of the phosphorylated residues refer to the amino acid sequence of PKC pi.
Regulation: Stimulation of phospholipase C (PLC) will induce the breakdown of phosphatidylinositol 4,5-bisphosphate **(PIP2)** into inositol 1,4,5-trisphosphate **(IP3),** which will induce the release of calcium from the intracellular stores, and diacylglycerol (DAG). Binding of DAG to the CI domain of PKC triggers its translocation to the membrane. Further association of the C2 domain with phosphatidylserine (PS), which is facilitated by the binding of Ca^{2+} in the case of conventional PKCs, will induce a conformational change that results in the release of the pseudosubstrate domain from the catalytic cleft and activation of the enzyme.

Asterisks indicate the hinge region, which becomes sensitive to proteolysis upon association of PKC with the membrane, and the pseudosubstrate domain, which become sensitive to proteolysis upon activation of PKC. (Adapted from Newton 1995).

1.1.3. PKC anchoring proteins.

A growing theme in signal transduction is the critical importance of a tight regulation of the subcellular localization of signaling molecules. The identification of a large number of proteins that can interact with PKCs and regulate their targeting to precise locations in the cell, their substrate specificity, and their activity has added another layer of complexity to the understanding of PKC signaling (reviewed in Mochly-Rosen and Gordon 1998, Ron and Kazanietz 1999, Jaken and Parker 2000).

Some PKC interacting proteins specifically bind inactive PKC, such as AKAP79 (A-kinase anchoring protein-79) and 14-3-3, possibly maintaining PKC in close proximity to its substrate and releasing it upon activation. Other proteins interact specifically with activated PKC and are termed RACKs (receptors for activated C-kinase), such as RACK1 and the (3-coatomer protein (fi-COP) (Mochly-Rosen et al. 1991, Ron et al. 1994, Csukai et al. 1996). The latter was found to act as a PKC e-specific RACK (Csukai et al. 1996). Although RACK1 was identified as a PKC p binding protein (Mochly-Rosen et al. 1991, Ron et al. 1994), it was subsequently found that it could bind other PKC isoforms, including a, y, 8, and e (Ron et al. 1995, Rotenberg et al. 1998, Pass et al. 2001). RACK1 is a 36-kDa protein formed of 7 WD-40 repeats and has a high degree of similarity to the P subunit of heterotrimeric G-proteins (Ron et al. 1994). WD-40 repeats are protein domains usually involved in protein-protein interactions. PKC interacts with RACK1 through its C2 domain in the case of cPKCs or the C2-like domain (VI region) of nPKCs. This binding region in PKC was termed the pseudo-RACK binding site (Mochly-Rosen et al. 1992, Ron et al. 1994, 1995, Ron and Mochly-Rosen 1995, Johnson et al. 1996, Hundle et al. 1997). A short sequence within the sixth WD-40 repeat of RACK1 (DIINALCF) induced PKC translocation and activation, and inhibited the binding of full length RACK1 to PKC, suggesting that this region of RACK1 mediates its binding to PKC (Ron and Mochly-Rosen 1994). Ron et al. (1999) recently reported that RACK1 was co-translocated with PKC p_2 upon its activation, suggesting a role for RACK1 as a shuttling protein.

In addition to its role as a PKC binding protein, RACK1 was found to interact with a number of other cellular proteins, suggesting that it could function as a scaffolding

protein, by bringing a number of signaling molecules to the same location. RACK1 can associate with phospholipase C y (Disatnik et al. 1994), the cAMP-specific phosphodiesterase PDE4D5 (Yarwood et al. 1999), and the type I interferon receptor (Croze et al. 2000). RACK1 was found to interact with the Src family kinases c-Src, Fyn, Yes, and Lck, and in doing so inhibited their kinase activity; RACK1 overexpression in NIH 3T3 cells inhibited Src activity and proliferation (Chang et al. 1998). RACK1 was constitutively bound to the common p chain of the IL-5/IL-3/GM-CSF receptors, and allowed the recruitment of PKC P to the receptor following IL-5 or PMA stimulation (Geijsen et al. 1999). Recently, it was found that RACK1 interacts with the membrane proximal region of the cytoplasmic tail of integrins Pi, **p2,** and ps, and that RACK1-integrin binding was dependent on the presence of PMA, suggesting the involvement of PKC in this interaction (Liliental and Chang 1998). However, the functional significance of the interaction between RACK1 and integrins was not investigated.

Interestingly, RACK1 was recently found to be dramatically upregulated during angiogenesis in vitro and in vivo, and in several types of human carcinomas, suggesting an important for role for RACK1, and possibly PKC, in angiogenesis and tumorigenesis (Berns et al. 2000).

Thus, RACK1 may act as a scaffolding or anchoring protein (similar to AKAPs) that mediates the localization of various signaling enzymes to specific subcellular compartments, thus allowing the formation of signaling complexes.

1.2. General aspects of the transduction of proliferative signals and of the cell cycle machinery.

Mitogenic signaling pathways and cell cycle regulation are tightly linked, as growth factor receptor stimulation initiates signaling cascades that lead to the activation of the cell cycle machinery, resulting in cell proliferation. The transition from one phase of the cell cycle to another is regulated by the activity of cyclin/cyclin-dependent kinase (CDK) complexes (Fig. 3) (Dirks and Rutka 1997). Cyclin-CDKs are themselves regulated by two families of cyclin-dependent kinase inhibitors (CKI), the INK4 family (p16/INK4A, pl5/INK4B, pl8/INK4C, and pl9/INK4D) and the Cip/Kip family (p21AVafl/Cipl, p27/Kipl, and p57/Kip2). Growth factor receptor signaling induces the expression of the

Gl cyclins (e.g. cyclin Dl-3). The Gl/S transition is regulated by cyclin D-CDK4/6 complexes that phosphorylate and inactivate the retinoblastoma (Rb) protein, releasing the E2F transcription factors, which in turn allow the transcription of genes required for the progression through S phase (such as cyclin E) (Fig. 4).

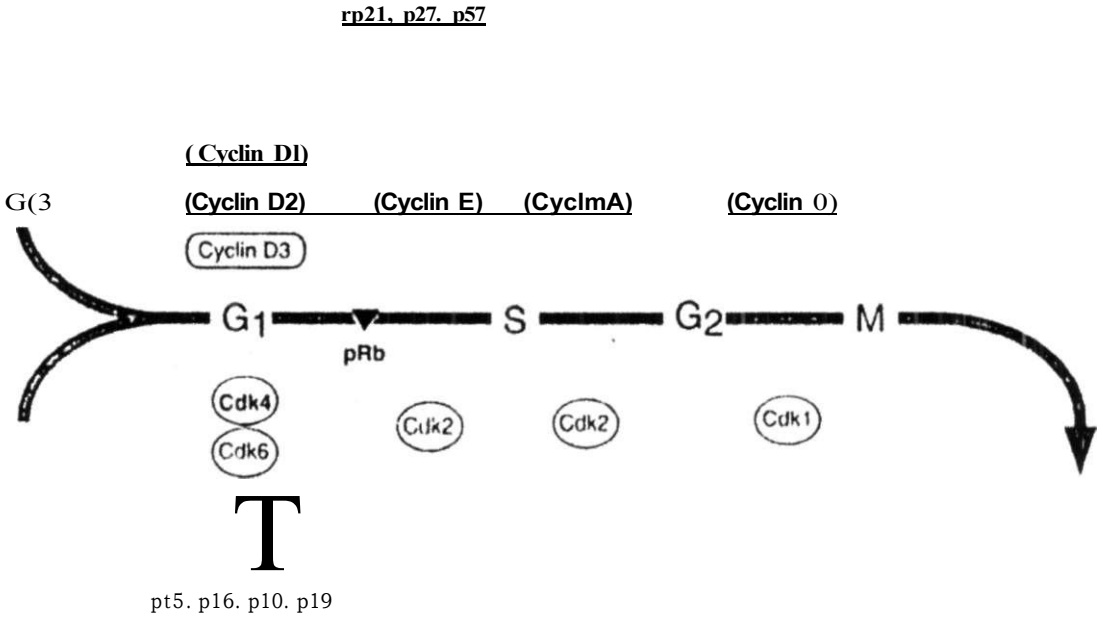


Figure 3: Regulation of the mammalian cell cycle.
The different phases of the cell cycle are Go (quiescence), Gi (Gap 1, or interphase), S (DNA synthesis), G_2 (Gap 2), and M (mitosis). During Gi and G_2, there is extensive "preparation" for the S and M phases, respectively. The triangle represents the 'restriction point', controlled by the retinoblastoma protein. Once past this point, cells are committed to divide, independently of the presence of extracellular stimuli such as growth factors. The transition from one phase to another is positively regulated by complexes formed between the cyclins (which provide substrate specificity) and their respective cyclin-dependent kinase (CDK) partners. Two families of CDK inhibitors regulate cyclin-CDK complexes. The Cip/Kip family (formed of p21/Wafl/Cipl, p27/Kipl, and p57/Kip2) can inhibit the activity of all cyclin-CDK complexes, and thus regulate the progression of each phase of the cell cycle. However, members of this family were also recently found to participate in the assembly and even activation of some cyclin-CDK complexes. The INK4 family members (formed of p16/INK4A, p15/TNK4B, p18/INK4C, and p19/INK4D) are specific for the inhibition of CDK4 and CDK6, and therefore regulate the Gi/S transition only. (Adapted F'rom Hall and Peters 1996).

Two pathways regulating cell cycle progression and playing a critical role in preventing tumor formation have been identified, namely the Rb and p53 pathways

(reviewed in Weinberg 1995, Sherr 1998). The Rb pathway is composed of p16/ENK4A, CDK4/6, and Rb, and disruption of the normal function of a single component of the pathway (i.e. loss of p16/INK4A or Rb, or amplification of CDK4/6) is sufficient to lead to deregulated growth control (Fig. 4). The p53 pathway is composed of p14/ARF (the human homologue of the mouse p19/ARF), the product of an alternative reading frame of the INK4a gene also encoding p16/INK4A (Sherr 1998), mouse double minute-2 (Mdm2), and p53. p14/ARF can activate p53 and promote the degradation of Mdm2. The latter sequesters and promotes p53 degradation, while p53 promotes Mdm2 expression and inhibits p14/ARF expression. These complex interactions aim at tightly regulating p53 level and activity (Fig. 4). p53 can block cell cycle progression through the induction of p21/Wafl/Cipl expression, and plays a critical role in the activation of the DNA repair machinery and induction of apoptosis. Either loss of p14/ARF, p53 mutation, or Mdm2 amplification can disrupt the p53 pathway. Several components of these pathways are often mutated in cancers, including gliomas (Fig. 4).

Other molecules commonly mutated in gliomas are growth factor receptors. Upon ligation of the RTK by its ligand, there is dimerization of the receptor, followed by trans-phosphorylation of the cytoplasmic tail of the receptor on specific tyrosine residues. Phosphorylated tyrosines provide docking sites for Src-Homology-2 domain (SH-2) and phosphotyrosine binding (PTB) domain-containing proteins, such as She, Grb2/Sos, and phospholipase Cy(PLCy), which in turn recruit and activate additional signaling molecules, thus generating a signaling cascade that leads to activation of transcription, translation and increased growth. Signaling pathways activated by RTK stimulation include the Ras/Raf/MAPK pathway, the PLCy/PKC pathway, PI 3-kinase/Akt, and Src family tyrosine kinases (see Fig. 5 for more detail). Ligand binding and receptor activation trigger endocytosis of the receptor and subsequent degradation in the lysosome.

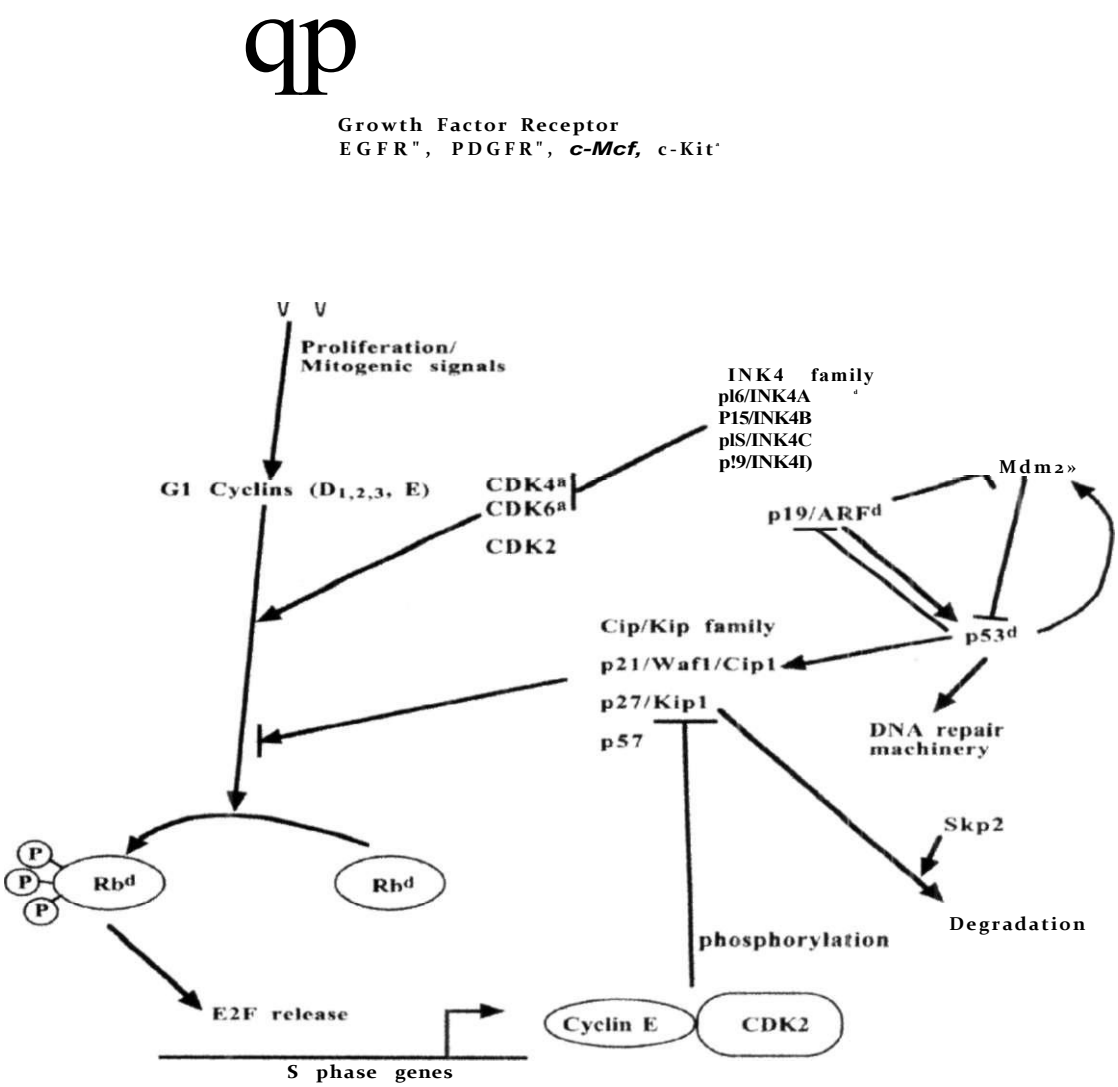


Figure 4: Genes mutated in astrocytomas involved in growth control.

[a] = Gene amplified, mutated (gain of function), or protein overexpressed in astrocytomas.

[d] = Gene deleted, mutated (loss of function), or expression repressed in astrocytomas.

A number of genes mutated in astrocytomas are involved in the regulation of growth control (through the regulation of the Rb and p53 pathways), either by controlling mitogenic signaling cascades, or cell cycle progression.

Mitogenic signals induce Gi cyclins expression; Gi cyclin-CDK complexes phosphorylate the Rb protein, which in turn releases E2F transcription factors, turning on transcription of genes required for entry into S-phase (e.g. cyclin-E). Members of the ENK4 family can inhibit CDK4/6, thus preventing hyperphosphorylation of Rb and progression into S-phase. Members of the Cip/Kip family can act both as assembly factors for cyclin-D/CDK4-6 complexes and as cyclin-CDK inhibitors, depending on their stoichiometry. p27/Kipl is phosphorylated by cyclin-E/CDK2, targeting it for ubiquitination and degradation. p21/Wafl/Cipl expression can be induced by p53, resulting in cell cycle arrest in Gi or **G2.** p53 is inhibited/destabilized by Mdm-2, and p53 induces Mdm-2 expression. p19/ARF (p14/ARF in human) can activate p53 functions and induce Mdm-2 degradation, resulting in p53 stabilization; the expression of p19/ARF itself is downregulated by p53.

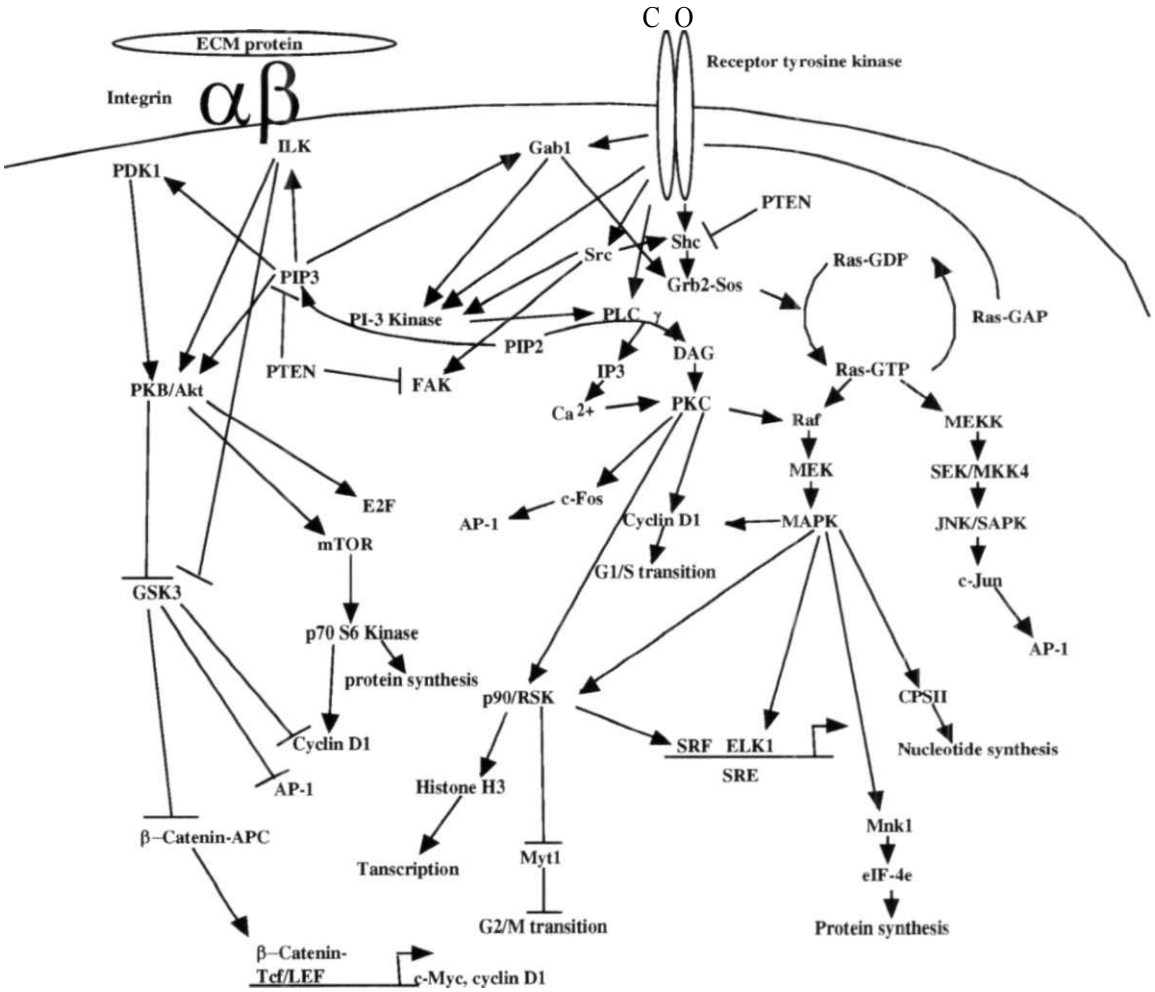


Figure 5: Some of the mitogenic signaling pathways activated downstream of Receptor Tyrosine Kinases (RTKs). RTK ligation by its cognate ligand induces trans-phosphorylation of the cytoplasmic tail of the RTK, providing docking sites for SH2 and PTB-containing proteins, including PI 3-kinase, Gabl, Src, PLC *y,* She, Grb2, and Ras-GAP. PI 3-kinase activation leads to increased levels of **PIP3,** which participates in the activation of Gabl, ILK, PDK1, PKB/Akt, and PLC *y.* Gabl participates in the activation of the ERK/MAPK and JNK pathways. Phosphorylation of She by RTKs induces association with Grb2 and Sos, which in turn activates Ras, leading to activation of the ERK and JNK pathways. Activated JNK phosphorylates c-Jun, increasing AP-1-mediated transcription. ERK phosphorylates and activates several targets involved in growth control: carbamoyl phosphate synthetase (CPS-II), an enzyme involved in pyrimidine-nucleotide synthesis; the ERK-interacting kinase Mnk1, which phosphorylates and activates the translation initiation factor eIF-4E; transcription factors, such as E1k1; p90-RSK, which participates in activation of the Serum Response Factor (SRF), phosphorylates histone H3, thus increasing accessibility of the transcription factor to DNA, and inactivates Myt1, a kinase that phosphorylates and inactivates CDK1/Cdc2 kinase, required for G2/M transition; and induces cyclin-D1 expression. Activation of PKB/Akt by **PIP3,** PDK1 and ILK leads to increased transcription, through inhibition of glycogen synthase kinase-3-PGSK-3p), which phosphorylates and targets p-catenin for degradation. PKB/Akt also phosphorylates and activates the transcription factor E2F and regulates protein synthesis through the activation of the mammalian target of rapamycin (mTOR), which itself activates p70-S6 Kinase.

1.3. Astrocytomas.

Astrocytomas are the most common primary brain tumor. The World Health Organization (WHO) has defined four categories of astrocytomas: pilocytic astrocytoma (WHO grade I), astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III) and glioblastoma multiforme (GBM)(WHO grade IV) (Kleihues et al. 1995). GBMs are the most common (approximately 70% of astrocytomas) and the most malignant of all astrocytomas and are characterized by a very poor prognosis. Current therapies, including surgery, chemotherapy, and irradiation have failed to improve the prognosis of patients with these tumors; therefore, there is a need for identifying new treatment strategies that could improve the outcome of the disease. Astrocytomas are thought to arise from cells of the astrocytic lineage. Based on genetic analyses, two pathways of GBM formation, with distinct pathogenesis, have emerged (Fig. 6) (Von Deimling et al. 1995, Lang et al. 1994).

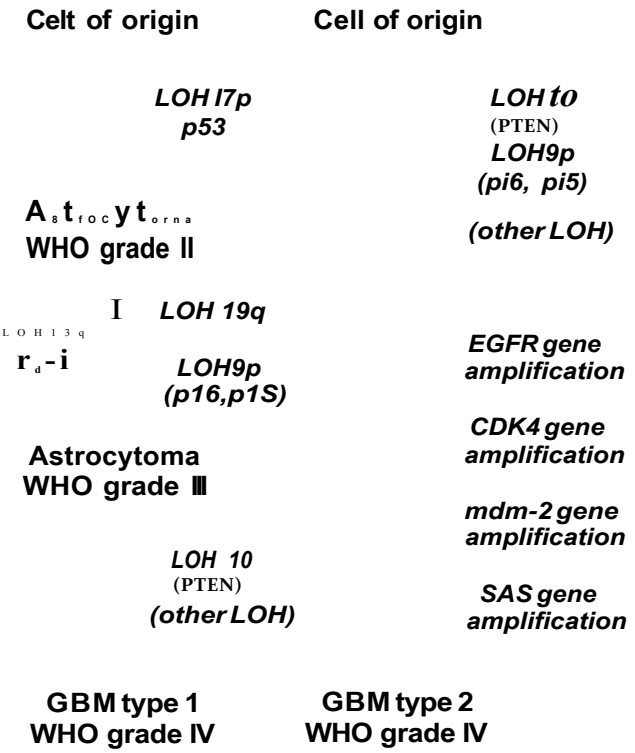


Figure 6: Pathways of glioblastoma formation. Most glioblastomas (GBMs) arise 'de novo', without any previous astrocytic glioma history (right pathway, GBM type-2, or primary glioblastoma). However, some GBMs arise in patients previously diagnosed with a lower grade astrocytoma (left pathway, GBM type-1, or secondary glioblastoma). Although these types of GBMs are not readily distinguishable by standard histopathological analysis, genetic analyses revealed that these tumors have distinct genetic alterations, as indicated on the schematic. Secondary GBMs have a better prognosis than primary (de novo) GBMs. GBMs type-1 are characterized by p53 mutations, while type-2 GBMs harbor EGFR mutations.

The 'de novo pathway' (GBM type-2) is characterized by the absence of p53 mutations, the presence of EGFR amplification/rearrangement, p16/INK4A and p!9/ARF

deletion (located on chromosome 9p), and loss of heterozygosity on chromosome 10 (where the PTEN tumor suppressor is located) (Biernat et al. 1997). The 'progressive pathway' leading to the development of GBMs (GBMs type-1, or secondary GBM) is characterized by early inactivation of p53, followed by the mutation of Rb, and Mdm2 and CDK4 amplifications as late events. The 'de novo pathway' occurs mostly in older patients and is associated with more aggressive tumors and poor prognosis, while the 'progressive pathway' occurs preferentially in younger patients and is associated with better prognosis (Von Deimling et al. 1995, Lang et al. 1994).

Genetic studies have shown the importance of cell cycle regulatory molecules in the development of astrocytomas (reviewed in Caskey et al. 2000). Mutations in the p53 gene have been reported in 30-50% of astrocytomas, in both low and high-grade tumors (Von Deimling et al. 1995). Mdm2 amplification and overexpression was found in about 10% of high-grade astrocytomas (grades III and IV) (Von Deimling et al. 1995, Ichimura et al. 2000). Recently, mutation/deletion of p14/ARF were found in 40% of GBMs (Ichimura et al. 2000). p14/ARF, p16/INK4A, and p15/TNK4B are frequently homozygously co-deleted since the INK4a gene, encoding both p16/BSfK4A and p14/ARF, and the ENK4b genes are very close on chromosome 9p21 (Ichimura et al. 2000). Mutations in p14/ARF, p53, and Mdm2 occur in a mutually exclusive manner (Reifenberger et al. 1993, Collins 1995, Ichimura et al. 2000). CDK4 amplification and overexpression has been detected in approximately 15% of GBMs (Collins 1995, Biernat et al 1997), while p16/TNK4A is homozygously deleted in 30-60% of tumors (Schmidt et al. 1994, Biernat et al 1997, Ichimura et al. 2000); loss or mutation of Rb was observed in 12-36% of GBMs (He et al. 1995, Ichimura et al. 1996, Biernat et al 1997, Burns et al. 1998). These mutations also occur in a mutually exclusive manner (Collins 1995, Biernat et al 1997). Amplification and overexpression of CDK6, and cyclin Dl and D3 were observed in a small fraction of astrocytomas. In total, inactivation of the Rb pathway occurs in 67-85% of GBM (Collins 1995, Ichimura et al. 2000). Interestingly, 96% of GBMs with altered Rb pathway had also deregulated p53 pathway (Ichimura et al. 2000). It is striking that during the pathogenesis of GBM, there is a nearly invariable inactivation of both the Rb and p53 pathways (through the inactivation or amplification of one of its components), indicating that inactivation of both the Rb and p53 pathways is a crucial event in astrocytic tumor

formation (Ichimura et al. 2000). Other genetic alterations target mostly growth factor receptors and their downstream signaling effectors, giving astrocytoma cells the impetus to proliferate.

Telomerase is a ribonucleoprotein enzyme complex that elongates telomeric DNA whose activity is thought to be necessary for cellular immortalization. Telomerase activity was detected in 72% of GBMs, 40% of anaplastic astrocytomas, and 0-20% of low-grade astrocytomas (Hiraga et al. 1998, Fathallah-Shaykh 1999).

All the growth factor receptors that have been implicated in astrocytoma pathogenesis are receptor tyrosine kinases. EGFR is thought to be a critical player in glioma pathogenesis. It is a 170 kDa transmembrane glycoprotein that belongs to the Erb-B subfamily of RTK. Physiological ligands of the EGFR include EGF, Amphiregulin, HB-EGF, and TGFa. The EGFR gene is amplified and the protein overexpressed in 40-50% of glioblastomas (Libermann et al. 1984, Bigner et al. 1988, Ekstrand et al. 1991, Von Deimling et al. 1995, Collins 1995, Worm et al. 1999). Amplification of the EGFR is associated with poor prognosis and shorter survival. In addition, a rearrangement of the EGFR gene can occur, resulting in the overexpression of transcripts lacking exons 2-7 and encoding a truncated receptor (p140/EGFR, or AEGFR) that lacks part of the extracellular domain (residues 6-273). Expression of AEGFR has been reported in the absence of EGFR amplification, and overall, AEGFR expression could be found in 49-62% of GBMs (Collins 1995, Wilkstrand et al. 1995, Worm et al. 1999). AEGFR is constitutively activated (Huang et al. 1997) and is unable to bind its physiological ligands (Ekstrand et al. 1994, 1995). AEGFR has an extended half-life and seems to escape the normal downregulation mechanisms (by the endosome-lysosome pathway) (Ekstrand et al. 1995, Huang et al. 1997). The effect of AEGFR overexpression on proliferation has been attributed to an increased activation of the Ras/ERK signaling pathway due to constitutive association of She and Grb2, two adaptor proteins involved in Ras activation, with phosphotyrosine residues on AEGFR (Montgomery et al. 1995, Prigent et al. 1996). The existence of an autocrine loop involving EGFR and its ligands has been well characterized in gliomas. The heparin binding EGF-like growth factor (HB-EGF) was abundantly expressed in GBMs and glioma cell lines, and was co-expressed with EGFR in 52% of GBMs (Mishima et al. 1998). Transforming growth factor a (TGF a)

expression was found in 88% of GBMs (Maruno et al. 1991). Recently, Holland et al. (1998, 2000a) developed a mouse model in which the animals spontaneously develop astrocytoma-like lesions. In this model, overexpression of a constitutively activated EGFR in cells of the astrocytic lineage induced rapid tumor formation in mice of ENK4A/ARF-/- background (lacking p16/INK4A and p19/ARF), or in mice of p53+/- background when activated EGFR was overexpressed along with CDK4. These results clearly demonstrate that, in order to be fully oncogenic, EGFR first requires the inactivation of both the p53 and Rb pathways.

Glioma cell lines commonly express abundant levels of PDGF-A, and both receptor chains (a and (5) (Nister et al. 1991, Westermark et al. 1995). Expression of the PDGF-B chain and the oc-PDGF-receptor was found in all astrocytomas, increasing with grade, while expression of the PDGF-A chain was found only in GBMs (Hermanson et al. 1992). These results indicate the existence of an autocrine loop involving PDGF and its receptors in glioma. PDGFRoc gene amplification was found in a small subset of GBMs, and interestingly, PDGFRoc and EGFR gene amplifications are mutually exclusive (Fleming et al. 1992).

c-Met is a RTK formed of a 50 kDa extracellular a subunit, and a 140 kDa (or 170 kDa) transmembrane (3 subunit with an intracellular kinase domain. Hepatocyte Growth Factor/Scatter Factor (HGF/SF) is the ligand for c-Met. HGF/SF plays a role in the regulation of cell growth, cell motility, morphogenesis, and is a potent angiogenic factor, possibly through the regulation of Vascular Endothelial Growth factor (VEGF) expression (Moriyama et al. 1998a). Expression of c-Met was found in 67-100%, and HGF/SF in 53-72% of GBMs; co-expression of both c-Met and HGF/SF was found in 40-86% of GBMs, suggesting the presence of an autocrine loop (Nabeshima et al. 1997, Moriyama et al. 1998b, Koochekpoor et al. 1997). Amplification of the c-Met gene has also been reported in a small fraction of high-grade astrocytomas.

Expression of VEGF, an angiogenic factor, was found in 37% grade II astrocytomas, in 67% grade III astrocytomas, and in 64% GBMs (Oehring et al. 1999). VEGF expression is associated with shorter survival. VEGF expression was induced by many growth factors in glioma cells, including EGF, PDGF, and HGF (Maity et al. 2000, Moriyama et al. 1998c).

Ras is a member of the small GTP-binding protein family; there are several Ras proteins in mammals (H-Ras, N-Ras, and K-Ras). In its GDP-bound state, Ras is inactive, while binding of GTP activates Ras and its GTPase activity (reviewed in Khosravi-Far et al. 1998, Marshall 1999). Ras is regulated by GDP-exchange factors (GEFs) (such as Sos), which facilitates the transition from the GDP-bound to the GTP-bound form, and by GTPase-activating proteins (GAPs) (such as RasGAPs, 4 isoforms known in mammals: p120-GAP, GAP1, GAP1-P4BP, and neurofibromin) which increase the GTPase catalytic activity, thus facilitating the return to a GDP-bound, inactive state. Ras is a key molecule in the activation of the MAPK pathway downstream of RTKs. Other targets activated by Ras include the JNK pathway (Minden et al. 1994), Rac and Rho GTPases, and PI 3-kinase. Activating mutations of Ras, resulting in decreased GTPase activity, are found in approximately 30% of all human cancers. However, such mutations were not detected in astrocytomas. Other mutations in the Ras pathway are activating mutations of GEFs or inactivating mutations of GAPs, both resulting in increased Ras activity. Although Ras is not mutated in astrocytomas, the overexpression and/or constitutive activation (through mutations or autocrine loop) of several RTKs is likely to result in increased Ras activation. Indeed, activated Ras (GTP-bound) levels were elevated in GBM specimens when compared to normal brain tissues (Guha et al. 1997, Feldkamp et al. 1999). Astrocytoma cell lines exhibit an increased amount of active Ras-GTP, similar to that observed in v-H-ras transformed fibroblasts, when compared to non-transformed NIH 3T3 cells (Guha et al. 1997). Ras activation correlated with the stimulation of RTKs such as EGFR and PDGFR (Guha et al. 1997). Thus, being activated downstream of many cellular receptors, Ras constitutes a target of choice for therapeutic purposes. Holland et al. (2000b) recently reported a mouse model in which expression of activated forms of K-Ras and PKB/Akt in neural progenitors (under the control of the Nestin promoter) gave rise to nearly 25% of GBMs in 9-week old mice. Expression of only one activated gene had no effect. These results underline the importance of these pathways in gliomagenesis.

PTEN (phosphatase and tensin homologue deleted from chromosome 10) was identified as the putative tumor suppressor frequently mutated on chromosome 10; loss of part or all of chromosome 10 is the most common genetic event in GBMs (reviewed in Besson et al. 1999). Mutations/deletions of the PTEN gene were reported in 27-44% of

GBMs (Wang et al. 1997, Lui et al. 1997, Bostrom et al. 1998). In another study, PTEN mutations were found to be restricted to primary (de novo) GBMs (32%), and were rarely found in secondary (arising from pre-existing low grade tumor) GBMs (4%) (Tohma et al. 1998). PTEN mutations are found only in high-grade astrocytomas (Wang et al. 1997, Lui et al. 1997, Bostrom et al. 1998, Tohma et al. 1998). PTEN is emerging as a critical regulator of cell growth, cell cycle progression, adhesion, migration, and apoptosis. PTEN is a dual-specificity protein phosphatase and a phosphoinositide 3-phosphatase. Therefore, PTEN can dephosphorylate both proteins and the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is the product of the reaction catalyzed by PI 3-kinase. PTEN was shown to directly dephosphorylate FAK on tyrosine residues, preventing p130-Cas recruitment and phosphorylation; this was associated with a decrease in adhesion and spreading (Tamura et al. 1998, 1999). PTEN could also dephosphorylate She directly, thus preventing subsequent association of She with Grb2 and Sos and activation of the Ras/Raf/MEK/ERK pathway following EGF stimulation (Gu et al. 1998, 1999). Recently, PTEN was shown to inhibit EGF signaling by preventing the recruitment of the docking protein Gabl to the EGF receptor, which is dependent on PIP_3 production by PI 3-kinase, and inhibiting the downstream activation of the ERK and JNK pathways (Rodrigues et al. 2000). By regulating PIP_3 levels, PTEN can negatively regulate all the pathways activated downstream of PI 3-kinase. For example, regulation of PIP_3 levels by PTEN is critical for the activation of PKB/Akt, which plays critical roles in the regulation of cell survival and proliferation. It is of interest that the activity of the three isoforms of PKB/Akt is elevated in glioblastoma cells lacking a functional PTEN, thus conferring on these cells a growth and survival advantage (Haas-Hogan et al. 1998, Morimoto et al. 2000). By decreasing the availability of PIP_3 for binding to their PH domains, PTEN was also shown to suppress the activation of PLC *y* and ILK (Morimoto et al. 2000). Thus, increased PIP_3 levels due to PTEN inactivation in gliomas may lead to increased activation of PLC y, which in turn may lead to PKC hyperactivation.

There is substantial evidence to suggest a critical role for PKC in the malignant phenotype of astrocytomas. PKC may constitute a relevant target for astrocytoma therapy since it is at the convergence of many signaling pathways. In astrocytomas, PKC has

been involved in the regulation of proliferation, cell cycle progression, invasiveness, apoptosis, and angiogenesis (reviewed in Baltuch et al. 1995, Bredel and Pollack 1997). Although PKC is not mutated nor amplified, high expression levels of several isoforms have been reported in astrocytoma cell lines, including PKC a, e, and £ (Mishra-Press et al. 1992, Xiao et al. 1994, Sharif and Sharif 1999). The specific involvement of PKC in the regulation of glioma cell proliferation and migration will be described in further detail below.

A number of signaling pathways are over-activated in gliomas, mainly due to the amplification or the presence of activating mutations in the genes encoding growth factor receptors (Fig. 4). Several of these pathways are also activated through the existence of autocrine or paracrine loops, in which either the receptor expressing cell or the neighboring cell, respectively, express the cognate ligand, thus permanently stimulating the receptor (Tang et al. 1997a). The transformed phenotype of astrocytoma cells is a complex interplay of several factors: the conventional cell cycle checkpoints are abrogated due to the inactivation of the key players of cell cycle control (Fig. 4), and mitogenic signals initiated by mutated, overactive growth factor receptors drive cells into an endless proliferative cycle. Also, activation of cell surface receptors leads to a cascade of signaling events that modulate invasion, migration, and angiogenesis.

1.4. Regulation of Glioma Proliferation and Cell Cycle Progression.

1.4.1. What drives gliomas to proliferate: an overview.

The activity and regulation of a number of mitogenic signaling pathways is aberrant in astrocytomas, and this is thought to play a crucial role in the development of these tumors (reviewed in Besson and Yong 2001). The cascade of events leading to the formation and the progression from low-grade to high-grade astrocytomas is well characterized. These events include activating mutations, amplification, and overexpression of various growth factor receptors (e.g. EGFR, PDGFR, c-Met, c-Kit), signaling intermediates (e.g. Ras and PKC), and cell cycle regulatory molecules (e.g. Mdm2, CDK4, and CDK6), which positively regulate proliferation and cell cycle progression (Fig.4). Inactivating mutations and deletions of signaling and cell cycle regulatory molecules that negatively regulate proliferation and cell cycle progression (e.g.

p53, p16/INK4a, p14/ARF, p15/INK4b, Rb, and PTEN) also participate actively in the development of the transformed phenotype. Several mitogenic pathways are also stimulated via an autocrine loop, with astrocytoma cells expressing both the receptors and the respective cognate ligand. Due to the multitude of factors involved in astrocytoma pathogenesis, attempts to target a single pathway for therapy have not given satisfactory results. The simultaneous targeting of several pathways or the targeting of signaling intermediates, such as Ras or PKC, situated downstream of many growth factor receptor signaling pathways may show more efficacy in astrocytoma therapy. Thus, it appears that in gliomas, abnormalities in proliferative signaling pathways, in conjunction with the inactivation of cell cycle control pathways, initiate and maintain astrocytoma cells in a permanent proliferative state, a crucial step in tumor formation.

1.4.2. Protein kinase C in the regulation of glioma proliferation and cell cycle progression.

There is substantial evidence to indicate that the PKC pathway is hyperactivated in gliomas, due to the constitutive activation of several pathways upstream of PKC and the inactivation of negative regulators such as PTEN, and that PKC plays an important role in the regulation of glioma cell proliferation (reviewed in Baltuch et al. 1995, Bredel and Pollack 1997). Although there is increasing evidence to indicate a major role for PKC in the regulation of the cell cycle machinery in many cell systems (reviewed in Livneh and Fishmanl997, Fishman et al. 1998, Black 2000), this aspect of PKC function in gliomas has not been thoroughly investigated.

PKC activity was markedly increased in astrocytoma cell lines when compared to non-transformed astrocytes and directly correlated with their growth rate (Couldwell et al. 1991). PKC activity was also elevated in astrocytoma specimens (Couldwell et al. 1992). Stimulation of PKC using phorbol esters resulted in dramatically increased proliferation and S-phase progression, as measured by ^{3}H-thymidine incorporation (Couldwell et al. 1991, 1992, Pollack et al. 1990a, 1990b, Baltuch and Yong 1996). On the other hand, downregulation of PKC with the phorbol ester PMA resulted in a decreased growth rate (Couldwell et al. 1990, 1991). Tamoxifen, at PKC inhibitory concentration, had a dramatic effect on glioma cell proliferation, and prevented PDGF or

serum-induced DNA synthesis (Pollack et al. 1990a). Similarly, the mitogenic effect of serum addition or stimulation with EGF, PDGF, or FGF on established glioma cell lines or low passage glioma cells was completely abrogated by PKC inhibition with various inhibitors (Pollack et al. 1990b, Couldwell et al. 1992, Baltuch et al. 1993a, Baltuch and Yong 1996). These results suggest that mitogenic signaling from tyrosine kinase receptors in astrocytoma cells is mediated by a PKC-dependent pathway. Various inhibitors of PKC resulted in a marked decrease in proliferation of glioma cell lines and low passage glioma cells *in vitro* (Pollack et al. 1990a, 1990b, 1996, Couldwell et al. 1991, 1992, Baltuch et al. 1993a, Baltuch and Yong 1996), or of tumor xenografts in mice (Pollack et al. 1996).

In a few studies, the impact of PKC inhibitors on cell cycle progression of glioma cells was investigated. CGP 41251 inhibited proliferation of 9 glioma lines; this was associated with a decrease in S-phase and accumulation in the G2/M phase, and with induction of apoptosis (Begemann et al. 1996). CGP 41251 also inhibited tumor growth of U87-MG and U373-MG implanted in the flank of nude mice (Begemann et al. 1996). The growth inhibition and cell cycle arrest caused by CGP 41251 and Ro 31-8220 (another staurosporine derivative) was later associated with decrease of CDK2 and CDK1/Cdc2 associated kinase activities and decreased CDK1/Cdc2 expression (Begemann et al. 1998a, 1998b); however, it appeared that these inhibitors could directly inhibit CDK2 and CDK1/Cdc2 associated kinase activities, therefore questioning the involvement of PKC in the growth inhibition observed using these inhibitors (Begemann et al. 1998a, 1998b). Nonetheless, the inhibitor Calphostin C, which binds to the regulatory domain of PKC (but did not inhibit directly CDK1/Cdc2 activity), had a similar effect on growth and CDK1/Cdc2 kinase activity, indicating a role for PKC in cell cycle regulation and proliferation of glioma cells (Begemann et al. 1998b).

Several studies have focused on characterizing the functions of specific PKC isoforms in glioma cell proliferation. For example, overexpression of PKC ***y*** increased growth rate, colony forming efficiency in soft agar, and mitogenic response to EGF and bFGF in glioma cells, while overexpression of PKC 6 had the opposite effects (Mishima et al. 1994). PKC ***r*** was recently found to mediate the PMA-induced proliferation in U1242 and U251 glioma cells (Hussaini et al. 2000). In another study, substance-P

stimulation of glioma cells induced ERK1/2 activation and c-Fos expression; PKC inhibitors inhibited both responses, and substance-P specifically induced PKC e translocation (Luo et al. 1997). Overexpression of a dominant-negative form of PKC e inhibited the proliferation of U373MG cells in vitro (Sharif et al. 2001). In 123-1N1 glioma cells, DNA synthesis induced by carbachol stimulation of the muscarinic receptor required PKC £ activation. The latter could be prevented by PI 3-kinase and phospholipase D inhibition (Guizzetti and Costa 2000). These results indicate that PKC plays a role downstream of G-protein coupled receptor-induced proliferation in gliomas.

Targeting of a specific PKC isoform has been achieved using antisense strategies. U87 cells overexpressing an antisense for PKC a exhibited reduced proliferation and serum-dependent growth (Ahmad et al. 1994), indicating a specific role for PKC a in regulating proliferation and mediating growth factor-induced mitogenic signals. Moreover, these cells failed to induce tumor formation in nude mice (Ahmad et al. 1994, Dean et al. 1996). Systemic administration of antisense oligonucleotide for PKC a successfully inhibited U87 tumor growth in the flank or in the brain of nude mice, doubling the median survival time, with 40% long-term survivors (Yazaki et al. 1996). Similarly, a ribozyme specific for PKC a inhibited glioma cell growth *in vitro* and injection of the ribozyme inhibited growth of pre-established tumors in the flank of rats (Sioud and Sorensen 1998). The PKC a ribozyme-induced growth inhibition was later associated with induction of apoptosis, possibly due to a decrease in BCl-XL expression in ribozyme-transfected cells (Leirdal and Sioud 1999). Other studies have associated the growth inhibitory effect of PKC a depletion, using antisense oligonucleotides, with induction of apoptosis (Dooley et al. 1998). Shen et al. (1999) found that PKC *a* oligonucleotide-induced apoptosis was due to p53-dependent induction of insulin-like growth factor-binding protein-3 (a protein that sequesters IGF, decreasing its availability, often associated with induction of apoptosis). Induction of apoptosis could be prevented by IGF treatment, while no change in BCl-XL, Bax, or p21/Wafl/Cipl was detected. Part of the antiproliferative effect of PKC inhibition *in vivo* may be due to inhibition of angiogenesis, as both PKC a and PKC £ were found to mediate PMA-induced VEGF mRNA upregulation in U373 glioma cells (Shih et al. 1999). Interestingly, the same PKC

isoforms were required for VEGF-induced proliferation of vascular endothelial cells (Wellner et al. 1999), suggesting that PKC may also play an important role in the angiogenic response. PKC activation in glioma cells has been associated with the induction of expression of a variety of proteins that positively regulate cell proliferation, including the proto-oncogenes c-Fos, c-Myc, and c-Sis, and bFGF (Blackshear et al. 1987, Murphy et al. 1988, Press et al.1989).

PKC has been involved in the downregulation of EGFR signaling through phosphorylation of the juxtamembrane domain of the receptor (T654), resulting in decreased affinity of the receptor for its ligand and inhibition of its tyrosine kinase activity (Lund et al. 1990, Jimenez-deAsua and Goin 1992, Morrison et al. 1996). This suggests that PKC could have an anti-proliferative role by inhibiting EGF signaling. However, more recent evidence suggests that although activation of PKC potently inhibits subsequent ligand-induced EGFR signaling, in cells that are pre-treated with EGF, PKC-mediated phosphorylation of the receptor stabilizes ligand-receptor interactions, and results in intensification of EGFR signaling (Gulliford et al. 1999). Another report indicated that PKC phosphorylation of EGFR decreases c-Cbl-induced ubiquitination of the receptor and its subsequent degradation through the endosome/lysosome pathway, and targets EGFR for recycling back to the cell surface (Bao et al. 2000). Therefore, in the astrocytoma context, where an autocrine EGF/EGFR loop exists and the receptor is permanently stimulated, PKC may synergize with EGFR in the mitogenic response by intensifying EGFR signaling and facilitating the recycling of the receptor.

Given the evidence that suggests a role for PKC in astrocytoma pathogenesis, clinical trials targeting PKC have been initiated. High-dose Tamoxifen, at PKC inhibitory concentration, was used in patients with recurrent Grade III and IV astrocytomas. There was a 25% response and 19% of patients exhibited stabilization of the disease with minimal side effects (Couldwell et al. 1996); this indicates that PKC inhibition produces a response in a group of tumors. Recently, high-dose tamoxifen was used in combination with carboplatin. The median survival was 13 months, while patients treated by surgery, radiotherapy, and carboplatin had a median survival of 9 months (Mastronardi et al. 1998). In contrast, a phase II trial with antisense oligonucleotide for PKC a (ISIS 3521)

in patients with recurrent high-grade glioma did not produce any response (Grossman et al. 1999).

There is considerable evidence to indicate a role for PKC in glioma proliferation. However, the mechanism involved has not been investigated. Moreover, how PKC drives cell proliferation with regards to the cell cycle machinery remains poorly understood.

1.5. Regulation of Glioma Migration.

1.5.1. Overview.

The ability of GBM cells to diffusely invade the brain parenchyma and migrate great distances from the primary tumor mass prevents complete resection of the tumor by conventional surgical methods and is thought to be a major cause of mortality for glioma patients (Holland 2000c). The invasive behavior of glioma cells may reflect the re-acquisition (by de-differentiation) of the migratory phenotype of glial progenitor cells or embryonic stem cells during development of the central nervous system. Glioma cells preferentially migrate along white matter tracks and the basement membrane of blood vessels (Laws et al. 1993).

The propensity of glioma cells to migrate along specific routes is probably a consequence of their ability to interact with extracellular matrix (ECM) proteins that are present along these paths, through a specific set of integrins and cell adhesion molecules. It also possibly reflects their ability to degrade these ECM proteins and other migration inhibitory proteins, through the expression of specific proteases, such as matrix metalloproteinases (MMPs) (Belien et al. 1999) and urokinase-type plasminogen activator (uPA) and uPA-receptor (uPAR).

The acquisition of a migratory and invasive phenotype likely results from the various genetic alterations that affect glioma cells during oncogenesis (see section 1.2.). The conjunction of activation of growth factor receptors (through amplification or activating mutations) and their downstream signaling cascades, and the inactivation of inhibitory pathways, such as PTEN, enables the cells to adopt a migratory phenotype. This may occur through different mechanisms. Indirectly, through activation/inhibition of transcription and translation of specific genes, it may lead to aberrant expression of proteases, integrins, and other proteins that will promote migration or to decreased

expression of inhibitory proteins. Directly, cross talk between growth factor receptors and integrins may lead to activation of the latter and of their downstream signaling pathways, thus inducing cell migration; the absence of inhibition of the signaling cascades downstream of integrins by PTEN may also contribute to enhancement of the migratory response. Thus, direct or indirect targeting of the pathways involved in the regulation of migration and invasion during oncogenesis leads to the acquisition of a migratory and invasive phenotype in gliomas.

Stimulation of various growth factor receptor pathways, such as FGF and EGF, potently induced glioma cell migration and invasion *in vitro* (Lund-Johansen et al. 1992, Berens et al. 1996). Expression of urokinase-type plasminogen activator (uPA) and uPA-receptor (uPAR) is induced by activation of the Ras/ERK, PKC/ERK, and Rac/JNK pathways, which are commonly hyper-activated in tumors (Aguirre Ghiso et al. 1999); uPA/uPAR are often overexpressed in a variety of tumors, including gliomas (Yamamoto et al. 1994).

1.5.2. General aspects of the regulation of cell adhesion and migration.

The tight control of cell adhesion and motility is crucial for a wide range of physiological and pathological processes, such as embryogenesis, inflammation, angiogenesis, wound healing, and tumor metastasis.

Migration is a complex and coordinated process that includes several steps: cell polarization, membrane extension, formation of cell-ECM attachment, contractile force and traction, and release of attachment (reviewed in Lauffenburger and Horwitz 1996, Horwitz and Parsons 1999). Cells emit a membrane protrusion driven by a network of actin filaments. At the tip of the protrusion forms an adhesive complex, or focal adhesion. Focal adhesions bundle the actin filaments and regroup a large number of proteins, including integrins. Focal adhesions also serve as points of traction over which the body of the cell moves. Traction is provided by contraction of actin and myosin. The small GTPases Rho, Rac, and Cdc42 are important regulators of migration due to their role in actin cytoskeleton assembly and regulation of myosin light chain activity, by regulating myosin light chain kinase (MLCK) and myosin phosphatase activities. Integrins mediate the attachment of the cell to the extracellular matrix (ECM) components, and the strength

of attachment to the ECM provides the tension required by the actin cytoskeleton for cell movement (stronger at the front of the cell, weaker at the rear). Regulation of focal adhesion formation and turnover is critical for the reorganization of focal contacts during migration. While there is formation of new focal adhesions at the front of the cell, the rear is where adhesion is released (by proteolysis or membrane 'ripping'). For example cells lacking Src family kinases or FAK have a migratory defect due to inhibition of focal adhesion turnover, while their formation is not affected (Klinghoffer et al.1999, Sieg et al. 1999). Indeed, FAK deficient cells fail to transiently inhibit Rho activity, which is required for focal adhesion turnover (Ren et al. 2000).

Integrins are heterodimeric cell surface receptors that mediate cell-cell and cell-ECM interactions, and are involved in the regulation of cell growth, migration, survival, and metastasis (Reviewed in Hynes 1992, Hughes and Pfaff 1998a, Aplin et al. 1999, Giancotti and Ruoslahti 1999). Eight integrin (3 subunits and 17 a subunits have been identified to date (Plow et al. 2000), and these can form over 20 distinct heterodimers. Integrin affinity and avidity can be modulated by intracellular signaling cascades, a process referred to as 'inside-out' signaling, leading to changes in adhesion and motility. Conversely, binding of integrins to ECM proteins elicits signals that are transduced into the cell, referred to as 'outside-in' signaling, to modulate integrin affinity and elicit various cellular responses, including spreading, migration, survival, apoptosis, and proliferation. The cytoplasmic tail of both integrin chains plays a crucial role in signal transduction and focal adhesion assembly. Integrins are central components of focal adhesions, in which they associate with cytoskeleton associated proteins such as vinculin, talin, and paxillin, and signaling molecules such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (Hemler 1998, Giancotti and Ruoslahti 1999). A number of intracellular signaling pathways have been involved in the regulation of integrin adhesive functions, including PI 3-kinase, Src, and the small GTP-binding proteins R-Ras, H-Ras, and Rho (Kolanus and Seed 1997, Hughes and Pfaff 1998b).

Among the proteins implicated in inside-out signaling, PKC has been found in many instances to play a crucial role in modulating integrin-mediated cell adhesion, spreading, and migration. However, the mechanism of action of PKC in these events remains elusive. The importance of PKC in integrin-mediated events has been described in a

number of reports. PKC activity was shown to be required for adhesion, spreading, migration, and focal adhesion and actin stress fiber assembly, on various ECM substrates (Woods and Couchman 1992, Vuori and Ruoslahti 1993, Lewis et al. 1996, Disatnik and Rando 1999). In addition to its role in focal adhesion formation, PKC activation induces the translocation of FAK (Vuori and Ruoslahti 1993, Lewis et al. 1996, DeFilippi et al. 1997, Disatnik and Rando 1999) and PYK2 (Litvak et al. 2000) to focal adhesions and their tyrosine phosphorylation in various cell systems.

The ERK pathway has also been implicated downstream of integrins and was found to be required for integrin-mediated motility. Activation of the ERK pathway following different stimuli at the cell surface was required for cell migration in various cell systems (Rigot et al. 1998, Klemke et al. 1997, Nguyen et al. 1999).

1.5.3. PKC in the regulation of glioma cell adhesion and migration.

Several lines of evidence indicate that activation of the PKC pathway regulates glioma cell migration and invasion, either through the regulation of integrin function and/or through the regulation of protease expression (Gomez et al. 1999). Much attention has concentrated on the role of PKC in glioma cell invasion through the induction of MMP expression (reviewed in Uhm et al. 1997, Yong et al. 1998). On the other hand, the possible function of PKC in the regulation of cell motility and integrin function has not been thoroughly investigated.

Inhibition of phospholipase C y, an activator of PKC, blocked glioma cell migration and invasion *in vitro* (Khoshyomm et al. 1999). Furthermore, various inhibitors of PKC could reduce glioma cell motility and invasion (Zhang et al. 1997), while PKC activation with phorbol esters promoted migration *in vitro* and PKC downregulation inhibited migration (Tysnes and Laerum 1993). The effect of PKC on glioma cell invasion was attributed to its role in the regulation of MMP-2 expression, which was required for glioma invasion *in vitro* (Uhm et al. 1996). More recently, PKC activation was found to upregulate MMP-2 and MT1-MMP expression and correlated with glioma cell invasion; these effects could be prevented by ERK or ornithine decarboxylase inhibition (Da Rocha et al. 2000). Also, PKC induced invasion correlated with increased MMP-2, MMP-9, and MT1-MMP expression, and inhibition of tissue inhibitor of matrix metalloproteinase-1

(TEVIP-l) and TEV1P-2 secretion (Park et al. 2000). One study indicated a specific role for PKC a in glioma invasion, as overexpression of the protein in U87 cells was associated with increased invasion in vitro (Cho et al. 1999).

Several integrins have been implicated in the regulation of glioma cell migration and invasion. Blockade of integrin Pi function using various strategies could inhibit glioma cell migration and invasion (Paulus and Tonn 1994, Paulus et al. 1996, Giese et al. 1996, Rooprai et al. 1999). Blocking antibodies against several integrins either inhibited migration/invasion, in the case of oC3, CC7, a_v, and p3 integrins, or increased migration, in the case of as and Of, integrins (Paulus and Tonn 1994). In contrast, inhibition of integrin ct_v function increased glioma cell migration on tenascin (Giese et al. 1996, Treasurywala and Berens 1998). Other cell adhesion molecules also affect glioma cell migration. Neural cell adhesion molecule (NCAM) overexpression inhibited rat glioma cell invasion (Owens et al. 1998). Interestingly, it was found that cells establishing more gap junctions were less migratory, and that stimulation of migration with EGF was associated with decreased expression of the gap junction protein Connexin 43 (McDonough et al. 1999).

So far, studies on the roles of PKC in the regulation of glioma migration and invasion have mainly focused on the regulation of the MMP/TTMP system. The relationship between PKC and other critical aspects of adhesion and migration, such as regulation of integrin function, have not been extensively studied. Therefore the role played by PKC in these processes and the mechanism involved remains unclear, as is the identity of the PKC isoforms involved.

1.6. General Hypothesis.

There is substantial evidence indicating that PKC plays an important role in the pathogenesis of gliomas and that PKC regulates several phenotypes of glioma cells. However, the identity of the PKC isoforms involved in these processes remains unclear, and the mechanism by which PKC regulates glioma phenotypes is poorly understood. Therefore, there is a need for a better understanding of the functions of PKC in gliomas, and of the mechanisms involved. This knowledge will be useful to design better strategies to improve glioma therapeutics and the prognosis of the disease.

In this thesis, we test the hypothesis that **specific isoforms of Protein Kinase C regulate distinct phenotypes of glioma cells.**

This hypothesis will be tested in the following specific aims:

1. **To characterize the isoforms of PKC expressed in glioma cells, and to identify the PKC isoform(s) that regulates cell cycle progression.**
2. **To determine the mechanism of regulation of cell cycle progression by this specific PKC isoform(s).**
3. **To identify the PKC isoform(s) that promotes glioma cell migration and adhesion.**
4. **To determine the mechanism by which this specific PKC isoform(s) regulates glioma cell adhesion and migration.**
5. **To determine whether PKC is activated downstream of the Hepatocyte Growth Factor Receptor/c-Met to control glioma cell migration.**

CHAPTER TWO

Materials and Methods

2.1. Solutions and Reagents.

2.1.1. Solutions.

-Antibody dilution buffer: Phosphate buffered saline (PBS) 1x; 3% bovine serum albumin; 0.05% Tween 20; 0.08% sodium azide.

-BES-buffered solution (BBS) 2x: 50 mM AT,A^bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), pH 6.95; 280 mM NaCl; 1.5 mM Na_2P0_4, filtered.

-$CaCl_2$: 2.5 mM, filtered.

-Cyclin-CDK Immunoprecipitation buffer: 50 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 0.1% Tween-20; 10% glycerol; complemented with 1 mM DTT; 10 mM ^-glycerophosphate; 1 mM NaF; 0.1 mM sodium orthovanadate; 10 pg/ml leupeptin; 10 pg/ml aprotinin; 10 pg/ml pepstatin A; and 1 mM PMSF.

-Digitonin Buffer: 20 mM Tris, pH 7.5; 2 mM EGTA; 2 mM EDTA; 0.5 mg/ml digitonin; 10 mM NaF; 4 mM PMSF; 10 pg/ml leupeptin; 10 pg/ml aprotinin; 10 pg/ml pepstatin A; 10 mM sodium orthovanadate.

-Digitonin-Triton lysis buffer: digitonin buffer supplemented with 1% Triton-XlOO.

-Geneticin: 70 mg/ml (50 mg/ml active) G418 in HEPES 100 mM, pH 7.3; filtered.

-Hypotonic buffer: 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KC1, 0.5 mM DTT.

-Immunoprecipitation buffer: 50 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 0.1% Tween-20; 1% Nonidet P-40; 10% glycerol; complemented with 1 mM DTT; 10 mM ^-glycerophosphate; 1 mM NaF; 0.1 mM sodium orthovanadate; 10 Ug/ml leupeptin; 10 pg/ml aprotinin; 10 ug/ml pepstatin A; and 1 mM PMSF.

-Kinase buffer: 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 mM p-glycerophosphate, 1 mM NaF, 2.5 mM EGTA, 0.1 mM sodium orthovanadate.

-Lysis buffer: 50 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 0.1% Tween-20; 1% Nonidet P-40; 0.2% SDS; 10% glycerol; complemented with 1 mM DTT; 10 mM P-glycerophosphate; 1 mM NaF; 0.1 mM sodium orthovanadate; 10 Ug/ml leupeptin; 10 pg/ml aprotinin; 10 pg/ml pepstatin A; and 1 mM PMSF.

-Luria Broth (LB) medium: 10 g/l bacto-tryptone; 5 g/l yeast extract; 10 g/l NaCl; 200 pl/l NaOH 10N.

-Phosphate buffered saline (PBS): 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; adjusted to pH 7.3.

-Protein loading buffer 4x: 200 mM Tris-HCl, pH 7.8; 400 mM DTT; 8% SDS; 0.4% bromophenol blue; 40% glycerol.

-Tris-Acetate-EDTA buffer: 40 mM Tris-base; 1.15 ml/l glacial acetic acid; 2 mM EDTA, pH 8.

-Tris-Glycine electrophoresis buffer: 25 mM Tris; 250 mM glycine, pH 8.3; 0.1% SDS.

-Transfer Buffer: 39 mM glycine, pH 8.3; 48 mM Tris-base, 0.037% SDS, 20% methanol.

2.1.2. Antibodies.

Table 1: list of antibodies.

Antibody specificity	Species	Type of analysis	Dilution	Source
Anti-Mouse HRP	Sheep	WB	1/10000	Jackson ImmunoResearch Lab
Anti-Rabbit HRP	Goat	WB	1/10000	Jackson ImmunoResearch Lab
Anti-mouse IgG+IgM (H+L) HRP	Goat	WB	1/10000	Jackson ImmunoResearch Lab
Anti-mouse IgG+IgM (H+L)	Goat	IP	5pg/IP	Jackson ImmunoResearch Lab
Anti-Rabbit Cy3	Goat	IF	1/500	Jackson ImmunoResearch Lab
Anti-Mouse Cy3	Goat	IF	1/500	Jackson ImmunoResearch Lab
Anti-Rabbit A488	Goat	IF	1/500	Molecular Probes
Anti-Mouse A488	Goat	IF	1/500	Molecular Probes
CDK4 (C-22)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
CDK6 (C-21)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
CDK2 (M-2)	Rabbit	WB	1/1000	Santa Cruz Biotechnology

cdc25B (C-20)	Rabbil	WB	1/500	Santa Cruz Biotechnology
cyclin A (H-432)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
cyclin A (BF683-AC)	Mouse	IP	4 pg/IP	Santa Cruz Biotechnology
cyclin B1 (H-433)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
cyclin B1 (GNS-AC)	Mouse	IP	4pg/IP	Santa Cruz Biotechnology
cyclin D1 (H-295)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
cyclin D1 (HD11-AC)	Mouse	IP	4pg/IP	Santa Cruz Biotechnology
FAK (clone 77)	Mouse	WB IF	1/1000 1/200	Transduction Laboratories
Integrin a_2 (MAB1950Z)	Mouse	IF	1/100	Chemicon
Integrin as (MAB1956Z)	Mouse	IF	1/100	Chemicon
Integrin a_v (MAB1953Z)	Mouse	IF	1/100	Chemicon
Integrin pi (MAB1951Z)	Mouse	IF IP WB	1/100 5pg/IP 1/1000	Chemicon
Integrin pi (AB1952)	Rabbit	IF WB	1/100 1/500	Chemicon
Integrin p_5 (AB1926)	Rabbit	IF WB	1/100 1/1000	Chemicon
Integrin a_v (blocking, MAB2021Z)	Mouse	IF block	1/100 5pg/TP	Chemicon
Integrin a_vPs (blocking, MAB1961)	Mouse	block	5ug/IP	Chemicon
Integrin a_2Pi (blocking, MAB1998)	Mouse	block	5ug/IP	Chemicon
Integrin a_vP3 (blocking, MAB1976Z)	Mouse	block	5pg/IP	Chemicon
Integrin asPi (blocking, MAB1969)	Mouse	block	5ug/IP	Chemicon
p34cdc2	Rabbit	WB	1/1000	Calbiochem
p21/Wafl (clone 70)	Mouse	WB IP	1/1000 5ug/IP	Transduction Laboratories
p21 (C-19)	Rabbit	WB	1/1000	Santa Cruz Biotechnology

p27 (F-8)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
p42/44 MAPK (ERK)(#9102)	Rabbit	WB IF	1/1000 1/250	New England Biolabs
phospho-p42/44 MAPK (Thr202/Tyr204)(#9101)	Rabbit	WB IF	1/1000 1/250	New England Biolabs
PKC a (clone 3)	Mouse	WB IP	1/1000 5pg/IP	Transduction Laboratories
PKC a	Rabbit	WB	1/1000	Dr. N. Groome
PKC a (662-672)	Rabbit	WB IP	1/1000 5 Ug/IP	Calbiochem
PKCy	Rabbit	WB	1/1000	Dr. N. Groome
PKCe	Rabbit	WB	1/1000	Dr. N. Groome
PKC e (clone 21)	Mouse	WB IP	1/1000 5 Ug/IP	Transduction Laboratories
PKCC	Rabbit	WB	1/500	Dr. N. Groome
PKC£	Rabbit	WB	1/1000	Gibco-BRL
PKCS	Rabbit	WB	1/500	Dr. N. Groome
PKCp$_2$	Mouse	WB	1/100	Dr. N. Groome
PKC i	Rabbit	WB	1/1000	Santa Cruz Biotechnology
PKC p (D-20)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
PKC 0	Mouse	WB	1/100	Santa Cruz Biotechnology
PKCe	Mouse	WB IP	1/500 5ug/IP	Transduction Laboratories
PKCpj	Rabbit	WB	1/1000	Calbiochem
PKCri (C-15)	Rabbit	WB	1/1000	Santa Cruz Biotechnology
RACK1 (clone 20, IgM)	Mouse	WB IP IF	1/2000 5 ug/IP 1/200	Transduction Laboratories
4G10 (anti-phospho-tyrosine)	Mouse	WB	1/1000	UBI
vinculin (hVIN-1)	Mouse	WB IF	1/4000 1/1000	Sigma-Aldrich

Note: WB= western blot, IF= immunofluorescence, IP= immunoprecipitation

2.1.3. Reagents.

The cDNA for human PKC a was kindly provided by Dr. G. Finkenzeller (Institut fur Molekulare Medizin, Freiburg, Germany). The cDNAs for human PKC e and $_{P}$21/Wafl/Cipl were from ATCC (#80050 and #79927, respectively); the cDNA for human RACK1 was obtained from Dr. D. Chang (UCLA, Los Angeles, USA). Phorbol 12-myristate 13-acetate (PMA), thymeleatoxin, Bisindolylmaleimide I, Calphostin C, G66983, PD98005, U0126, poly-L-lysine, laminin, vitronectin, and fibronectin were from Calbiochem. GRGDSP peptides were from Gibco-BRL. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue (MTT) was from Sigma-Aldrich.

2.2. Tissue Culture.

The established human glioma cell lines used were U251N, U373, A172, U178, and U563, which are well characterized. They were kindly provided by V.P. Collins (Ludwig Institute for Cancer Research, Stockholm, Sweden) (Collins, 1982). Human fetal astrocytes were obtained from therapeutic abortions and prepared as described previously (Vecil et al. 2000). Cells were grown in minimum essential medium (MEM) containing 10% fetal bovine serum, 0.1 mM non-essential amino acids, 0.1% dextrose, 2 pg/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine. A11 medium constituents were from Gibco-BRL. Cells were cultured at 37°C, 5% $C0_{2}$, 100% humidity, and were split by trypsinization and dilution 3 times a week. A11 experiments shown in this thesis were performed on U25 IN cells, unless otherwise stated.

2.3. Transfection.

Stable transfections were performed using the calcium-phosphate method adapted from Chen and Okayama (1987) and Jordan et al. (1996). Exponentially growing cells were trypsinized, seeded at 20-30% confluency in 100 mm dishes, and incubated overnight in 10 ml of growth medium at 37°C, 5% $C0_{2}$. Before transfection, cells were equilibrated for 3 h at 35°C, 3% $C0_{2}$ in 5 ml of growth medium. Twenty ug of plasmid DNA was mixed gently with 0.25 ml of 250 mM $CaCl_{2}$ and 0.25 ml of BBS 2x, and the mixture was incubated for 20 min at room temperature. The calcium-phosphate-DNA

solution (0.5 ml) was added dropwise to the plates, and incubated for 24 h at 35°C, 3% **CO2.** Medium was removed and cells rinsed once with PBS, refed, and incubated for an additional 24 h at 37°C, 5% $C0_2$. Cells were split (at least 1/10) and selected for stable transfection with growth medium containing 250 pg/ml of active G418, until the formation of isolated clones (approximately 2 weeks). Following selection, clones were isolated with cloning rings (Bellco Glass Inc.). Stably transfected cells were kept at all times in the presence of 250 pg/ml of active G418 (Calbiochem).

Transient transfections were performed using the Fugene-6 reagent (Roche) according to the manufacturer's instructions, for 48 h before the experiment. The plasmid pTracer (Invitrogen), which encodes the green fluorescent protein, was used to monitor transient transfection efficiency; an efficiency of 50-60% was commonly obtained.

2.4. Expression Vectors and Antisense Constructs.

2.4.1. Sense PKC a and 8 expression constructs.

The cDNAs for PKC a and e were inserted into the pBKRSV vector (Invitrogen), in which the ***lac*** promoter has been deleted, as recommended by the manufacturer, to allow high expression levels under the control of the RSV promoter. As20 and As27, and Es1 and EslO are clones stably overexpressing PKC a or PKC e, respectively.

2.4.2. Antisense PKC a, p21/Wafl/Cipl, PKC s, and RACK1.

Full-length cDNAs for human PKC a, p21/Wafl/Cipl, and PKC e were subcloned in antisense orientation into a pREP9 episomal vector (Invitrogen). The full length RACK1 cDNA was inserted in antisense orientation into the pcDNA3.1 vector. The antisense construct or control vector (pREP9 or pcDNA3.1) was transfected into U251N cells, using the calcium-phosphate method for stable transfectants, or using the Fugene 6 reagent (Roche) for transient transfections. Cells transfected with the empty pREP9 or pcDNA3.1 vector were used as control. Clones were screened by Western blotting for decreased expression of the protein of interest. Stably transfected cells were maintained permanently under selection pressure (400 pg/ml G418).

2.5. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR).

Total RNA was extracted using TRIZOL reagent (Gibco-BRL) according to the manufacturer's instructions. Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One pg of RNA was used as a template for each RT-PCR reaction. The reverse transcription step was carried at 50°C for 15 min using AMV-RT (Gibco-BRL). PCR (50 cycles, to maximize detection) was executed as follows: Denaturation 45 sec, annealing (63°C for PKC **y,** 62°C for PKC a, 58°C for PKC Pi and P2) for 60 sec, and elongation 72°C for 90 sec. PCR products were analyzed by agarose (2%) gel electrophoresis and ethidium bromide staining. Primer sequences for PKC a, Pi, p_{2}, and **y** were as follows:

Table 2: PCR primers for PKC isoforms.

PKC isoform	Primer	PCR product size
PKC a, sense	5' -GGCTGAGGTTGCTGATGAAA	295 bp
PKC a, antisense	5' -CGAAACTCCAAAGGAAAGGG	
PKC pi, sense	5' -GGAGAAACTTGAACGCAAAG	169 bp
PKC Pi, antisense	5' -GTATAAGAGAAGCCAGCAAA	
PKC p_{2}, sense	5' -GGAGAAACTTGAACGCAAAG	136 bp
PKC P2, antisense	5' -ATTCCTGATGACTTCCTGGT	
PKC **y,** sense	5' -CCCACAGCAGATGAGATCCA	391 bp
PKC **y,** antisense	5' -CAGTTGTCAGCATCAGCCAC	

2.6. Western Blotting.

Cells were lysed either in Digitonin-Triton lysis buffer or in lysis buffer, and scraped from the culture dish with a cell scraper. Lysates were homogenized by pipeting several times in a 1 ml syringe with a 26 gauge needle and incubated on ice for 10 min. Lysates were cleared by centrifugation for 5 min at 10,000 rpm, and supernatants were kept for analyses. Protein concentration was determined using the Bio-Rad protein assay (Bradford method). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) for 2 h at 250 mA. Membranes were blocked in PBS containing 0.5% Tween-20 and 10% milk for 1 h.

Membranes were incubated with primary antibodies overnight at 4°C, and with secondary antibodies for 1 h at room temperature. All antibodies were diluted in PBS containing 0.1% Tween-20 and 3% milk, except for the 4G10 and anti-ERK antibodies, which were diluted in PBS containing 0.5% Tween-20 and 5% bovine serum albumin. ECL (Amersham) was used for immunodetection.

2.7. **Cell Cycle Analysis by Flow Cytometry.**

Cells were trypsinized, rinsed once in PBS, and resuspended in 300 pi of PBS. Cells were fixed by the addition of 1 ml of 100% ethanol and mixed gently. Samples were kept at 4°C. Fixed cells were pelleted and resuspended in PBS containing 50 pg/ml RNase A and 50 pg/ml propidium iodide and incubated for 30 min at 37°C. Cell cycle analysis was performed on a FACScan (Becton-Dickinson) by determining the DNA content of each cell.

2.8. Immunofluorescence.

2.8.1. DNA staining.

Glioma cells were seeded in culture dishes containing glass coverslips and allowed to grow for at least 24 h. Cells were fixed in 70% ethanol, rinsed three times in PBS and incubated for 5 min in PBS containing 1 pg/ml of propidium iodide. After three rinses in PBS, coverslips were mounted onto glass slides. Observation was done on a Leica DMRBE microscope, and images were acquired using a Spot charge-coupled-device (CCD) camera.

2.8.2. **Staining of cellular proteins.**

Cells were grown on glass coverslips for 24 h before treatment. Following the appropriate treatment, cells were rinsed in PBS and fixed in 1% paraformaldehyde at 37°C for 20 min. Coverslips were stored in PBS at 4°C until stained. Cells were permeabilized for 3 min in 0.2% Triton-XlOO, rinsed 3 times in PBS, and incubated for 1 h with primary antibodies diluted in antibody dilution buffer at 37°C (in the case of ERK and phospho-ERK-specific antibodies, incubation was overnight at 4°C). The coverslips were rinsed 3 times in PBS. Incubation with secondary antibodies (at a 1/500 dilution in

antibody dilution buffer) was for 30 min at 37°C. The coverslips were rinsed 3 times in PBS and mounted on glass slides using gelvatol. Images were obtained on a Leica DMRBE microscope using a Spot CCD camera, or on an Olympus Fluoview 300 confocal laser-scanning microscope.

2.9. Subcellular fractionation.

2.9.1. Fractionation into Digitonin-soluble and particulate fractions.

Cells were partitioned into soluble (cytosolic) and particulate fractions using a method adapted from Frey et al. (1997). Briefly, cells were lysed in Digitonin lysis buffer and homogenized by pipeting several times in a 1 ml syringe with a 20'/2-gauge needle. Digitonin-soluble (cytosolic) and insoluble (particulate) fractions were separated by ultracentrifugation at lOOOOOg (29,000 rpm) for 45 min at 4°C. The supernatant formed the cytosolic fraction. The pellet was resuspended in Digitonin-Triton lysis buffer, incubated on ice for 30 min and cleared by centrifugation for 10 min at 10,000g at 4°C. Proteins were quantified using the Bio-Rad protein assay. Samples were subjected to SDS-PAGE; 30 |ig of proteins were loaded per well.

2.9.2. Fractionation into cytoplasmic and nuclear fractions.

Cells were trypsinized, pelleted, and rinsed once in hypotonic buffer. Cells were resuspended in hypotonic buffer containing 0.1% NP-40 and incubated for 10 min at 4°C, with occasional mixing and pipeting (with 1 ml tip). Lysis of the plasma membrane and integrity of the nuclei were checked under a phase-contrast microscope. Nuclei were pelleted by centrifugation at 2,000 rpm for 5 min, rinsed once in hypotonic buffer, and lysed in lysis buffer. The nuclear fraction was sonicated for 10 sec and debris were cleared by centrifugation for 5 min at 10,000 rpm. Protein concentration was determined with the Bio-Rad protein assay.

2.10. Evaluation of the growth rate of PKC a antisense transfected cells.

Twenty five thousand cells per well were plated in 24-well plates in 1 ml of medium. Four days later, cells were trypsinized and the entire content of each well (in 500 pi of PBS) was then transferred to vials (each containing 9.5 ml PBS) and counted using a Z2

Coulter Counter (3 pm gate). The resultant values obtained represented the total number of cells per well. Results were analyzed using 1-way ANOVA with Bonferroni multiple comparisons.

2.11. Multiprobe RNase Protection Assay.

In vitro transcription kit for probe synthesis, RPA kit, and probe sets hCC-1, hCYC-1, and hCC-2 were from Pharmingen. Cellular RNA was extracted from cells at the appropriate time, using the TRIZOL reagent (Gibco-BRL), according to the manufacturer's instructions, and quantified by spectrophotometry (OD 260 nm). 5 pg of RNA was used per RPA reaction. The experimental procedure was done as described by the manufacturer, with the exception that [a-^{33}P] UTP (10 mCi/ml) was used in place of [oc-^{32}P] UTP.

2.12. Immunoprecipitations.

2.12.1. Cyclin-CDK complexes Immunoprecipitation.

Cyclin A, Cyclin B1, and Cyclin Dl were immunoprecipitated using monoclonal antibodies conjugated to agarose beads (BF683-AC, GNS-AC, and HD11-AC, respectively) (Santa-Cruz Biotechnology) that do not interfere with kinase activity. p21 immunoprecipitations were carried out using agarose conjugated polyclonal anti-p21 (C-19) antibodies, from the same source. Immunoprecipitations and kinase assays were performed as described by Matsushime et al (1994). Semi-confluent U251N glioma cells were scrapped at the appropriate time into 1 ml of cyclin-CDK IP buffer. The cells were lysed on ice for 30 min with vortexing every 5 min. Lysates were clarified by centrifugation at 10,000g for 10 min at 4°C. Protein concentration was determined using the Bio-Rad protein assay. 250 or 500 pg of proteins (volumes were adjusted to 500 pi with cyclin-CDK IP buffer) were used for each immunoprecipitation. Because of the large amount of proteins required for each time point, and for a better consistency in the results, immunoprecipitations were carried out in a sequential manner, each cyclin being sequentially immunoprecipitated from the same cellular extract. Protein extracts were incubated with 10 pg of the indicated primary antibodies for 1 h at 4°C. The immunoprecipitated complexes were then washed three times with 1 ml of cyclin-CDK

IP buffer, and once with kinase buffer. Half of the samples were submitted to Western blotting, the other half was subjected to kinase assay. For kinase assays, control immunoprecipitation with protein-G coated agarose beads only were carried out and processed similarly to samples.

2.12.2. Co-immunoprecipitations.

Cell lysates were prepared as described above. For each immunoprecipitation, 5 pg of the appropriate antibody, 300 pg of proteins, 30 pi of protein A/G agarose beads (Santa Cruz Biotechnology) were incubated for 3 h at 4°C. For control immunoprecipitations, the primary antibody was substituted with a goat anti mouse IgG + IgM (H+L). In the case of mouse IgM anti RACK1 antibodies, 5 pg of goat anti mouse IgG + IgM (H+L) were added to allow binding of the IgM to the beads. Immunoprecipitates were rinsed 3 times in immunoprecipitation buffer. The immunoprecipitates were subjected to Western blotting as described above.

2.13. Cyclin-CDK complex kinase assay.

The immunoprecipitated complexes were resuspended in 40 pi of kinase buffer. Five pCi of y-^{32}ATP (NEN, 3000 Ci/mmole) and 2 pg of histone HI or 1 pg of Rb fragment (46 kDa)(769, Santa Cruz Biotechnology), as substrate, were added. The reaction was incubated for 30 min at 30°C. Samples were boiled in SDS sample buffer, subjected to SDS-PAGE, and the dried slab of gels was autoradiographed on Kodak Blue XB-1 film.

2.14. Migration assay.

Cells were seeded at 80% confluency (approximately 2x10^{6} cells) in 60 mm dishes and grown for an additional 24 h. A linear scratch was done with a rubber policeman across the diameter of the plate, the plate rinsed with PBS and refed with growth medium supplemented (or not) with the appropriate activator or inhibitor. Cells were incubated for a given time, rinsed with PBS, and fixed 10 min in 95% ethanol-5% acetic acid at room temperature. Fixed dishes were then stained with hematoxylin overnight and rinsed with ddHiO. For each plate, pictures were taken on an inverted microscope (Olympus) at 40

times magnification. The distance migrated from the scratch line by the cells at each time point was then measured (in mm) on the prints.

2.15. Adhesion assays.

The adhesion assay was adapted from Current Protocols in Cell Biology. Plates were coated for 1 h at 37°C with poly-L-lysine at 10 pg/ml in PBS. Where needed, further coating with ECM proteins (laminin, vitronectin, or fibronectin) at 10 pg/ml in PBS was carried out overnight at 37°C. Cells were trypsinized, counted, and diluted to a concentration of 5×10^5 cells per ml in serum-free medium. When needed, aliquots of cells were incubated with the appropriate antibody or inhibitor at the indicated concentration for 15 min at 4°C (with the exception of PMA, which was also added 12 h prior to the experiment and replenished at the time of the experiment). In each well of a 24-well plate, 250,000 cells were seeded and incubated for 10 min at 37°C. Plates were rinsed twice with PBS, and the remaining adhering cells were incubated for 90 min in medium containing 0.1 mg/ml of MTT. After three rinses with PBS, the MTT stain was solubilized by incubation for 10 min in dimethylsulfoxyde (300 pi for 24-well plates, 100 pi for 96-well plates), and OD was measured at 550 nm or 600 nm. Adhesion assays with blocking antibodies or inhibitors were performed in a similar manner but in 96-well plates, with only 3×10^4 cells seeded per well.

CHAPTER THREE

Aiml: PKC a Regulates Cell Cycle Progression of Human Glioma Cells.

3.1. Introduction.

PKC is a multigene family of phospholipid-dependent serine-threonine kinases, which plays a central role in signal transduction and has been implicated in a wide range of physiological or pathological cellular functions, such as cell growth, transformation and differentiation. There are 12 members of the PKC family known so far and these are divided into three groups based on their requirements for activation (reviewed in Newton 1997, Mellor and Parker 1998). The conventional PKCs (cPKCs) a, p\, fc, and y, require Ca^{2+}, diacylglycerol (DAG) and phosphatidylserine for full activation. The novel PKCs (nPKCs) 8, e, n, 8, v and p do not require Ca^{2+} for activation. Finally, the atypical PKCs (aPKCs) £ and ***i/X*** are both Ca^{2+} and DAG insensitive. Conventional and novel PKC isoforms are activated by the tumor promoting phorbol esters, while the atypical isoforms are not. The distribution of PKC isoforms is both tissue specific and cell type specific. Also, the roles of a specific PKC isoform can be different from one cell type to another. The different functions of each PKC isozyme in cells is thought to be mainly due to a tight control of their subcellular localization, by a set of anchoring proteins, and substrate availability (reviewed in Jaken 1996).

Malignant gliomas are the most common brain neoplasms and are the second highest cause of death from neurological diseases after stroke. High-grade gliomas, glioblastoma multiforme (GBM), have a very poor prognosis, with less than 10% of patients surviving beyond two years. Although classical anti-cancer therapies are ineffective, it has recently been shown that PKC inhibitors such as tamoxifen, at PKC inhibitory concentrations, produced a 40% response rate in patients with recurrent malignant gliomas (Baltuch et al. 1993b, Couldwell et al. 1996. Mastronardi et al. 1998). The use of PKC inhibitors in clinical trials for patients with gliomas stems from previous observations that PKC activity is deregulated in gliomas (reviewed in Baltuch et al. 1995). Moreover, PKC inhibitors could reduce glioma cell proliferation by over 90% (Couldwell et al. 1992, Baltuch et al. 1993a, Begemann et al. 1996, Pollack and Kawecki 1997). Specific inhibition of PKC a, using an antisense oligonucleotide strategy, inhibited U87 glioma

cell growth *in vitro* (Ahmad et al. 1994) and in a mouse model *in vivo* (Dean et al. 1996, Yazaki et al. 1996). Also, the use of a PKC a-specific ribozyme blocked glioma cell growth (Sioud and Sorensen 1998). Collectively, the data suggest that PKC plays an important role in the regulation of glioma cell proliferation, although the identity of the PKC isoform and the mechanisms by which PKC accomplishes these functions remain to be clarified.

Here, we have characterized the pattern of PKC isozyme expression and activation in several glioma cell lines, and assessed which of these could be responsible for increasing the proliferation rate of glioma cells. Our results indicate that the a isoform of PKC controls proliferation and positively regulates cell cycle progression in human glioma cells.

Figure 7: Human glioma cell lines express the PKC isoforms a, 8, e, r|, p, **and** C
The expression of 11 PKC isoforms was examined in four different human glioma cell lines (U251N, U178, U563, and A172) and in two human fetal astrocyte primary cultures by Western blotting using isoform specific PKC antibodies. The molecular masses (kDa) of the PKC species were: a (82 kDa), pi (80 kDa), p, (80 kDa), ***y*** (80 kDa), 8 (78 kDa), e (90 kDa), ***r***| (78 kDa), **6** (79 kDa),p (115 kDa), t (74 kDa), and C (72 kDa). Protein extract from adult human brain was used as a positive control. Equal amounts of protein (100 pg) were loaded in each well.

3.2. Results.

3.2.1. Glioma cell lines express the PKC isoforms a, 8, e, r\, p, and

Using Western blot analysis, we determined that PKCs a, 5, £, T), p and C were expressed in all 4 human glioma cell lines (U251N, U178, U563, and A172) tested, while the isoforms pi, **p2, y,** t, and 6 could not be detected (Fig. 7). RT-PCR, using conventional PKC isoform specific primers, confirmed the lack of isoforms **pi, P2,** and ***y*** (Fig. 8). It is of note that all four glioma cell lines tested exhibited the same pattern of PKC expression. A similar expression pattern was also observed in two human fetal astrocyte primary cultures. Such a similarity between glioma cells and astrocytes is not surprising since glioma cells are commonly thought to arise from cells of the astrocytic lineage. In view of the similarities in PKC isoform expression by all 4 glioma cell lines, subsequent experiments focused on the U178 and U251N glioma lines.

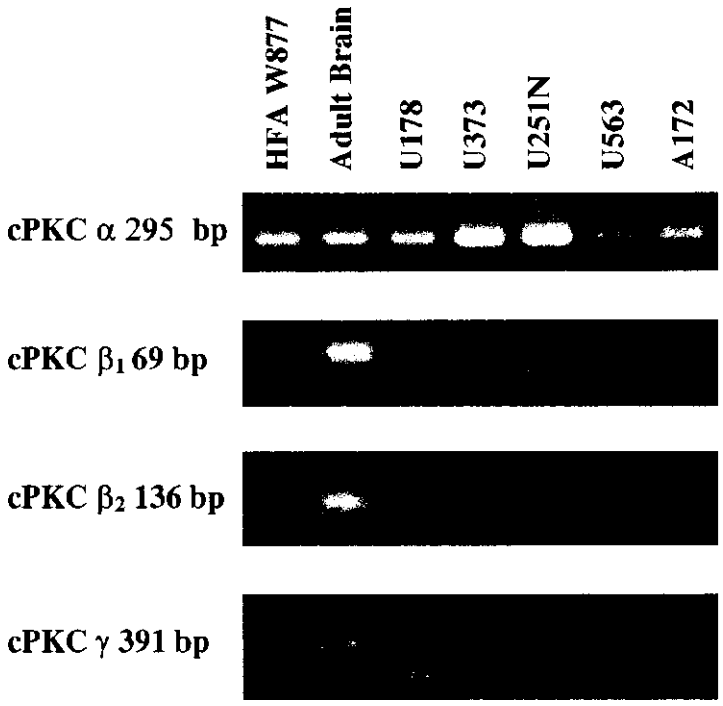


Figure 8: RT-PCR analysis of conventional PKC isoform expression. Expression of the four conventional PKC isoforms (PKC a, **pi, p**$_2$, and ***y)*** was analyzed by RT-PCR using isoform specific primers, in five human glioma cell lines (U251N, U178, U563, U373 and A172), and one human fetal astrocyte primary culture. RNA extracts from human adult brain were used as positive control. One pg of RNA was used per reaction. The PCR reaction was carried out for 50 cycles.

3.2.2. PKC activation with a phorbol ester increases progression of human glioma cells through the cell cycle.

Previous work had shown that PKC inhibitors dramatically affect the growth rate of glioma cells (Couldwell et al. 1992, Baltuch et al. 1993a, Begemann et al. 1996, Pollack and Kawecki 1997); however, the identity of the PKC isoform involved remains unclear.

In order to establish which isoform(s) of PKC was potentially involved, we tested the effect of a phorbol ester, phorbol 12-myristate 13-acetate (PMA), a potent activator of conventional and novel PKC isoforms, on the cell cycle progression of the human glioma cell line U251N. Upon PMA (100 nM) addition, U251N cells rapidly entered S phase (Fig. 9B), with a corresponding drop of the Go-Gi content. This was followed by a marked progression into the **G2**-M phases of the cell cycle, between 6 h and 12 h of treatment. By 24 h of treatment, the distribution of the cells between the different phases of the cell cycle was very similar to that of the control cells. Similar results were obtained using the human glioma cell lines U178, A172 and U563 (data not shown). Control cells, treated only with vehicle (DMSO), showed no significant change in their distribution between the different phases of the cell cycle during the 24 h time course examined (Fig. 9A).

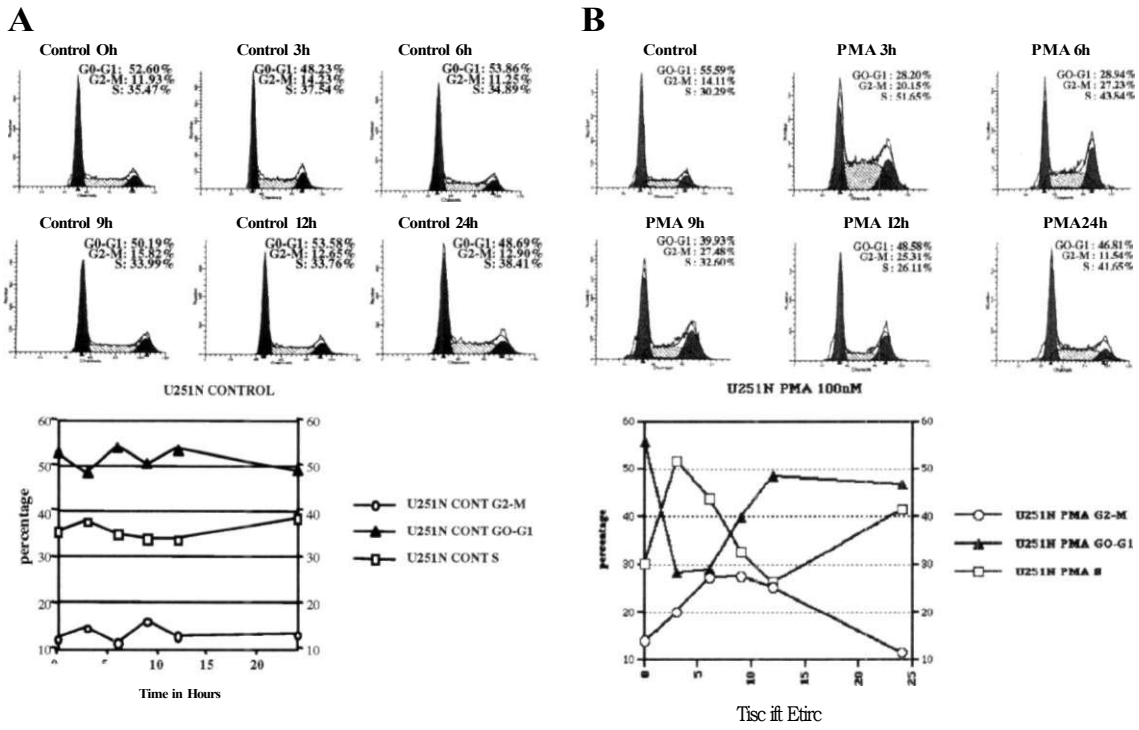


Figure 9: PKC activation increases progression of human glioma cells through the S and G2-M phases of the cell cycle. (A) Flow cytometry analysis of vehicle treated U251N cells. (B) Flow cytometry analysis of U251N cells treated with the phorbol ester PMA. Asynchronously growing U251N cells were treated with 100 nM PMA, or with vehicle only, collected at various time points, and stained with propidium iodide. For each side scatter plot, the y-axis is the number of cells, while the x-axis is the DNA content. Values from each scatter plot are then graphed in the bottom panels.

To confirm that cells were not blocked in the G_2 phase, propidium iodide-stained U251N cells grown on glass coverslips were analyzed by immunofluorescence microscopy at various times following PMA or vehicle treatment. Cells at all stages of mitosis could be observed in both PMA-treated cells (Fig. 10), and vehicle treated cells, indicating that the cells were progressing normally through mitosis. The percentage of cells showing a mitotic appearance (condensed chromatin and chromosome alignment) correlated with the G_2-M content given by flow cytometry analysis in both PMA treated and vehicle treated cells. Therefore, PKC activation seems to play a role in the regulation of cell cycle progression.

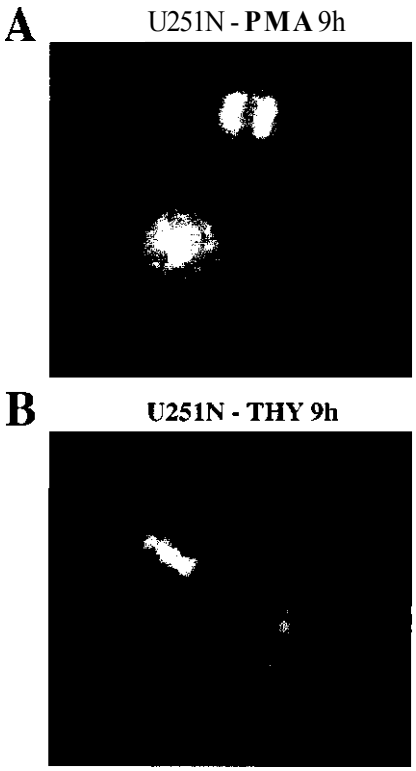


Figure 10: Glioma cells treated with PMA or THY progress normally through mitosis.
Immunofluorescence of cellular DNA stained with propidium iodide showing cells in interphase or at different stages of mitosis (anaphase in A, and metaphase in B). U251N cells were grown on glass coverslips for 24 h, treated either with PMA (A) or thymeleatoxin (B) for 9 h and then fixed.

To identify the isoform of PKC responsible for the increased progression of the cells through the cell cycle, we monitored the effect of PMA on the subcellular localization of the six PKC isoforms expressed in glioma cells. Translocation of PKC from the cytosol to the membrane is a hallmark of its activation (Newton 1997). Upon PMA treatment, only PKC a and e were translocated from the Digitonin-soluble (cytosolic, C) to the Digitonin-insoluble (particulate, P) fraction (Fig. 11); PKC 8, T|, p, and *C,* remained unaffected by PMA. PKC a was totally downregulated by proteolytic degradation

following 24 h of treatment, while PKC £ was still present, and translocated, in the cells at that time. Collectively, the data indicate that of the six PKC isoforms expressed in glioma cells, only PKC a and £ were significantly activated by PMA stimulation.

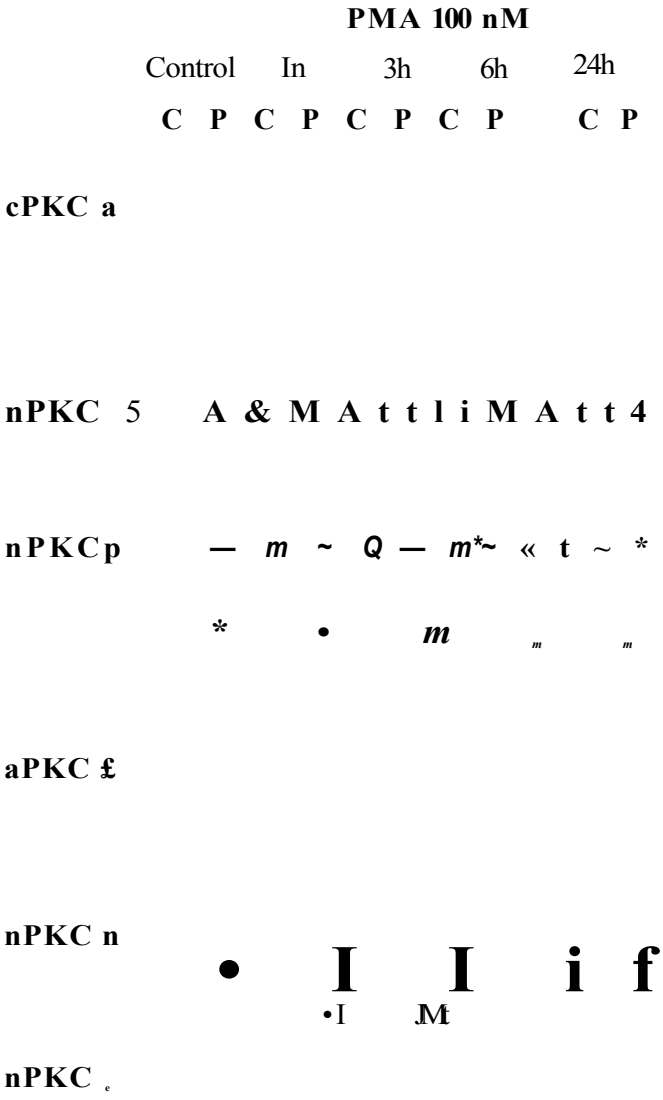
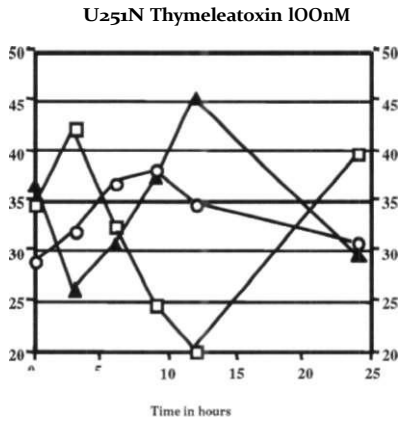


Figure 11: PKC a and 8 are the only isoforms translocated by PMA in glioma cells.
Western Blot analysis of the subcellular distribution between cytosolic and membrane fractions of the six PKC isoforms expressed in glioma cells following phorbol ester treatment using isoform specific antibodies. U251N protein extracts collected at various times following PMA treatment were fractionated into Digitonin-soluble (cytosolic, C) and Digitonin-insoluble (particulate, P) fractions. Thirty pg of proteins were loaded in each well. Similar distribution following PMA treatment was also obtained for the U178 glioma cell line (data not shown). Note that in the doublet obtained for PKC e, only the upper band (90 kDa) is the active form of the enzyme.

3.2.3. PKC a is necessary and sufficient to increase progression through the cell cycle.

To differentiate between PKC a and £, we used the conventional isoform-specific PKC agonist thymeleatoxin (Ryves et al. 1991, Kazanietz et al. 1993); in glioma cells, thymeleatoxin should activate only PKC a, since it is the only conventional PKC isoform

expressed in those cells. Flow cytometry analysis of thymeleatoxin (100 nM)-treated U251N cells (Fig. 12A) revealed a cell cycle progression profile similar to that obtained with PMA (Fig. 9B). Similar results using thymeleatoxin were obtained with the glioma cell lines U178, A172, and U563 (data not shown). Western blot analysis confirmed that PKC a was specifically translocated (and activated) by thymeleatoxin, whereas PKC £ remained unaffected (Fig. 12C). In addition, the increased progression of glioma cells through the cell cycle correlated with the time frame of activation/translocation of PKC a. The analysis of later time points revealed that PKC a was still downregulated at 48 h and 72 h following either PMA or thymeleatoxin treatment (Fig. 12B-C).



U251NTHYG2_M

u25iNTHYGo-Gi$_{\text{were co}}$ii$_{\text{ec}}$ted

U251N THY S

B

PMA 100 nM

Oh 1h 3h 6h 12h 24h 48h 72h

PKC : p c P c P c P

mm 1

PKC

Thymeleatoxin 100 nM

Oh Ih 6h 24h 48h 72h

PKC C P C P C P C P C P C P

PKC

Figure 12: PKC a activation is sufficient to increase cell cycle progression.

(A) Flow cytometry analysis of U251N cells stimulated with thymeleatoxin (100 nM). Cells were collected at various time points, and stained with propidium iodide for DNA content analysis.

(B) and (C) Western Blot analysis of the subcellular localization of PKC a and £ upon PMA (B) or thymeleatoxin (C) treatment between 0 h and 72 h. PKC a was rapidly translocated to the membrane by both PMA and thymeleatoxin. and downregulated by 24 h. PKC £ was translocated only upon PMA addition, and was not downregulated at later time points. U251N protein extracts collected at various times following PMA or thymeleatoxin treatment were fractionated into cytosolic ***(C)*** and particulate ***(P)*** fractions. 30 pg of proteins were loaded in each well.

Further confirmation of the specific role of PKC a in the regulation of cell cycle progression of glioma cells was provided by PKC a depletion experiments. U178 (or U25 IN, data not shown) human glioma cells were pre-treated with PMA for 48 h in order to deplete the cells of their endogenous PKC a. Cells were then re-stimulated with 100 nM PMA and their distribution between the different phases of the cell cycle was analyzed by flow cytometry between 0 h and 30 h following re-stimulation (Fig. 13). In the absence of a detectable level of PKC a, there was no significant change in the cell cycle progression of glioma cells following PMA stimulation over the 30 h time course (Fig. 13).

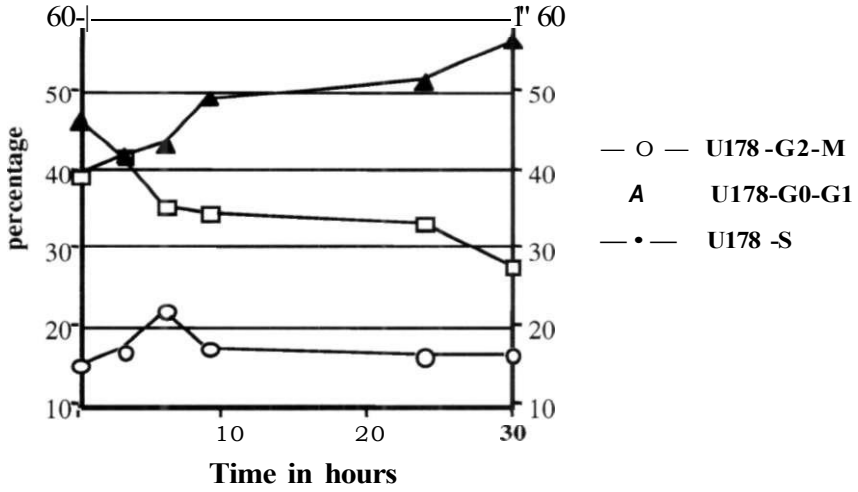


Figure 13: PKC a is required for PMA-induced increased cell cycle progression of glioma cells.
Flow cytometry analysis of U178 glioma cells depleted of their endogenous PKC a by 48 h of PMA treatment and re-stimulated with 100 nM PMA (time 0 to 30 h). Cells were collected at various time points following re-stimulation, and stained with propidium iodide for DNA content analysis.

The role of PKC a in cell proliferation was further addressed using an antisense strategy to partially deplete glioma cells of their endogenous PKC a. Equally seeded cultures of the different clones were grown for four days and counted, giving a direct reading of their growth rate. Two antisense PKC a clones, ASa1 and ASa2, exhibited a growth rate of less than half the rate of the wild type U251N or the empty vector transfected cells (Fig. 14), thus indicating that PKC a levels are directly proportional to the basal proliferation rate of glioma cells.

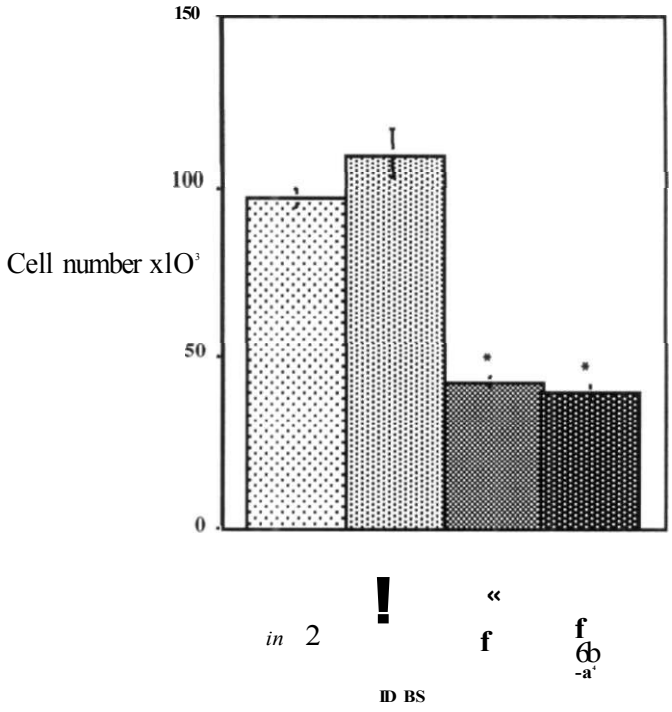


Figure 14: Decreased cell number in antisense PKC a transfected cells.

Twenty five thousand cells were seeded for each cell line (U25 IN, Control vector, ASocl, and ASoc2). Four days later, cells were counted using a Coulter counter; cell numbers are displayed in the top panel. Each clone was analyzed in quadruplicates. Results were analyzed using a 1-way ANOVA with Bonferroni multiple comparisons.
*=p<0.0001.
Bottom panel: Western blot using a monoclonal anti-PKC a antibody (clone 3, Transduction Laboratories) shows the endogenous PKC *a* level in the respective clones. 100 pg of proteins were loaded in each well.

Altogether, the data strongly suggest that PKC a specific activation is necessary and sufficient for the increased progression of human glioma cells through the cell cycle induced by PKC agonists such as PMA or thymeleatoxin. Moreover, our data indicate that PKC a directly controls glioma cell proliferation, as decreased PKC a expression correlates with decreased proliferation.

3.3. Discussion.

PKC plays a major role in the regulation of cell growth and differentiation; its specific function depends on the cellular context, localization, and substrate availability (reviewed in Jaken 1996). In a number of cell systems, it has been shown that either activation or inhibition of PKC could influence cell cycle progression by a variety of mechanisms (reviewed in Livneh and Fishmanl997, Fishman et al. 1998, Black 2000).

We found that the human glioma cell lines U251N, U178, U563, and A172 expressed 6 PKC isoforms, namely PKCs a, 8, e, n, p and £ . There has been some controversy in the literature concerning which isoforms of PKC were expressed in glioma cells, possibly

due to the lack of adequate reagents to test for specific PKC isoform expression. There might also be some variations between different cell lines. To our knowledge, this is the first time that expression of all PKC isoforms was tested (PKC **v** expression was not tested due to the unavailability of reagents). Several groups also reported that high expression levels of several isoforms were detected in astrocytoma cell lines, including PKC a, e, and £ (Mishra-Press et al. 1992, Xiao et al. 1994, Sharif and Sharif 1999). However, during our screen, we did not observe any striking difference in the expression levels of PKCs between the various glioma lines tested and adult brain extracts or human fetal astrocyte primary cultures.

We have found that PKC plays a role in the regulation of cell cycle progression in glioma cells, as PKC activation by phorbol esters increased glioma cell progression through the cell cycle. This is consistent with previous data showing the correlation between PKC activity and glioma cell proliferation (Couldwell et al. 1992) and the clinical results showing that inhibitors of PKC may have some efficacy in patients with gliomas (Baltuch et al. 1993b, Couldwell et al. 1996, Mastronardi et al. 1998). Moreover, we have determined that PKC a activation was necessary and sufficient to increase cell cycle progression of glioma cells. We have also shown that the expression level of PKC a directly correlates with the proliferation rate of glioma cells.

PKC **T)** was recently found to mediate the PMA-induced proliferation in U1242 and U251 glioma cells (Hussaini et al. 2000). We cannot exclude that in our cells PKC **T|** is also involved in the regulation of cell proliferation, as we did not address specifically the role of this isoform in our study. It is possible that since PKC r] is totally translocated to the membrane (Fig. 11), even in the absence of PMA stimulation, this isoform may be constitutively activated in glioma cells.

Recently, PKC has been associated with regulation of cell cycle progression (reviewed in Livneh and Fishmanl997, Fishman et al. 1998, Black 2000) either during the Gi to S progression or during the **G2**/M transition. PKC has been shown to regulate Gi progression through the modulation of CDK activity, either by modifying cyclin or CDK expression levels or by modifying the expression of the cyclin-CDK inhibitors (CKIs). In Swiss 3T3 cells, phorbol esters accelerate growth factor-induced cell cycle entry and progression into S phase by elevating cyclin Dl levels, and downregulating

p27/Kipl expression (Mann et al. 1997). Predominantly, however, PKC plays an inhibitory role on cell cycle progression. PMA treatment of IMR-90 cells resulted in **Gi** arrest due to the downregulation of CDK7 and cyclin H, thus preventing the activation of CDK2 (Hamada et al. 1993). Overexpression of PKC r) caused delayed entry into S phase and prolonged the **Gi** phase in NIH-3T3 cells; this correlated with increased expression of p21/Wafl/Cipl and p27/Kipl, decreased CDK2 associated activity, and decreased Rb phosphorylation (Livneh et al. 1996). In intestinal epithelial cells, PKC a specific activation resulted in **Gi** arrest, delayed transit through S and **G2**/M phases through upregulation of p21AVafl/Cipl and p27/Kipl, resulting in hypophosphorylation of Rb (Frey et al. 1997). It was also demonstrated that PMA-induced **Gi** arrest could be mediated via phosphorylation of p53 by PKC and activation of p53 DNA binding (Delphin and Baudier 1994).

Increasing evidence also implicates PKC in the inhibition of the **G2**/M transition, often by modulating cdc2 (CDK1) activity by influencing the expression levels of cdc25 or the CKIs. Growth arrest in **G2**/M following PMA treatment was observed in Demel melanoma cells (Arita et al. 1998), U937 leukemia cells (Hass et al. 1993), and vascular endothelial cells (Kosaka et al. 1996). This growth inhibition correlated with the downregulation of cyclin B and/or cdc25, thus causing the inhibition of cdc2 kinase activity. Also, PKC p_2 activity was shown to be required for the G_2/M transition in HL60 cells, by acting as a lamin B kinase; lamin B phosphorylation is required for nuclear envelope breakdown to occur at the onset of mitosis (Thompson and Fields 1996).

Thus, in contrast to most cell types, the activation of PKC, and specifically PKC a, in glioma cells facilitates progression of cells through the cell cycle.

CHAPTER FOUR

Aim 2: Involvement of p21/Wafl/Cipl in PKC a-Induced Cell Cycle Progression.

4.1. Introduction.

The transition from one phase of the cell cycle to another is regulated by the activity of cyclin-CDK complexes (Fig. 5) (Roberts 1999, Sherr and Roberts 1999). Expression levels of cyclins vary during the cell cycle, and are tightly regulated. Cyclin D is induced by growth factor or serum stimulation of quiescent cells and remains expressed throughout the cell cycle. The **Gi**/S transition is regulated by cyclin D-CDK4/6 complexes that phosphorylate and inactivate the Rb protein, releasing the E2F transcription factors, which in turn allow the transcription of genes required for the progression through S phase (such as cyclin E). Cyclin E levels rise in late **Gi** and remain elevated until the end of S phase; exit from S phase requires cyclin E degradation. Cyclin A is expressed from early S phase until mitosis. Cyclin B is expressed from late **G2** and is degraded at the end of mitosis. In gliomas, the timing of cyclins expression was found to be aberrant (Dirks et al. 1997).

CDK levels do not change during the cell cycle, as their activity and substrate specificity is regulated by the cyclins, which are found in limiting amounts. Several CDKs are overexpressed in gliomas (Sonoda et al. 1995, He et al. 1995, Costello et al. 1997, Burns et al. 1998). For example, CDK4 amplification and overexpression were detected in approximately 15% of GBMs (Collins 1995, Biernat et al 1997).

The activities of cyclin-CDK complexes are themselves regulated by two families of cyclin-dependent kinase inhibitors (CKI), the FNK4 family (p16/INK4A, p15/INK4B, p18/INK4C, and p19/INK4D) and the Cip/Kip family (p21/Wafl/Cipl, p27/Kipl, and p57/Kip2). Generally, p27/Kipl expression is elevated in quiescent cells and the protein is degraded as the cells enter the cell cycle. The p21/Wafl/Cipl protein is induced by p53 upon DNA damage and other cellular stresses. A number of studies have reported the induction of p21AVafl/Cipl expression by PKC in other cell types (Hass et al. 1993, Frey et al. 1997, Zezula et al. 1997, Arita et al. 1998). The CKIs p16/TNK4A and p15/TNK4B

were either deleted, mutated, or the genes silenced through promoter hypermethylation in 30-60% of glioblastomas (Schmidt et al. 1994, Sonoda et al. 1995, Srivenugopal and Ali-Osman 1996, Herman et al. 1996, Biernat et al 1997, Ichimura et al. 2000).

The large number of aberrations in cell cycle regulatory proteins predisposes glioma cells to defects in the regulatory mechanisms of cell cycle progression. Here we found that PKC activation induced p21/Wafl/Cipl upregulation in human glioma cells. Increased p21/Wafl/Cipl levels facilitated the formation of active ternary cyclin/CDK/p21 complexes, resulting in cell cycle progression.

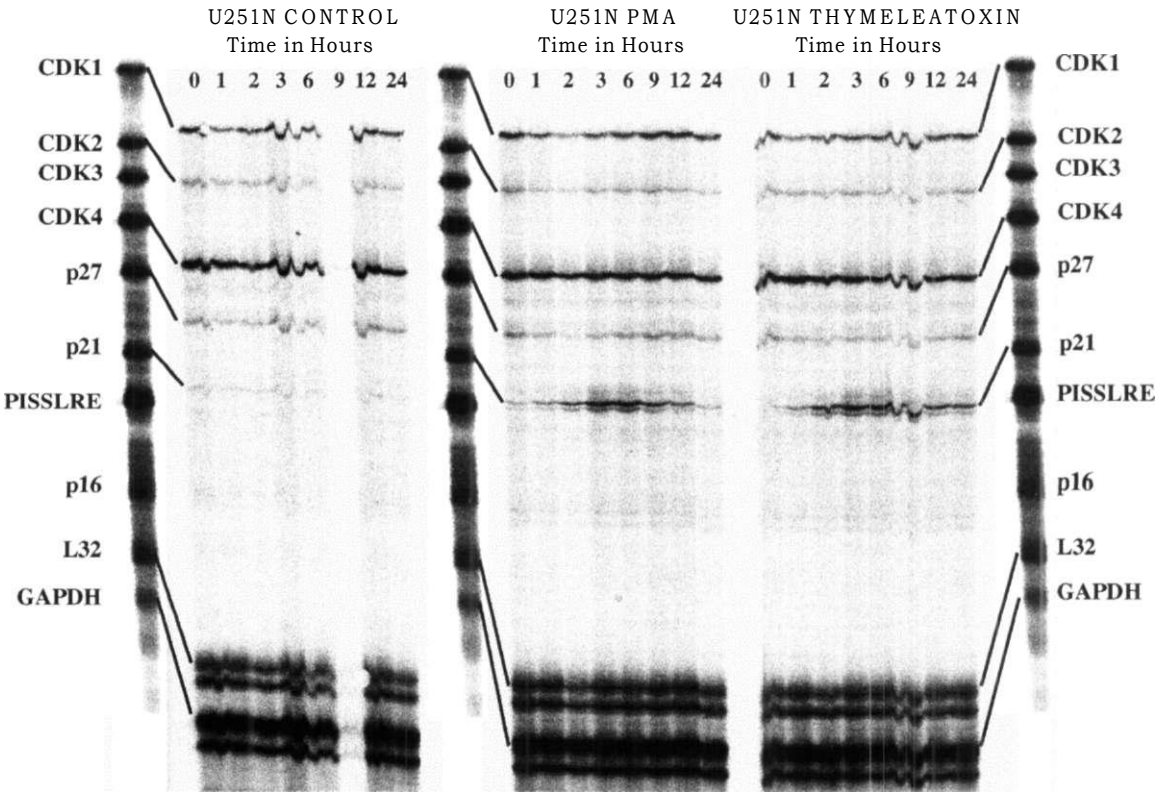


Figure 15: p21/Wafl mRNA is upregulated by PKC a activity.
Multiprobe RNase protection assay (RPA) on U251N glioma cell RNA extracts in untreated (control), PMA (100 nM), or thymeleatoxin (100 nM) treated cells at various timepoints. Five pg of RNA were used for each reaction. RPA using the hCC-1 probe set shows a marked upregulation of p21 mRNA between 1 h and 12 h following PMA or thymeleatoxin addition. p16 and CDK3 mRNAs were undetectable at all times. No change was detected in the various CDK mRNA levels.

4.2. Results.

4.2.1. p21AVafl/Cipl is upregulated following PKC a activation.

To evaluate the molecular mechanism by which PKC a induces cell cycle progression, we examined the transcript levels of several cell-cycle regulatory proteins using a multiprobe RNase protection assay (RPA). Using the probe set hCC-1, we determined that p21/Wafl/Cipl mRNA was strongly and rapidly upregulated (Fig. 15) between 1 h and 12 h following PMA or thymeleatoxin treatment, with a maximum increase at 3 h and 6 h. Other mRNA (CDKl/cdc2, CDK2, CDK4, and p27) levels remained unaffected by PKC a activation. CDK3, and PISSLRE (a cdc2 related kinase acting at G_2/M)(Li et al. 1995) mRNAs could not be detected in this assay.

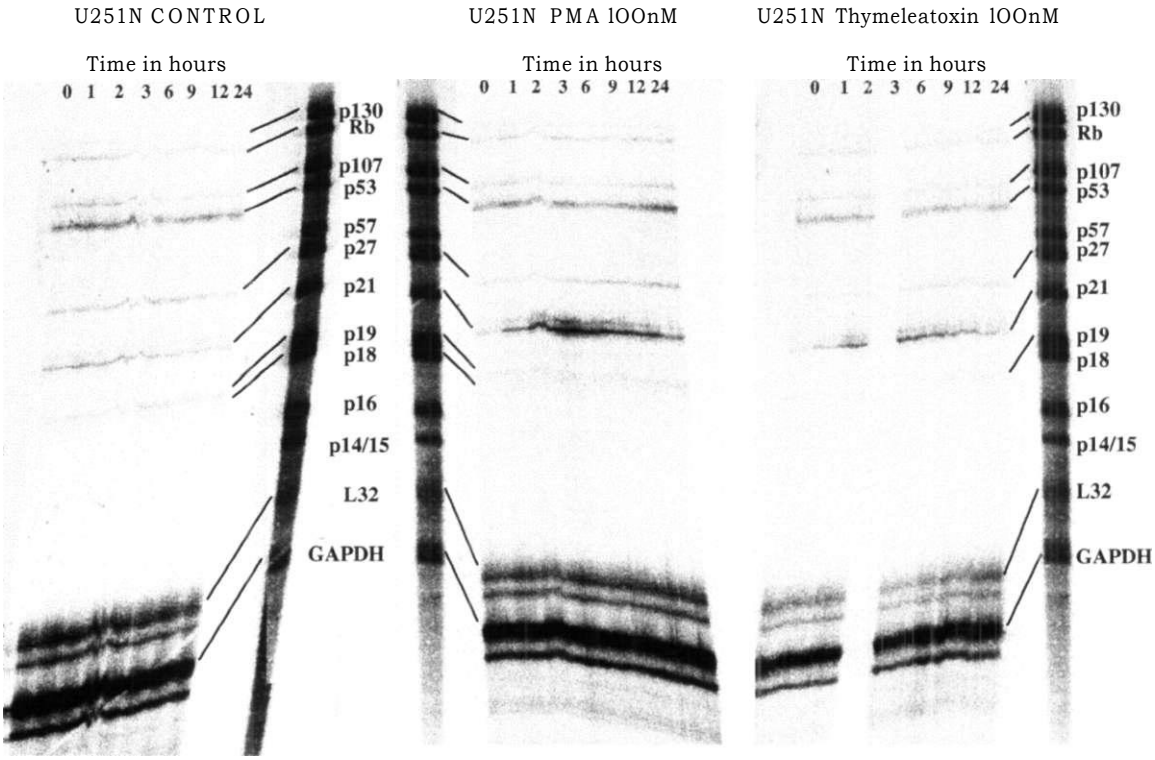


Figure 16: p21/Wafl/Cipl is the only cell cycle regulator upregulated following PKC activation. Multiprobe RNase protection assay (RPA) on U251N glioma cell RNA extracts in untreated (control), PMA (100 nM), or thymeleatoxin (100 nM) treated cells at various timepoints. Five pg of RNA were used for each reaction. RPA using the hCC-2 probe set shows the upregulation of p21 between 1 h and 12 h following PMA or thymeleatoxin addition. mRNAs for p57, p19, p16, and p15 were undetectable at all times.

Using the probe set hCC-2 and hCYC-1 (Fig. 16 and 17, respectively), we determined that the mRNA levels of p130, Rb, p107, p53, p27/Kipl, p18/INK4C, cyclins A, B, C, Dl and D3 were not affected by PKC activation. On the other hand, the transcripts for p57/Kip2, p19/TNK4D, p16/TNK4A, p15/TNK4B, and cyclin D2 and cyclin Al could not be detected using this assay.

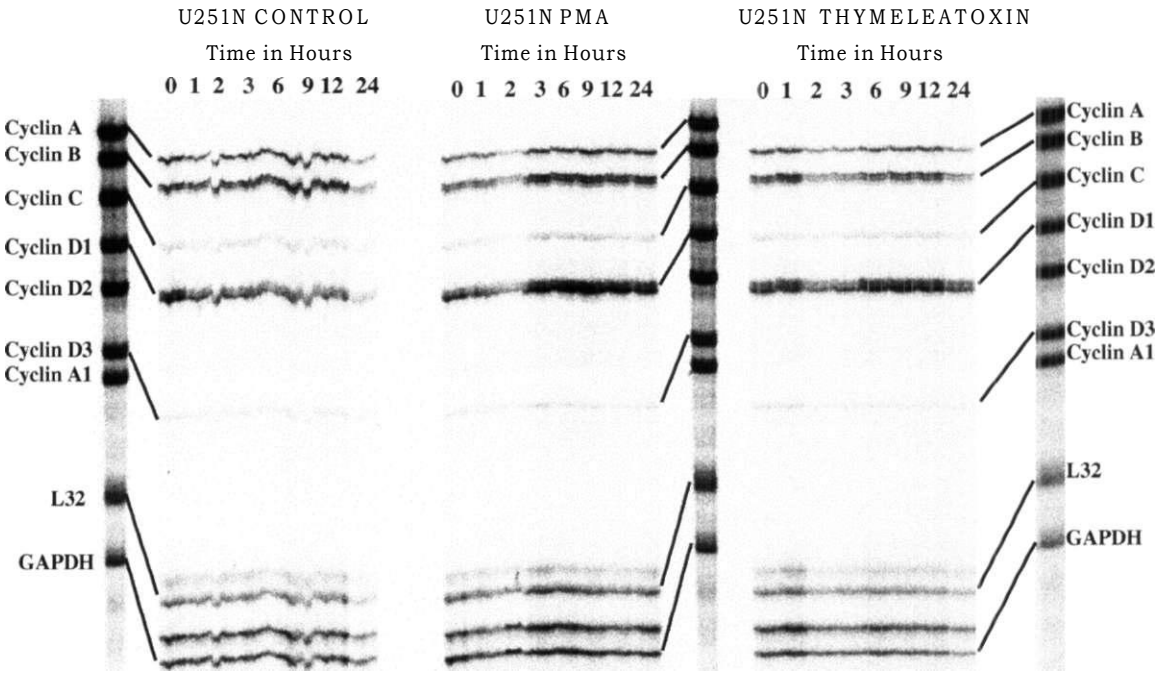


Figure 17: No change in cyclin mRNA levels following PKC activation.
Multiprobe RNase protection assay (RPA) on U251N glioma cell RNA extracts in untreated (control), PMA (100 nM), or thymeleatoxin (100 nM) treated cells at various timepoints. Five pg of RNA were used for each reaction. RPA using the hCYC-1 probe set shows no change in mRNA levels of the various cyclins. Cyclin A, B, and Dl mRNA levels were elevated at all times. The mRNAs for cyclin D2 and cyclin Al could not be detected in this assay.

Consistent with a change at the mRNA level, the p21/Wafl/Cipl protein was also upregulated (Fig. 18). In untreated U251N cells, p21/Wafl/Cipl protein level remained constant, while in cells treated either with PMA or thymeleatoxin, p21/Wafl/Cipl was strongly upregulated by 2 h, with a maximum at 9 h after treatment. The expression level of p21/Wafl/Cipl was monitored over a 72 h period following PMA treatment of U251N cells; Fig. 18B shows that p21/Wafl/Cipl induction is transient and correlates with the time frame of PKC a activation. Western blot analyses were also performed for a variety

of other cell cycle regulators. In correspondence with the RNA results, protein levels of p27/Kipl, cyclins A, **B,** Dl and E, and CDK2, CDK4, and CDK6, were not affected (Fig. 19). These data indicate that the CKI p21/Wafl/Cipl is the only cell cycle regulatory molecule upregulated following PKC a activation.

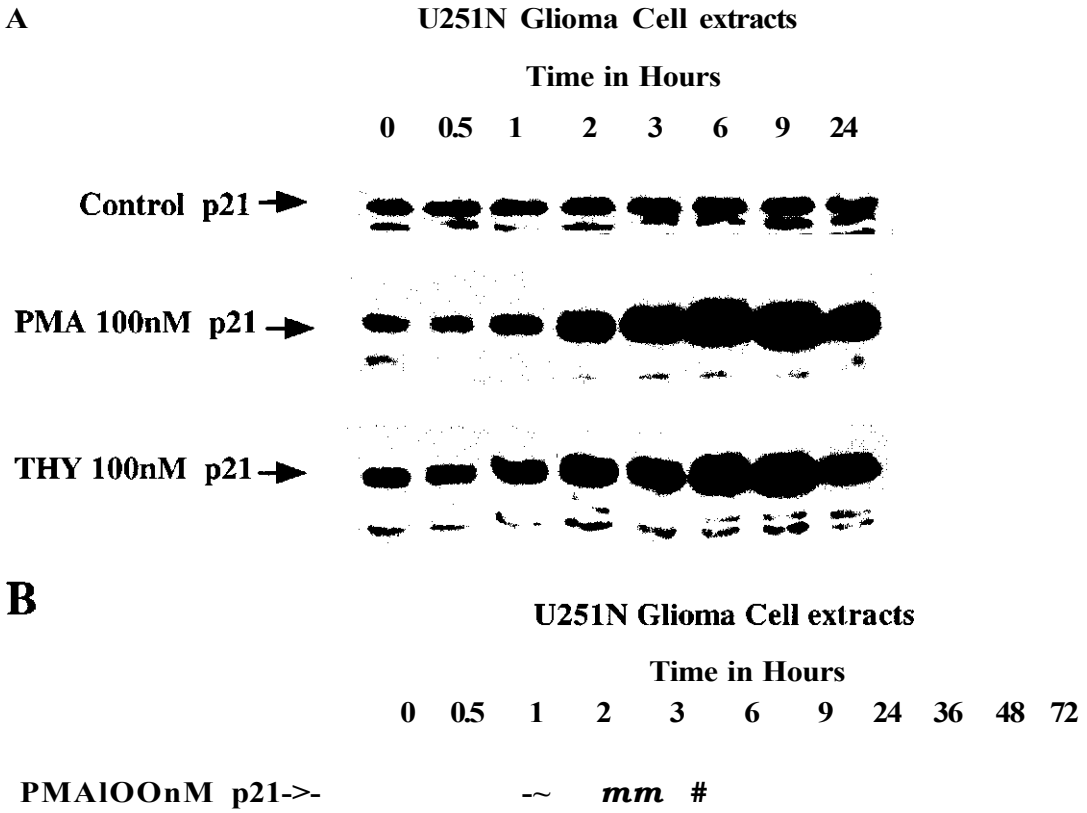


Figure 18: Upregulation of the p21AVafl/Cipl protein following PKC a activation. (A) Western blot analysis of U251N glioma cells treated with PMA (100 nM), thymeleatoxin (100 nM), or untreated, shows a strong upregulation of the p21 protein following PMA or thymeleatoxin treatment. **(B)** p21 levels over a 72 h period following PMA treatment of U25 IN cells. Forty pg of proteins were loaded per well. Note that the exposure time in **(B)** was shorter than in (A) in order to get a better idea of the magnitude of p21 induction, thus explaining the apparently low p21 levels at times 0 h, 36 h, 48 h, and 72 h.

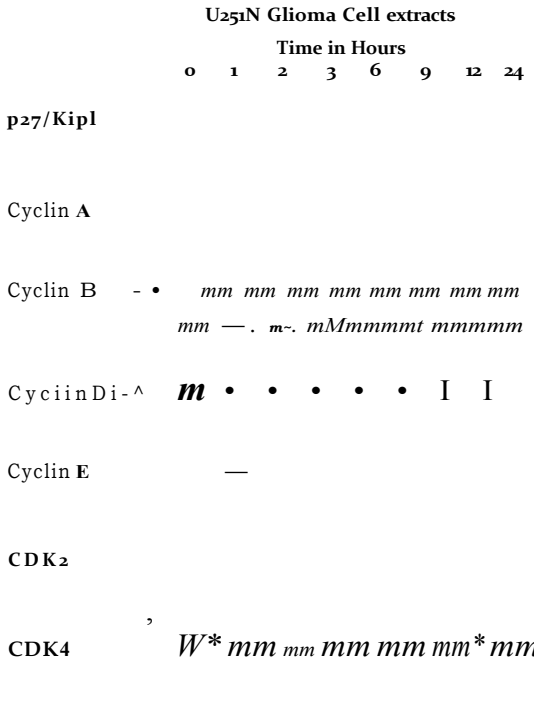


Figure 19: No change in cyclin or CDK protein levels following PKC activation.

Western blot analysis of various cell cycle regulatory proteins (p27/Kipl, Cyclin A, B, D1, E, CDK2, 4, and 6) following PMA treatment of U251N glioma cells. The data obtained by RPA was confirmed at the protein level. PMA treatment did not alter the protein levels of various cell cycle regulators. Sixty Dg of proteins was loaded per well. The third lane of the CDK2 Western blot was not loaded.

4.2.2. p21/Wafl/Cipl upregulation is associated with the formation of active ternary cyclin-CDK-p21 complexes.

Although the upregulation of p21/Wafl/Cipl would appear paradoxical with increased progression through the cell cycle, a number of reports have shown that p21/Wafl/Cipl can be upregulated during cell proliferation (Michieli et al. 1994, Nourse et al. 1994, McLeod et al. 1995, Mantel et al. 1996, Hiyama et al. 1998), and that p21/Wafl/Cipl can act as an assembly and activity-promoting factor for cyclin-CDK complexes (LaBaer et al. 1997, Cheng et al. 1999). In order to establish whether p21/Wafl/Cipl was associated with active cyclin-CDK complexes, we performed coimmunoprecipitations and kinase assays, using cyclin-specific antibodies, at various times following PKC activation.

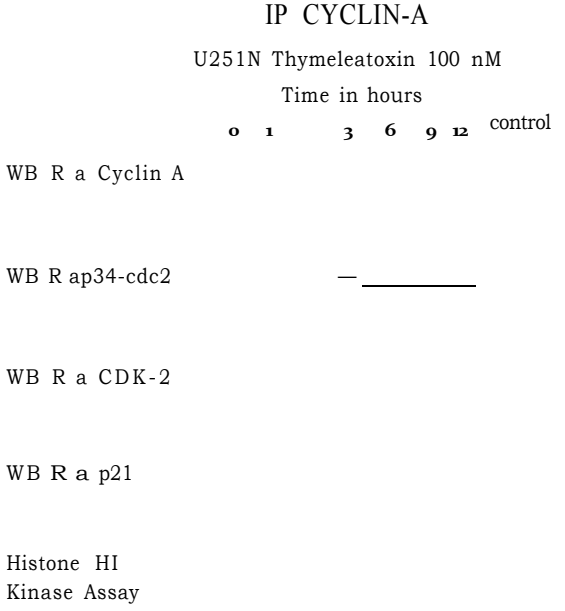


Figure 20: Increased association of p21/Wafl/Cipl with cyclin A/CDK2 or Cdc2 complexes following PKC activation.

Cyclin-A immunoprecipitates at various times following thymeleatoxin stimulation of U251N cells were subjected to SDS-PAGE and blotted for cyclin A, Cdc2, CDK2, and p21/Wafl/Cipl. An approximately equal amount of cyclin A was present in the cells throughout the duration of the experiment. For each immunoprecipitation, the kinase activity of the complex was measured by its ability to phosphorylate histone HI *in vitro*. The control lane in the kinase assay panel refers to an extract subjected to protein-G coated agarose beads immunoprecipitation only. The formation of a ternary complex was accompanied by an increase in the kinase activitv of the complexes.

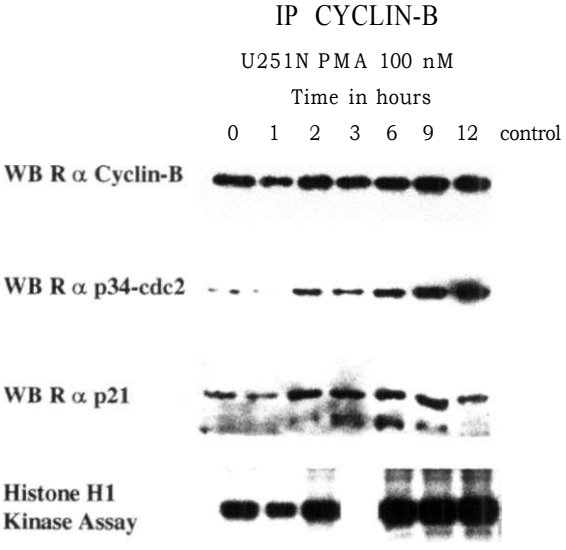


Figure 21: Increased association of p21/Wafl/Cipl with cyclin B/Ccd2 complexes following PKC activation. The formation of a ternary complex was accompanied by an increase in the histone kinase activity of the complexes. Cyclin B immunoprecipitates at various times following PMA stimulation of U251N cells were subjected to SDS-PAGE and blotted for cyclin B, Cdc2, and p21/Wafl/Cipl. They show that approximately equal amounts of cyclin B were present in the cells throughout the duration of the experiment. For each immunoprecipitation, the kinase activity of the complex was measured by its ability to phosphorylate histone HI *in vitro*. The cyclin-B associated kinase activity at 3 h was not measured. The control lane in the kinase assay panel refers to an extract subjected to protein-G coated agarose beads immunoprecipitation only.

Upon PKC activation, a rapid (within 1 hour) and sustained (up to 12 h) association of the immunoprecipitated cyclin with its respective CDK(s) partner(s) and with p21/Wafl/Cipl, to form a ternary complex, was observed (Fig. 20-22); the amount of cyclin immunoprecipitated remained constant throughout the experiment. We ensured that we were not working in the presence of saturating amounts of the respective cyclins by performing IPs in the presence of a double amount of antibodies. Thus, changes in the amount of CDK and p21/Wafl/Cipl coimmunoprecipitated, as well as changes in the kinase activity of the complex, were not attributable to variations of the amount of cyclin immunoprecipitated. Kinase assays were performed on the immunoprecipitates, using histone HI as a substrate for cyclin A and cyclin B complexes (Fig. 20 and 21, respectively), and Rb as a substrate for cyclin-Dl complexes (Fig. 22). There is an apparent correlation between the formation of the ternary cyclin-CDK-p21 complexes and the increased kinase activity of these complexes. Changes in kinase activity of cyclin-E immunoprecipitates were not significant (data not shown).

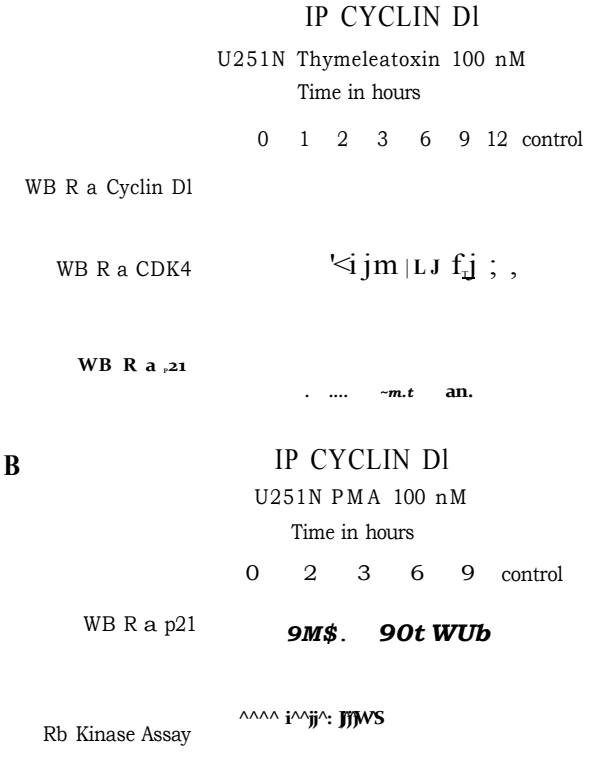


Figure 22: Increased association of p21/Wafl/Cipl with cyclin D1/CDK4 complexes following PKC activation. (A) Cyclin Dl immunoprecipitates at various times following thymeleatoxin stimulation of U251N cells were subjected to SDS-PAGE and blotted for cyclin D1, CDK4, and p21/Wafl/Cipl. Approximately equal amounts of cyclin Dl were present in the cells throughout the duration of the experiment. (B) The formation of a ternary complex was accompanied by an increase in the Rb kinase activity of the complexes. For each immunoprecipitation, the kinase activity was measured by the ability to phosphorylate Rb. The control lane in the kinase assay panel refers to an extract subjected to protein-G coated agarose beads immunoprecipitation only.

To confirm that p21-containing cyclin/CDK complexes could exhibit kinase activity, reciprocal immunoprecipitations of p21/Wafl/Cipl at various times following PMA stimulation were performed (Fig. 23), and the Rb-kinase activity co-immunoprecipitated with p21/Wafl/Cipl was measured. Figure 23 shows an increasing amount of p21AVafl/Cipl immunoprecipitated following PKC stimulation in glioma cells, in agreement with the upregulation of the protein observed previously (Fig. 18); the p21-associated kinase activity increased correspondingly. These results suggest that p21/Wafl/Cipl associates with cyclin/CDK complexes and this is accompanied by an increased kinase activity of these complexes.

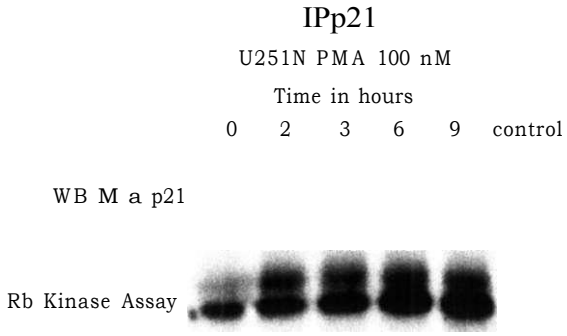
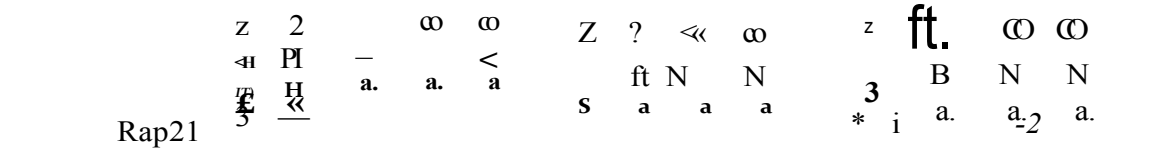


Figure 23: p21/Wafl/Cipl-containing cyclin/CDK complexes are active. As expected, an increasing amount of p21/Wafl/Cipl was immunoprecipitated following PMA stimulation. p21/Wafl/Cipl was detected using a monoclonal anti-p21 antibody. For each immunoprecipitation, the kinase activity was measured by its ability to phosphorylate Rb *in vitro*. The p21 associated kinase activity appears correlated to the amount of p21 immunoprecipitated. The control lane in the kinase assay panel refers to an extract subjected to protein-G coated agarose beads immunoprecipitation only.

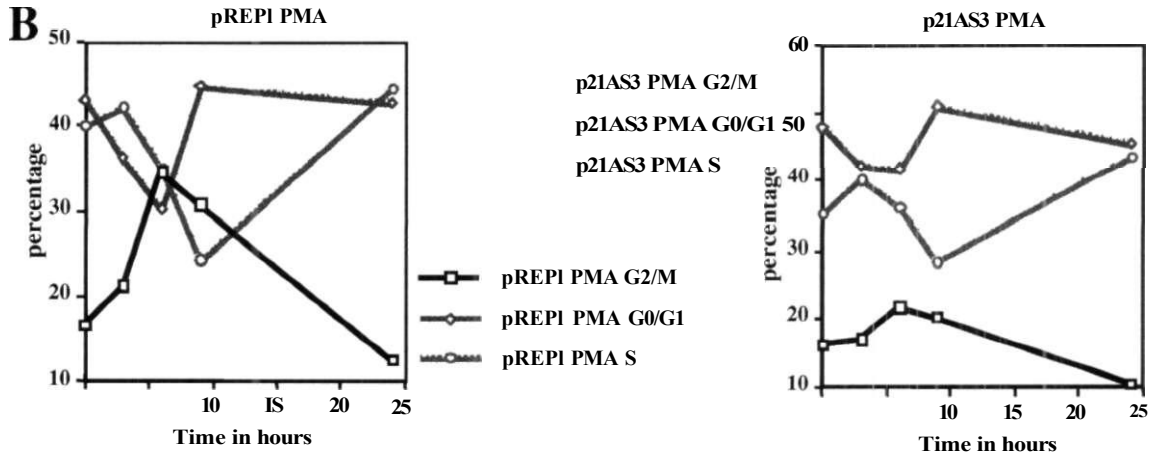
4.2.3. p21/Wafl/Cipl upregulation is required for the PKC-induced cell cycle progression.

To establish the requirement of p21/Wafl/Cipl upregulation in PKC a induced cell cycle progression, we used an antisense approach to decrease the p21/Wafl/Cipl protein level and to prevent, at least partially, its upregulation. The p21AVafl/Cipl cDNA was cloned in antisense orientation in the episomal vector pREP9 and transfected into U251N glioma cells; the endogenous p21/Wafl/Cipl protein level of several clones is shown (Fig. 24A). Upon PMA treatment, the induction of progression through **G2**/M is markedly reduced when compared to the control vector (Fig. 24B); however, the initial entry into S

phase still occurs. The induction of the p21/Wafl/Cipl protein by PMA is clearly reduced compared to empty vector-transfected cells (Fig. 24B). Figure 25 shows the percentage of cells induced to progress through G2/M (AG2/M= %G2/M PMA treated - %G2/M untreated) in wild type, empty vector transfected cells, and five individual antisense p21 clones. In response to PMA treatment, antisense p21 overexpressing cells exhibited a marked reduction in the percentage of cells induced to progress through G2/M (AG2/M) compared to wild type or empty vector transfected cells. The average inhibition of progression for the five p21 antisense clones compared to the three control lines was 57%. These results indicate that p21 upregulation is required for PKC a induced cell cycle progression.



Rap21



pREPl PMA p21AS3 PMA

6 9

Rap21

Figure 24: p21/Wafl/Cipl upregulation is required for PKC-induced cell cycle progression. (A) Endogenous p21 protein level in the wild-type (U251N), empty vector transfected cells (pREP1 and pREP4), and p21 antisense transfected cells (p21AS). 50 pg of proteins were loaded per well. (B) Flow cytometry analysis of pREP1 and p21AS3 clones following PMA treatment. There is a marked reduction in the number of p21AS3 cells induced to progress through G2/M when compared to empty vector cells (pREPl). Bottom panel: Western blot for the p21 protein. Extracts were collected at the same time as the FACS samples. 50 pg of proteins were loaded per well.

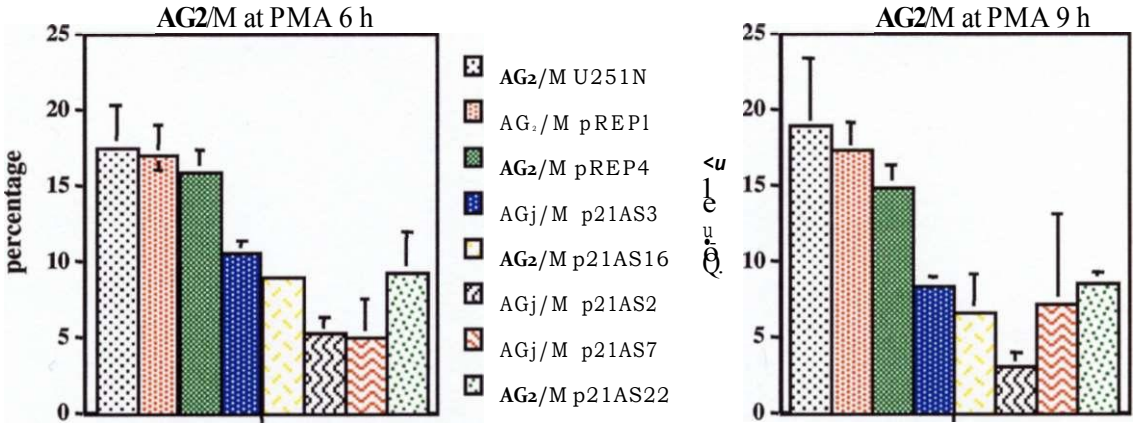


Figure 25: Antisense-p21 decreases PKC-induced G2/M progression.
Flow cytometry analysis of wild type (U251N), empty vector (pREP1 and pREP4), and several clones transfected with p21 antisense (p21AS), at 6 h (left panel) and 9 h (right panel) following PMA treatment. p21AS clones exhibit a marked reduction of the number of cells induced to progress through **G2**/M.
AG_2/M= $\%G_2/M$ PMA treated - **%G2**/M control. Each bar is the mean of three independent experiments; the SEM for each cell line is plotted on the graph.

4.3. Discussion.

In summary, our results suggest that in glioma cells, PKC a activation upregulates the p21AVafl/Cipl protein, which is incorporated into active ternary cyclin-CDK-p21 complexes, thus facilitating cell cycle progression (Fig. 26). p21AVafl/Cipl may therefore represent an important therapeutic target to control the growth rate of glioma cells.

To elucidate the mechanism by which PKC increased glioma cell cycle progression, we analyzed the expression of various cell cycle regulatory proteins following PKC activation. The only cell cycle regulatory protein upregulated by PKC activity was p21/CiplA¥afl. A number of studies have reported the PKC-induced upregulation of p21/Cipl/Wafl in other cell types, but these were associated with cell cycle block, unlike the case for glioma cells reported here (Hass et al. 1993, Frey et al. 1997, Zezula et al. 1997, Arita et al. 1998). PKC-induced p21/Cipl/Wafl upregulation was shown to be p53 independent in some cases, and to occur in cells expressing a mutant p53 (Zeng and El-Deiry 1996). This is very likely the case here, since the U251N glioma cell line expresses a mutant, transcriptionally inactive form of p53 and no change in p53 amounts, at least at the mRNA level, was detected at any time (Fig. 16). Jung et al. (1995) reported that

p21/Cipl/Wafl expression was consistently elevated in human glioma specimens, independent of the presence of a functional p53. In view of our results, the high p21/Cipl/Wafl levels in gliomas could be a consequence of the high PKC enzyme activity in these cells.

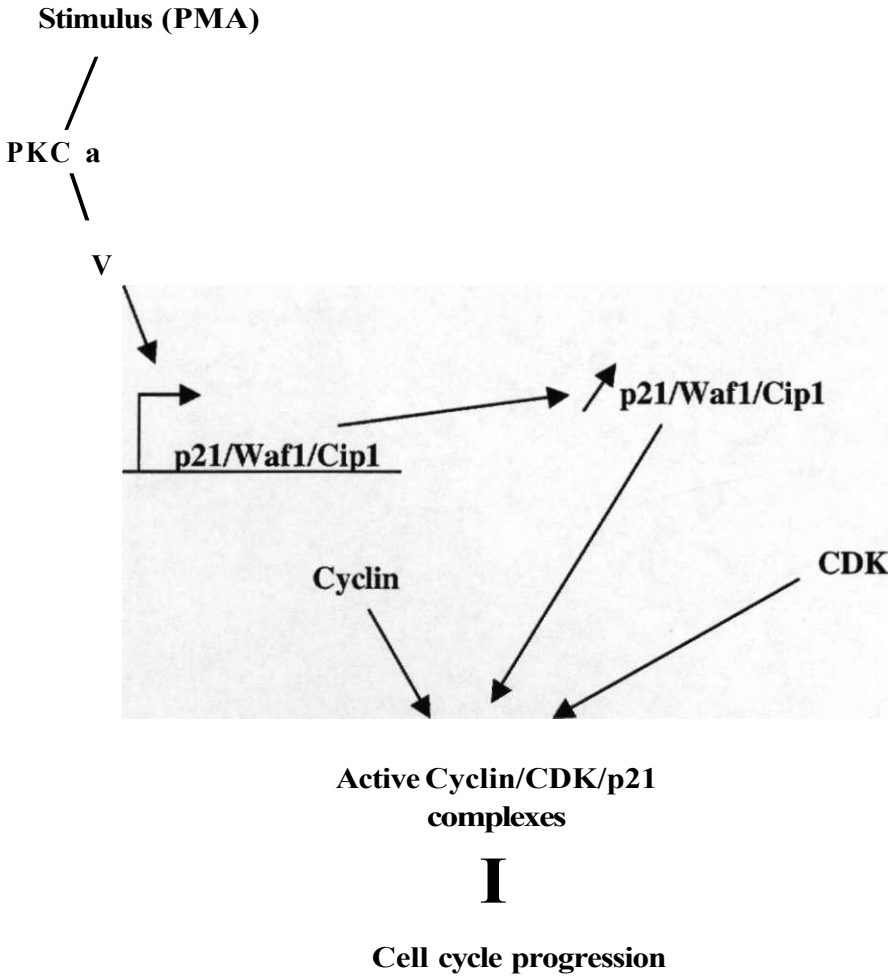


Figure 26: Summary of the data obtained in aims 1 and 2.
Stimulation of PKC a with a phorbol ester induces the upregulation of the p21/Wafl/Cipl protein. Increased p21/Wafl/Cipl levels facilitate the assembly of active ternary cyclin/CDK/p21 complexes, thus inducing cell cycle progression.

Our results indicate that p21/Cipl/Wafl upregulation was accompanied by an increase in ternary cyclin-CDK-p21 complex formation, and by an increase in their associated kinase activity. Moreover, the upregulation of p21/Cipl/Wafl appeared to be required for the PKC-induced cell cycle progression. Our results are supported by the findings that p21/Cipl/Wafl upregulation could occur in response to mitogenic signals

(Michieli et al. 1994, Nourse et al. 1994, McLeod et al. 1995, Mantel et al. 1996, Hiyama et al. 1998). More specifically, p21/Cipl/Wafl upregulation was required in order to promote proliferation of myeloid cells following steel factor (SLF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation, as bone marrow cells from p21-/- mice could not be induced to proliferate following SLF and GM-CSF stimulation (Mantel et al. 1996). Similarly, p21/Wafl/Cipl was required for PDGF-induced vascular smooth muscle cell proliferation *in vitro* (Weiss et al. 2000). Also, p21/Wafl/Cipl was required for intestinal cell mitogenic response *in vivo,* as enterocytes from p21-/- mice failed to proliferate following small bowel resection (Stern et al. 2000).

Other reports have suggested a role for p21/Cipl/Wafl as an assembly factor for cyclin-CDK complexes (reviewed in Roberts 1998, Sherr and Roberts 1999). p21/Cipl/Wafl could be associated with both catalytically active and inactive cyclin-CDK complexes (cyclin-A/CDK2 and cyclin-B/Cdc2), and a model was proposed according to which the stoichiometry of p21/Cipl/Wafl was critical to allow or inhibit kinase activity (Zhang et al. 1994, Harper et al. 1995). When one p21/Cipl/Wafl molecule was binding to cyclin/CDK, the complex was catalytically active, while binding of several p21/Cipl/Wafl subunits inhibited the complex. LaBaer et al. (1997) have shown that p21/Cipl/Wafl could function as an assembly and activity-promoting factor for cyclin D1-CDK4, cyclin D3-CDK4 and cyclin E-CDK2 complexes when p21/Cipl/Wafl levels were below a certain threshold, after which the presence of excess p21/Cipl/Wafl became inhibitory. However, several reports have contested this model, by demonstrating that a single p21/Wafl/Cipl molecule inhibits cyclin A/CDK2 complexes (Cai and Dynlacht 1998, Hengst et al. 1998, Adkins and Lumb 2000). On the other hand, Cheng et al. (1999) have shown compelling evidence for the roles played by p21/Cipl/Wafl and p27/Kipl in the regulation of cyclin/CDK complex assembly and activity. In that study, mouse embryo fibroblasts deficient for both p21/Cipl/Wafl and p27/Kipl fail to assemble detectable levels of cyclin D/CDK4 complexes, and to efficiently target cyclin-D to the nucleus (Cheng et al. 1999). Both the assembly and activity of cyclin D/CDK4 complexes could be restored by re-introducing either p21/Cipl/Wafl or p27/Kipl into those cells (Cheng et al. 1999). However, another group recently contested these results: active complexes between cyclin D1, cyclin D3 and

CDK4 were found in p21/p27-/- fibroblasts, and addition of p27/Kip1 inhibited cyclin D3/CDK4 complexes (Kumar Bagul et al. 2000).

Another attractive hypothesis that could account for the absence of inhibition of cyclin-CDK complexes by p21/Waf1/Cip1 is the existence of a class of p21-binding proteins that could modulate its inhibitory activity. The human papillomavirus type-16 (HPV-16) E7 oncoprotein can interact with p21/Waf1/Cip1 and abrogate p21-mediated inhibition of cyclin A and E-associated kinase activities (Funk et al. 1997, Jones et al. 1997). Another oncoprotein, SET, was found to bind directly to the C-terminus of p21/Waf1/Cip1 and to reverse the inhibition of cyclin E/CDK2 complexes (Estanyol et al. 1999). High levels of the SET protein were detected in U251N glioma cells (data not shown). Another protein that also binds the C-terminus of p21/Waf1/Cip1, TOK-1 (p21 and CDK interacting protein-1) was found to enhance the inhibitory effect of p21AVaf1/Cip1 toward CDK2 complexes (Ono et al. 2000). The protein CARB (Cip1-associated regulator of cyclin B) is both a p21/Waf1/Cip1 and a cyclin B binding protein. Its function may be to sequester cyclin B in the cytosol. p21/Waf1/Cip1 would then compete with CARB for binding to cyclin B and allow its nuclear translocation during G_2 (McShea et al. 2000). Calmodulin was found to bind to the C-terminus of p21/Waf1/Cip1 and to regulate its subcellular localization, as calmodulin inhibition prevented the nuclear translocation of p21/Waf1/Cip1, and therefore possibly the nuclear translocation of cyclin/CDK complexes (Taules et al. 1999). Another protein, Ciz-1 (Cip1 interacting zinc finger protein-1) was found to bind to the N-terminal domain of p21/Waf1/Cip1 (the CDK binding domain), and Ciz-1 overexpression resulted in cytoplasmic accumulation of p21/Waf1/Cip1 (Mitsui et al. 1999). Two types of p21 -interacting proteins seem to exist: some that bind to p21/Waf1/Cip1 and modulate its inhibitory activity toward cyclin/CDK complexes; and those that play a role in the regulation of subcellular localization of either p21/Waf1/Cip1 or cyclins, thereby indirectly modulating cyclin/CDK activity. Thus, one could speculate that the absence of inhibition of p21-containing cyclin-CDK complexes observed in glioma cells in response to PMA could be due to the presence of one or more of these proteins that can modulate the inhibitory activity of p21/Waf1/Cip1.

A role for phosphorylation events in the regulation of p21/Waf1/Cip1 activity is emerging. Recently, PKC r) was found associated with cyclin E/CDK2/p21 complexes in

murine and human keratinocytes; p21/Wafl/Cipl in these complexes was phosphorylated in a PKC activator-dependent manner, and this was correlated with inhibition of CDK2 activity (Kashiwagi et al. 2000). p21/Wafl/Cipl was phosphorylated on Ser-146 and Ser-160 by PKC and on Thr-145 by PKA. The Ser-146 phosphorylation of p21/Wafl/Cipl by PKC inhibited p21/Wafl/Cipl binding to proliferating cell nuclear antigen (PCNA) (Scott et al. 2000). Binding of p21/Wafl/Cipl to PCNA inhibits PCNA activity and PCNA-dependent DNA synthesis. These reports suggest that PKC, through the phosphorylation of p21/Wafl/Cipl, can modulate both the cyclin/CDK and PCNA inhibitory activities of p21/Wafl/Cipl.

Altogether, the mechanism of regulation of cyclin/CDK complexes by cyclin/CDK inhibitors appears to be a lot more complex than first anticipated, and resolving these issues will require further investigation.

The expression of a number of cell cycle regulatory proteins is altered in gliomas. Several CDKs are overexpressed in a large subset of gliomas and glioma cell lines (Sonoda et al. 1995, He et al. 1995, Costello et al. 1997, Burns et al. 1998), and our results indicate high mRNAs levels for Cdc2/CDK1, CDK4 (Fig. 15), cyclin A, cyclin B, and cyclin Dl (Fig. 17) at all times in U251N cells. On the other hand, mRNAs for p57/Kip2, pl9/INK4D, pl6/INK4A, and pl5/INK4B could not be detected in U251N cells (Fig. 16). The INK4a (encoding p16/INK4A and p19/ARF) and INK4b (encoding pl5/INK4B) genes are either deleted, mutated, or hypermethylated (thus preventing transcriptional activity) in a wide range of tumors, including gliomas (Sonoda et al. 1995, Srivenugopal et al. 1996, Herman et al. 1996). One may speculate that due to the abundance of various cyclins and CDKs, and lack of several CKIs in glioma cells, the upregulated levels of p21/Wafl/Cipl induced by PKC activation do not become inhibitory, p21/Wafl/Cipl molecules being "mopped up" by cellular cyclins and CDKs, thus resulting in increased assembly of active cyclin-CDK-p21 complexes, rather than inhibiting them, and leading to increased cell cycle progression. Our findings raise the possibility that p21/Wafl/cipl may be an active player in the pathology of glioma cells and participates to maintain a hyper-proliferative state in those cells. Furthermore, it was reported recently that the p21/Wafl/Cipl protein was elevated in 50% of cases of patients with astrocytomas, especially within the higher grades, and that p21/Wafl/Cipl

expression was associated with a shorter overall survival in patients with gliomas (Korkolopoulou et al. 1998). Interestingly, similar results were reported in bladder and prostate cancer (Lipponen et al. 1998, Aaltomaa et al. 1999). High p21/Wafl/Cipl levels in glioblastoma cells were also associated with increased resistance to chemotherapeutic agents (Ruan et al. 1998, 1999).

The transformed phenotype of glioma cells appears to be a complex interplay of numerous factors: aberrations in signaling cascades due to growth factor receptor amplification or mutations; increased PKC activity; absence of several tumor-suppressor proteins; aberrant timing of cyclin expression (Dirks et al. 1997); overexpression of several CDKs and cyclins; and abundance of p21AVafl/Cipl. All these factors may contribute to a deregulated growth control in gliomas.

CHAPTER FIVE

Aim 3: Opposite Roles for PKC a and sin the Regulation of Integrin-Mediated Adhesion and Migration.

5.1. Introduction.

The tight control of cell adhesion and motility is crucial for a wide variety of physiological and pathological processes, such as embryogenesis, inflammation, angiogenesis, wound healing, and tumor metastasis. Integrins are heterodimeric cell surface receptors that mediate cell-cell and cell-ECM interactions, and have been involved in the regulation of cell growth, migration, survival, and metastasis (reviewed in Hynes 1992, Hughes and Pfaff 1998, Aplin et al. 1999, Giancotti and Ruoslahti 1999). Eight integrin (3 subunits and 17 a subunits have been identified to date (Plow et al. 2000), and these can form over 20 distinct heterodimers. Integrin affinity and avidity can be modulated by intracellular signaling cascades ("inside-out" signaling), leading to changes in adhesion and motility; conversely, binding of integrins to ECM proteins elicits signals that are transduced into the cell ("outside-in" signaling). Integrins are central components of focal adhesions, in which they associate with cytoskeleton associated proteins such as vinculin, talin, and paxillin, and signaling molecules such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (Hemler 1998, Giancotti and Ruoslahti 1999). A number of intracellular signaling pathways have been involved in the regulation of integrin adhesive functions, including PI 3-kinase, Src, and the small GTP-binding proteins R-Ras, H-Ras, and Rho (Kolanus and Seed 1997, Hughes and Pfaff 1998). Among the proteins implicated in inside-out signaling, PKC has been found in many instances to play a crucial role in modulating integrin-mediated cell adhesion, spreading, and migration. However, the mechanism of action of PKC in these events remains elusive.

The importance of PKC in integrin-mediated events has been described in a number of reports. PKC activity was shown to be required for adhesion, spreading, migration, and focal adhesion and actin stress fiber assembly, on various ECM substrates (Woods and Couchman 1992, Vuori and Ruoslahti 1993, Lewis et al. 1996, Disatnik and Rando

1999). In addition to its role in focal adhesion formation, PKC activation induces the translocation of FAK (Vuori and Ruoslahti 1993, DeNichilo and Yamada 1996, Lewis et al. 1996, Disatnik and Rando 1999) and PYK2 (Litvak et al. 2000) to focal adhesions and their tyrosine phosphorylation in various cell systems. In some studies, the identity of the PKC isoform involved in integrin-mediated processes has been investigated. It appears that, depending on the cell type, different PKC isoforms are involved in the regulation of integrin-mediated processes (Harrington et al. 1997, Tang et al. 1997b, Haller et al. 1998, Laudanna et al. 1998, Ng et al. 1999). For example, both PKC *a* and 8 were translocated to focal adhesions during vascular smooth muscle cell adhesion on fibronectin, and their specific inhibition by antisense oligonucleotide inhibited cell spreading (Haller et al. 1998). In breast carcinoma cells, an interaction between integrin Pi and PKC a was demonstrated, and overexpression of PKC a stimulated Pi-dependent migration by facilitating integrin pi endocytosis and recycling to the plasma membrane (Ngetal. 1999).

Few studies have focused on the events upstream of PKC in the regulation of integrins. The CDK inhibitor p16/INK4a could block a,**p3**-dependent cell spreading on vitronectin upstream of PKC, by blocking PKC-dependent localization of a,**P3** to focal adhesions; this inhibition could be reversed by CDK6 overexpression (Fahraeus and Lane 1999). The epidermal growth factor receptor (EGFR) could induce ct,**p5**-mediated migration on vitronectin in a PKC-dependent manner (Klemke et al. 1994). Similarly, PKC a was required for EGF-induced integrin Of^-dependent migration on laminin-1; this was associated with the phosphorylation on serine of the **P4** integrin by PKC (Rabinovitz et al. 1999). Despite considerable evidence describing the importance of PKC in integrin-mediated adhesion, the mechanism by which PKC regulates these processes remains poorly understood.

The ability of GBM cells to migrate great distances from the primary tumor mass prevents complete resection of the tumor by conventional surgical methods and is thought to be a major cause of mortality for glioma patients (Holland 2000c); however, the mechanism that these cells utilize to migrate is poorly understood. Inhibition of PLC *y,* an activator of PKC, blocked glioma cell migration and invasion *in vitro* (Khoshyomm et al. 1999). Furthermore, various inhibitors of PKC could reduce glioma cell motility and

invasion (Zhang et al. 1997), while PKC activation with phorbol esters promoted migration *in vitro* and PKC downregulation inhibited migration (Tysnes and Laerum 1993). Thus, PKC may be an important player in the regulation of invasive and migratory processes.

In Chapter one, we have shown that U251N glioma cells express the PKC isoforms a, 8, e, T|, p, and and that PKC a controls cell cycle progression and proliferation (Besson and Yong 2000). Here, we report that PKC £ positively regulates integrin-dependent adhesion and motility in glioma cells, while PKC a plays an opposite role. PKC £ activation induces focal adhesion and lamellipodia formation, and integrin clustering. Thus, our data indicate an important role for PKC in the regulation of integrin-mediated adhesion and motility in human glioma cells.

5.2. **Results.**

5.2.1. PKC a and £ play opposite roles in the regulation of glioma cell migration.

To determine whether PKC could play a role in glioma cell migration, we evaluated the motility of U251N glioma cells, in the presence or absence of the phorbol ester PMA, a potent activator of conventional and novel PKC isoforms. PMA treatment increased the motility of cells over the 72 h period analyzed (Fig. 27). We have previously shown that U251N glioma cells express the PKC isoforms a, 8, £, rj, p, and but not pi, p_2, l, and 0 (Fig. 7) (Besson and Yong 2000). To determine which isoform was responsible for increasing cell motility, we analyzed the translocation pattern, following PMA stimulation, of the 6 PKC isoforms expressed over a 72 h period. Translocation of PKC from the cytosol to the membrane is a hallmark of its activation (Jaken 1996, Newton 1997). Upon PMA treatment, PKC a and £ were the only isoforms translocated from the cytosolic to the particulate fraction in these cells (Fig. 28), as previously observed (Fig. 11) (Besson and Yong 2000). PKC 8, r), p, and £ remained unaffected by PMA (neither translocated nor downregulated). PKC a was totally downregulated by 24 h of treatment, and remained absent at 72 h. On the other hand, PKC £ was only partially downregulated and remained translocated at 72 h.

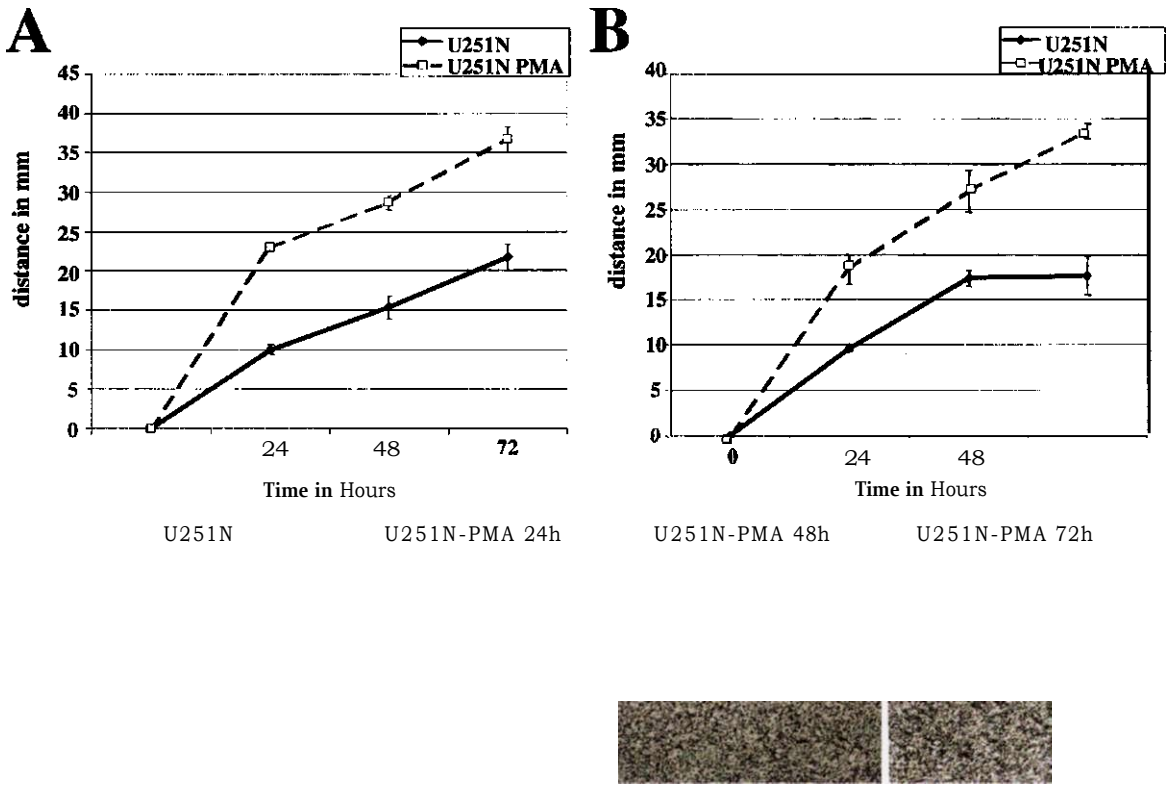


Figure 27: PKC activation induces migration of human glioma cell. (A-B) Two individual migration assays on U251N cells in the absence or presence of the phorbol ester PMA (100 nM). Cells were fixed at the indicated time and stained with hematoxylin. Pictures were taken at a 40x magnification and the distance migrated was measured on the prints, in mm. Results for this, and all subsequent graphs, are plotted as mean +/- SEM. (C) Representative pictures of a migration assay. Cells were fixed and stained with hematoxylin. Pictures were taken at a 40x magnification on an inverted microscope.

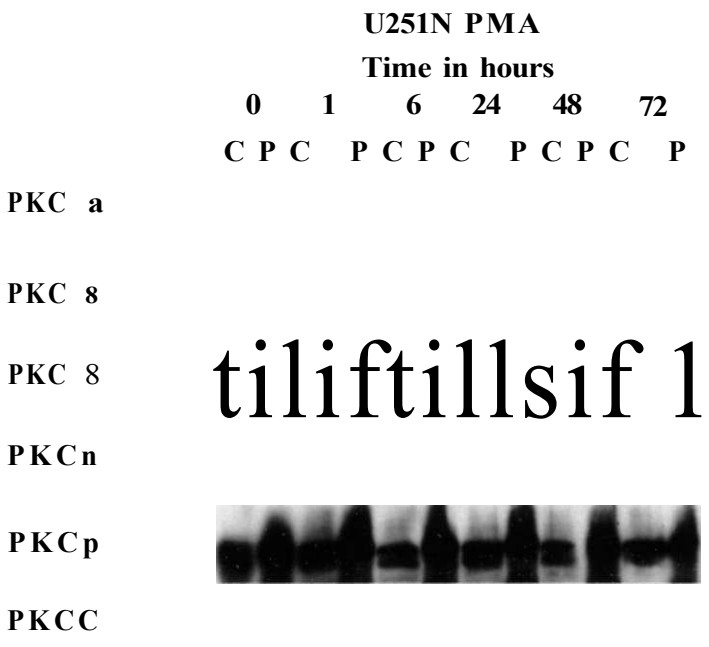


Figure 28: Translocation of PKC a and e by PMA. Cells were treated with 100 nM PMA, collected at various times, and fractionated into Digitonin-soluble (cytosolic, C) and Digitonin-insoluble (particulate, P) fractions. Both PKC a and e were translocated in response to PMA. PKC a was totally downregulated by 24 h. In contrast, PKC e was only partially downregulated and remained translocated at 72 h. PKC 8, rj, p, and £ were neither translocated nor downregulated by PMA. PKC a was 80 kDa, e was 90 kDa, 8 was 78 kDa, T| was 78 kDa, p was 115 kDa, and £ was 72 kDa. Fifty pg of proteins were loaded per well.

To dissect the roles of these 2 isoforms, we generated clones stably overexpressing either PKC a or £. Two representative clones for each isoform were used in this study; their respective expression levels are shown in Fig. 29.

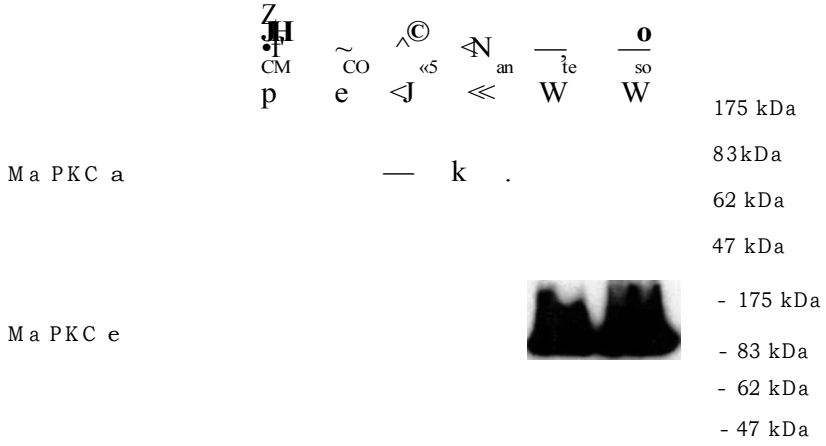


Figure 29: Overexpression of PKC a and s.
Western blot analysis of wild type cells (U251N), control vector transfected cells (pBK), 2 clones overexpressing PKC a (As20 and As27) and 2 clones overexpressing PKC £ (Esl and EslO), probed for PKC a (top panel), or PKC £ (bottom panel). Endogenous levels of PKC a and £ seem very low due to a short exposure to avoid saturating the signal in overexpressing clones. Fifty pg of proteins were loaded per well.

The effect of PKC overexpression was compared to the wild type U251N and vector transfected cells (pBK) in a motility assay (Fig. 30). In the absence of PMA stimulation, the PKC a overexpressing clones (As20 and As27) migrated slightly slower than the wild type or control vector cells, and the PKC £ overexpressing clones migrated slightly faster (Fig. 30, left panel). However, when cells were incubated with 100 nM PMA, these differences were exacerbated and it became clear that PKC £ overexpression increased motility, while PKC a overexpression decreased cell motility (Fig. 30, right panel). Together, these results suggest that PKC £ positively regulates glioma cell migration, while PKC a plays an opposite role. To verify that the increased motility induced by PMA was due to the activation of PKC, as PMA has been reported to activate other targets (Kazanietz 2000), we performed motility assays in the presence of two different PKC-specific inhibitors, Calphostin C and Bisindolylmaleimide I. The presence of either of these inhibitors substantially decreased the basal migration level (in the absence of PMA) of pBK and EslO cells, and completely abolished the increased motility induced

by PMA in both cell lines (Fig. 31), indicating that the effect of PMA on motility was indeed a consequence of PKC activation.

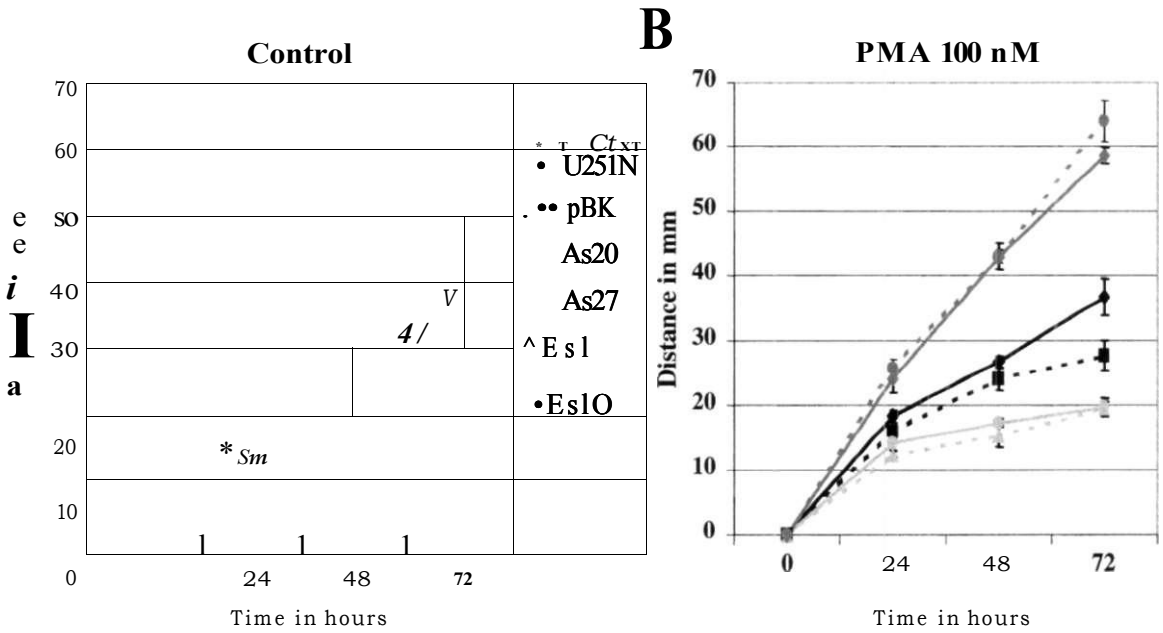


Figure 30: PKC E positively regulates migration while PKC a has an opposite role. Migration assay on wild type U251N cells, control vector transfected cells (pBK), 2 clones overexpressing PKC a (As20 and As27) and 2 clones overexpressing PKC e (Esl and EslO), in the absence of PMA (left panel) or in the presence of 100 nM PMA (right panel) over a 72 h period. PKC e overexpressing cells migrate faster than control cells, while PKC a overexpressing cells have a reduced migration comnared to control cells.

5.2.2. PKC e activation increases focal adhesion formation.

Focal adhesions are the sites of interaction between a cell and its extracellular environment and play a critical role in cell adhesion and migration. PKC has previously been reported to regulate focal adhesion formation in other cell types (Woods and Couchman 1992, Vuori and Ruoslahti 1993, Lewis et al. 1996, Disatnik and Rando 1999); we therefore addressed whether PKC activation in glioma cells was affecting focal adhesions. Following PKC activation with PMA, there was an increase in the number of focal adhesions after 2 h, as seen by vinculin and FAK staining (Fig. 32). By 24 h, these focal adhesions were clustered at the edges of the lamellipodia, suggestive of a motile phenotype. PMA treatment was also accompanied by an increase in tyrosine phosphorylation levels of vinculin and FAK, as assessed by Western blot analysis using the phosphotyrosine-specific antibody 4G10 (Fig. 33).

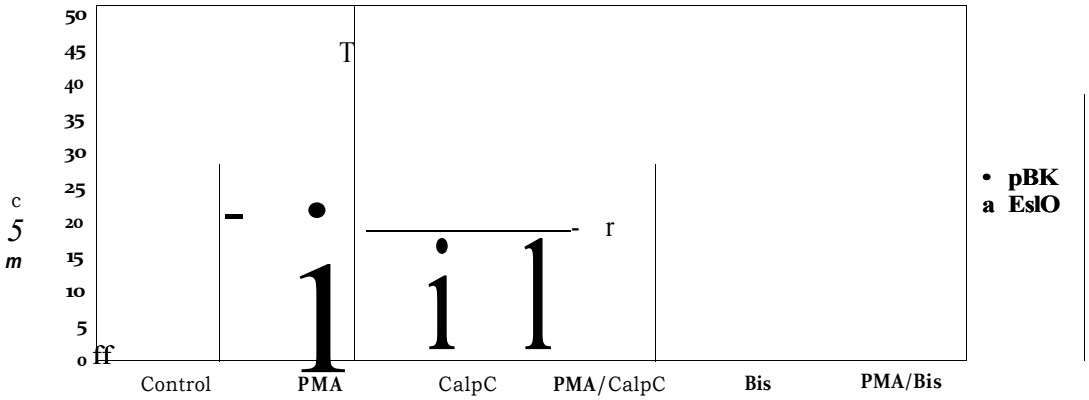


Figure 31: Inhibition of PMA-induced migration by PKC inhibitors, indicating that PMA induces migration through PKC activation. The inhibitor (Calphostin C [CalpC], 200 nM; or Bisindolylmaleimide I [BisI], 5 pM) was added at the beginning of the experiment or 1 h prior PMA stimulation of EslO (gray bars) or pBK cells (black bars). Cells were allowed to migrate for 48 h, and then fixed and stained.

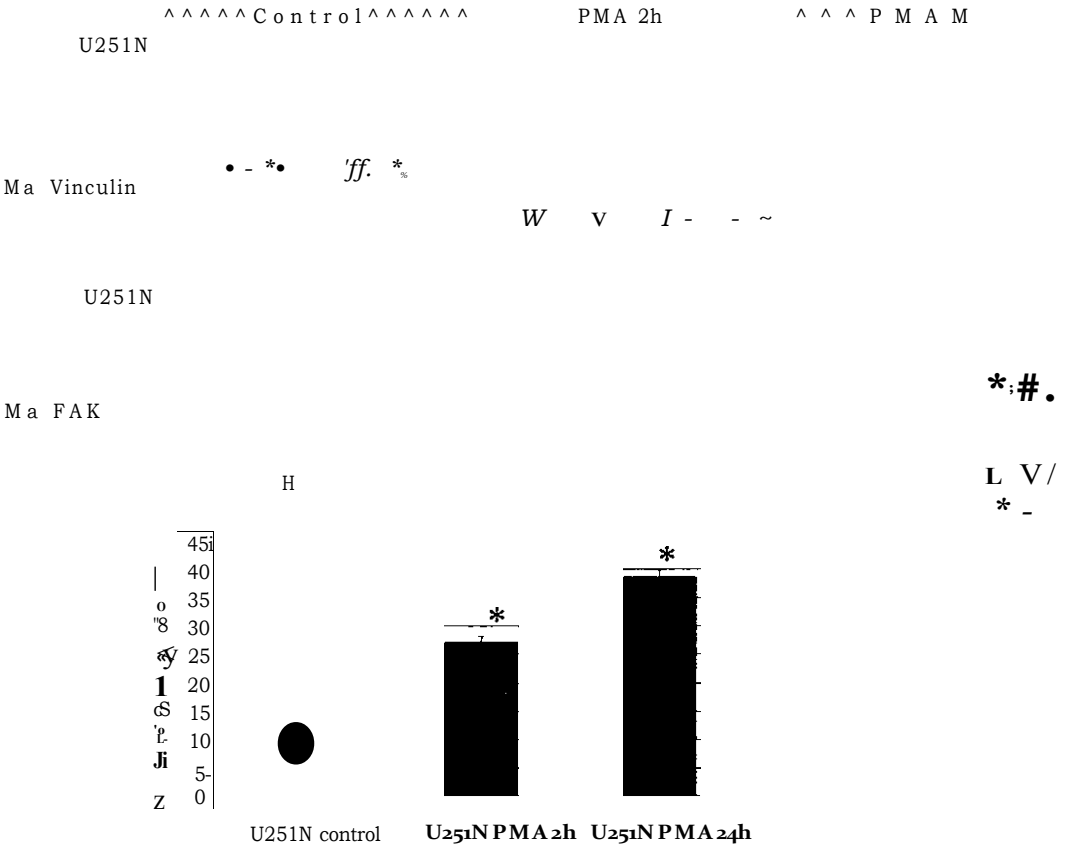


Figure 32: PKC activation increases focal adhesion formation.
U251N glioma cells were stimulated (or not, Control) with 100 nM PMA for 2 h or 24 h, fixed, and stained with a mouse anti-vinculin (1/500) antibody (A) or with a mouse anti-FAK (1/200) antibody (B) to visualize focal adhesions. (C) Count of the number of focal adhesions per cell, using the Image Pro image analysis program. A minimum of 180 cells was counted for each timepoint. *= $p < 0.001$ when compared to control in a one way ANOVA with Bonferroni multiole analvsis comparison test.

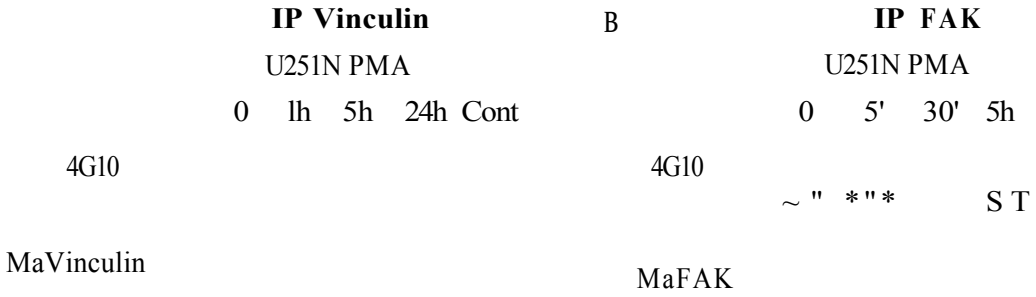


Figure 33: PKC activation induces the tyrosine phosphorylation of vinculin and FAK. Vinculin (A) or FAK (B) were immunoprecipitated from U251N cell lysates at various times following PMA (100 nM) stimulation. Immunoprecipitates were run on SDS-PAGE and tyrosine phosphorylation levels were monitored by probing with a monoclonal 4G10 (1/1000) antibody. Membranes were reprobed with a mouse anti-vinculin (A) or mouse anti-FAK (B) to control for loading. A goat anti-IgG/IgM antibody was used in the control immunoprecipitation (Cont).

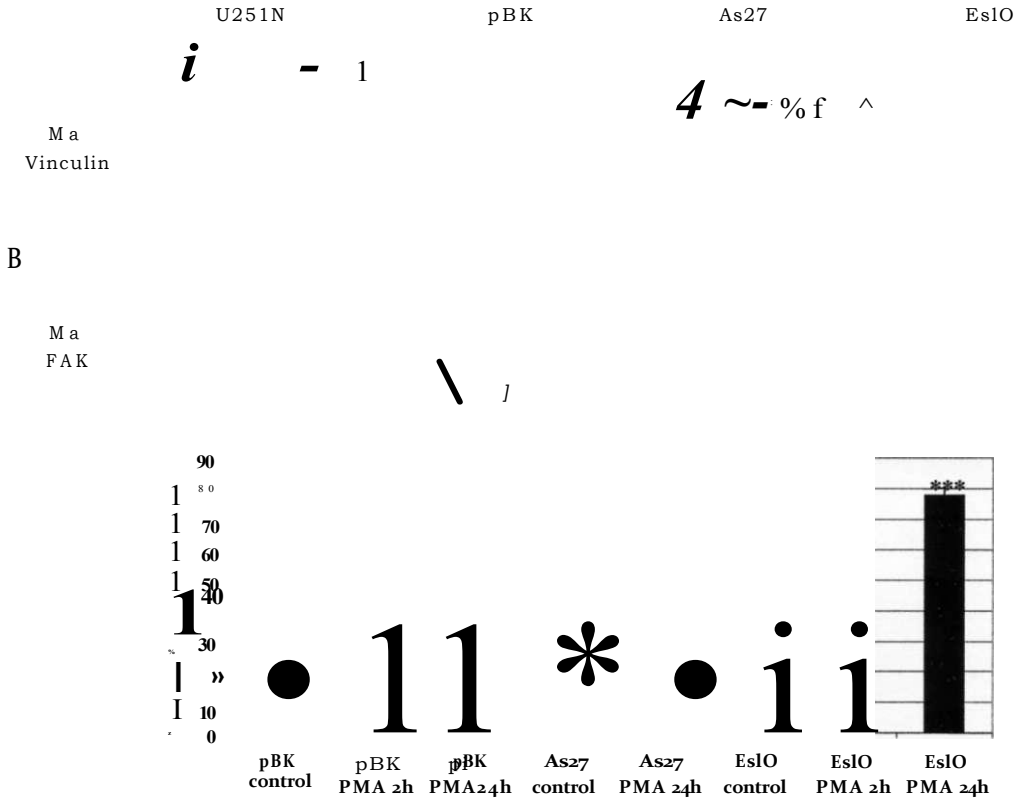


Figure 34: Increased number of focal adhesions in PKC s overexpressing cells. Focal adhesions were visualized by vinculin (A) or FAK (B) staining in unstimulated U251N, pBK, As27, and EslO cells. EslO cells exhibit an increased number of focal adhesions in the absence of PMA stimulation compared to U251N or pBK cells. The reverse is observed in As27 cells. (C) Count of the number of focal adhesions per cell, using the Image Pro image analysis program. A minimum of 160 cells was counted for each timepoint. *= p< 0.001 when compared to pBK control; **= p<0.001 when compared to pBK PMA 2 h; ***= p<0.001 when compered to pBK PMA 24 h in a one way ANOVA with Bonferroni multiple analysis comparison test.

To address more specifically the role of individual PKC isoforms in focal adhesion formation, we monitored the number of focal adhesions by vinculin and FAK staining, in the absence of PMA, in wild type, control vector (pBK), As27, and EslO cells (Fig. 34). Interestingly, the slower migrating As27 cells had a reduced number of focal adhesions. In contrast, the faster migrating EslO cells, that overexpress PKC e, exhibited an increased number of focal adhesions compared to wild type or pBK cells. These differences were still apparent following PMA stimulation of these cells (data not shown). Taken together, the data indicate that PKC £, which positively regulates glioma cell migration, facilitates focal adhesion assembly. On the other hand, PKC a overexpression reduces both the migrating ability of these cells and the number of focal adhesions.

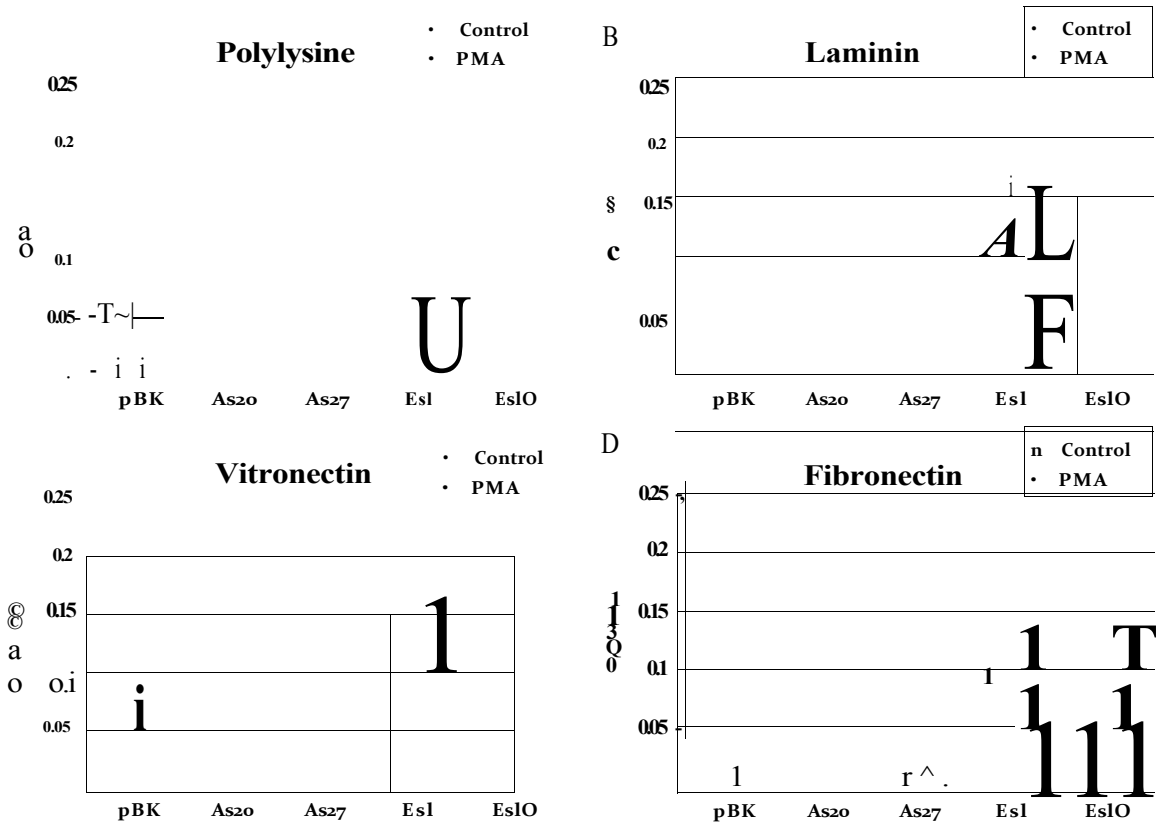


Figure 35: Opposite roles for PKC a and e in the regulation of integrin mediated adhesion. Adhesion assay on control vector transfected cells (pBK), PKC e overexpressing cells (Esl and EslO), and PKC a overexpressing cells (As20 and As27) on Poly-L-lysine (A), used as a non-integrin substrate, laminin (B), vitronectin (C), and fibronectin (D). PKC e overexpressing cells exhibited an increased adhesion on all integrin substrates tested and this was further increased following PMA (100 nM) treatment. On the other hand, PKC a overexpressing cells had a decrease in adhesion on vitronectin and fibronectin upon PMA treatment.

5.2.3. Opposite roles for PKC a and £ in the modulation of integrin-mediated adhesion.

Integrins are crucial components of focal adhesions and mediate cellular attachment to ECM proteins. Since PMA stimulation increases focal adhesion assembly, we investigated whether integrin-mediated adhesion was altered following PKC activation in glioma cells, and more particularly by the overexpression of either PKC a or £. We performed adhesion assays on various integrin substrates (laminin, vitronectin, and fibronectin) while poly-L-lysine was used as a control for integrin-independent adhesion (Fig. 35). Adhesion on poly-L-lysine was not significantly affected by PMA treatment or by the overexpression of either PKC a or £ (Fig. 35A). However, adhesion on laminin, vitronectin, and fibronectin was increased following PMA stimulation (Fig. 35B-D, black bars). More importantly, Es1 and EslO cells that overexpress PKC £ exhibited an increased adhesion on all substrates tested, and adhesion was further enhanced by PMA (Fig. 35B-D). On the other hand, PKC a overexpressing cells, As20 and As27, exhibited a reduced adhesion on fibronectin (Fig. 35D), and to a lesser extent on vitronectin (Fig. 35C), following PMA stimulation.

To confirm that the PMA-induced increase in adhesion was indeed a consequence of PKC activation, adhesion assays were performed on EslO cells in the presence of the PKC inhibitors Calphostin C or Bisindolylmaleimide I (Fig. 36). Bisindolylmaleimide I did not decrease the basal adhesion level, but completely abolished the PMA induced adhesion. In contrast, Calphostin C decreased both the basal and PMA-induced adhesion. These experiments indicate that the effect of PMA on adhesion is a consequence of PKC activation. Interestingly, PMA stimulation also increased cell spreading on integrin substrates, and PKC £ overexpressing cells could spread more rapidly than wild type or pBK cells. On the other hand, cells overexpressing PKC a had an impaired spreading (data not shown). Collectively, the data indicate that PKC £ positively regulates integrin-mediated adhesion, while PKC a seems to negatively regulate adhesion on specific integrin substrates, such as fibronectin.

shown). To address more specifically the role of individual PKC isoforms in integrin clustering, wild type, pBK, As27, and EslO cells were stained for specific integrins. EslO cells, but not the others, had a partial clustering of the above-mentioned integrins in the absence of PMA (data not shown), suggesting the involvement of PKC e in integrin clustering.

To confirm the involvement of specific integrins in PMA-induced adhesion, we performed adhesion assays in the presence of blocking antibodies targeted against the integrin chains affected by PMA. The addition of blocking antibodies completely abolished the PMA-induced integrin-mediated adhesion on their respective substrate, while an isotype control antibody had no effect on adhesion (Fig. 38). Similarly, addition of RGD peptides (100 nM) abrogated the effect of PMA on adhesion on fibronectin and vitronectin (Fig. 38B-C). The data suggest that PKC activation regulates the clustering of specific integrin heterodimers, leading to increased adhesion, spreading, and migration.

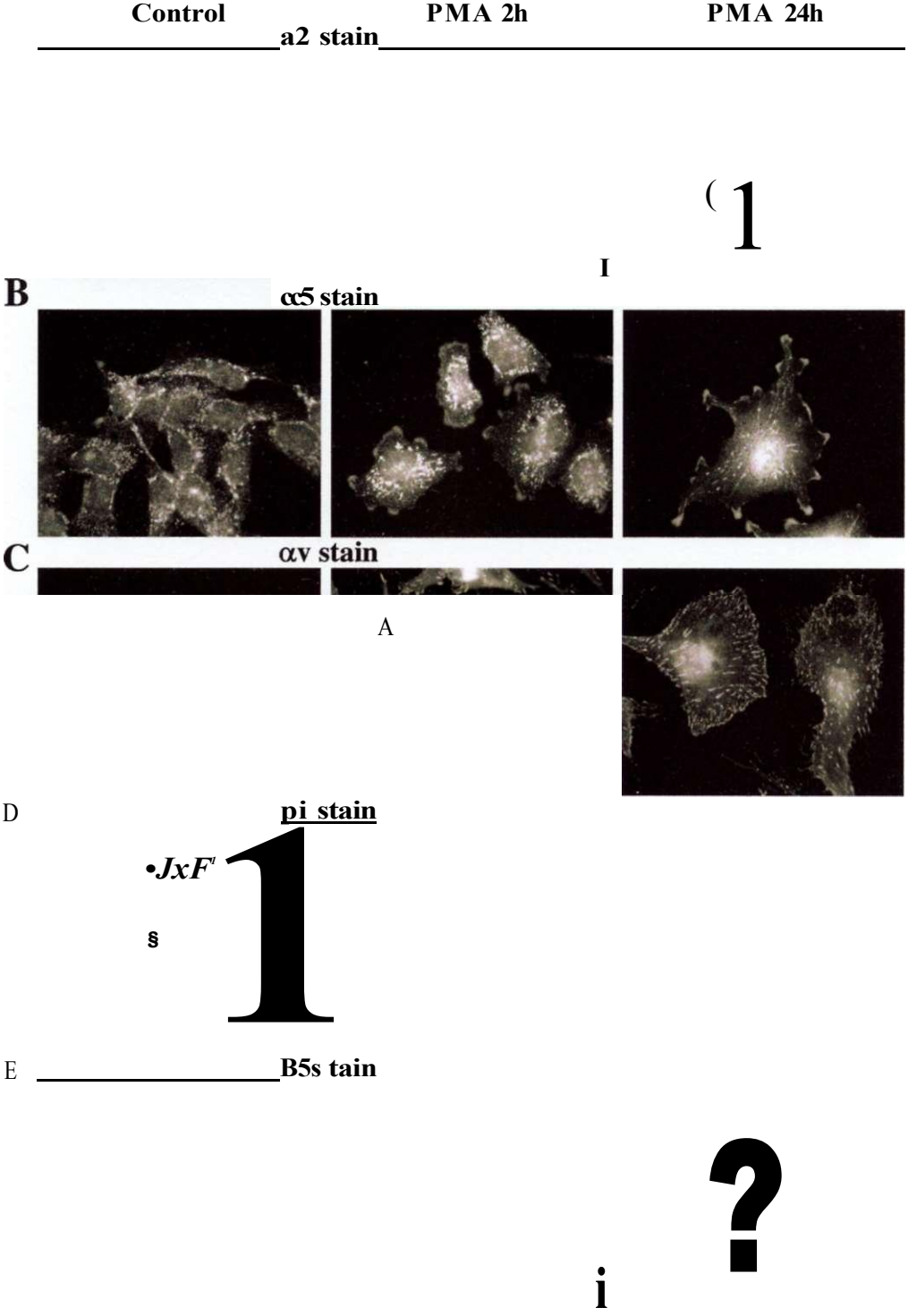


Figure 37: PKC activation induces the clustering of select integrins.
Upon PMA stimulation, several integrin chains are relocated to either the lamellipodia, in the case of integrin a_2, as, pi, and Ps, or to focal adhesions, in the case of cc_v and Pi. Cells plated on glass coverslips were treated with PMA (100 nM) for 2 h (middle column) and 24 h (right column), or with DMSO (control, left column), fixed, permeabilized, and stained for specific integrin chains at a 1/100 dilution: (A) Mouse anti-a_2. (B) Mouse anti-a_5. (C) Mouse anti-a_v. (D) Mouse anti-Pi. (E) Rabbit anti-p_5.

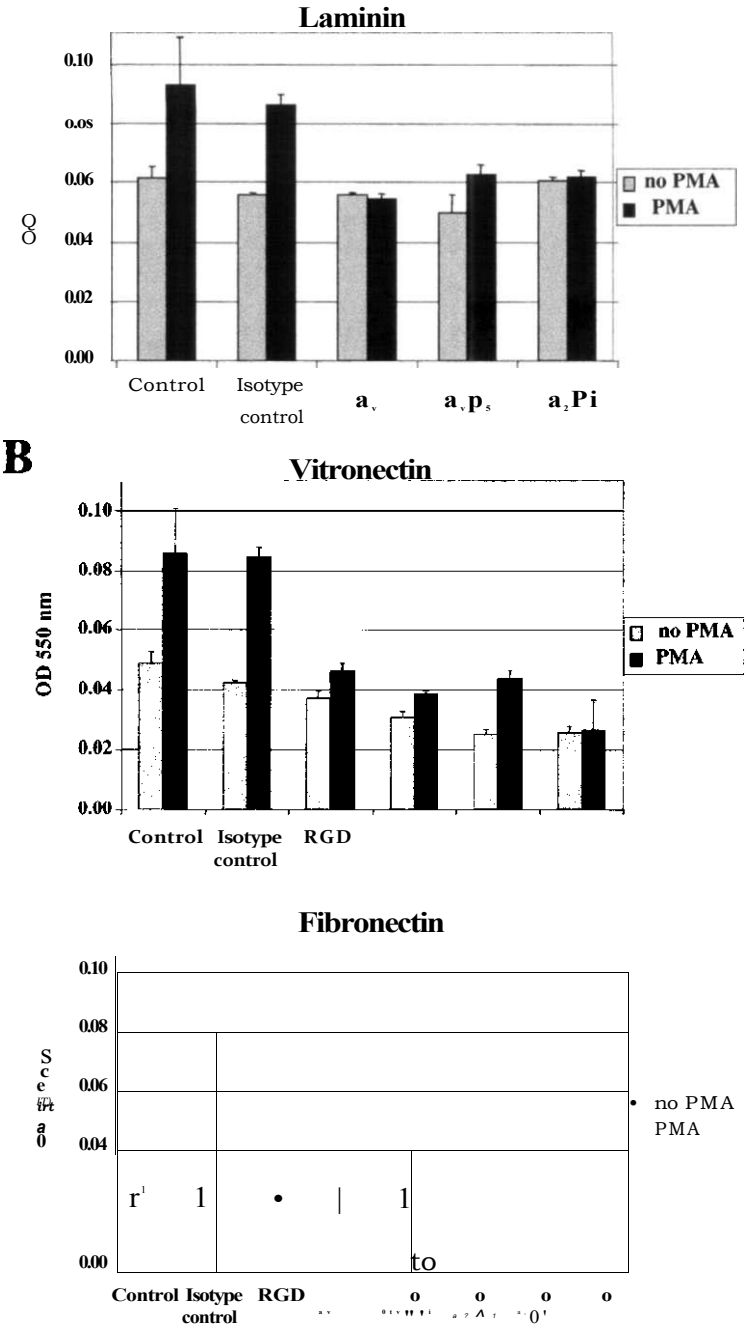


Figure 38: Blockade of PKC-induced adhesion by interfering with integrin function.
Adhesion on laminin (A), vitronectin (B), or fibronectin (C) of EslO cells in the presence or absence of 100 nM PMA, with pre-incubation of the cells with RGD peptide or blocking antibodies for the integrins previously shown to be clustered following PMA treatment. No effect on adhesion was observed with an isotype control antibody. Integrin blocking antibodies were tested on the substrate known to bind to the specific integrin (Plow et al., 2000). Cells were pre-incubated with the blocking antibody or isotype control, at 10 mg/ml, or with RGD peptide (100 nM) for 15 min at 4°C before the adhesion assay.

5.3. Discussion.

PKC activity plays a critical role in integrin-mediated adhesion, spreading, migration, and focal adhesion assembly in other cell types (Woods and Couchman 1992, Vuori and Ruoslahti 1993, Lewis et al. 1996, Disatnik and Rando 1999). However, it remains

unclear whether PKC plays a role in the regulation of integrin function in gliomas, and the identity of the PKC isoforms involved is unknown. Here, we demonstrate that PKC e is involved in the regulation of integrin-mediated adhesion, migration, and focal adhesion formation in human glioma cells.

In human glioma cells, PKC a and e appear to play opposite roles in the regulation of adhesion, migration, and focal adhesion formation. Similarly, in vascular endothelial cells, different PKC isoforms were found to exert different roles in adhesion or migration. PKC a and 6 were found to increase cell migration, without effect on adhesion, and PKC 8 overexpression increased adhesion on vitronectin; furthermore, both PKC a and 9 increased cell cycle progression and PKC 8 inhibited proliferation (Harrington et al. 1997, Tang et al. 1997b). In our system, PKC e acts as a positive regulator of integrin-mediated adhesion and migration of glioma cells, while PKC a inhibits these processes. It remains unclear at this point whether the negative role of PKC *a* in integrin-mediated events involves an active process that leads to focal adhesion disassembly or inhibition of integrin signaling. Another possibility is that PKC a counteracts adhesion and migration by gearing the cell toward a proliferative pathway, likely to be incompatible with motility (go or grow hypothesis), as we have previously shown that PKC a is required for cell cycle progression and proliferation in glioma cells (Besson and Yong 2000). In contrast, PKC e overexpression or depletion had no effect on cell proliferation (data not shown). Also, biochemical fractionation of the cells into cytoplasmic and nuclear fractions revealed that both isoforms are targeted to different subcellular localizations upon activation with PMA: PKC a is translocated to the nucleus (nuclear envelope) while PKC e is retained in the cytoplasmic fraction (plasma membrane), providing further evidence for the different roles played by these two isoforms (see Fig. 50 in the next chapter).

Inhibition of PKC by 2 distinct inhibitors, Calphostin C, which binds to the regulatory domain of PKC, and Bisindolylmaleimide I, which binds to the ATP binding site, led to different cellular responses in the adhesion experiments (Fig. 36). Calphostin C inhibited both basal and PMA-induced adhesion, while Bisindolylmaleimide I inhibited only PMA-induced adhesion. One may speculate that calphostin C inhibits functions that are linked to the regulatory domain of PKC, thus preventing potential roles of PKC that are

independent of its catalytic activity, or preventing other proteins from associating with PKC through its regulatory domain (see Discussion in Chapter 6: Aim 4).

It remains unclear why PKC activation induces the clustering of some integrins (ot2, oC5, a_v, Pi, and p_s) but not others (oti, CC3, 04, 0t6, and P4). This likely reflects the diversity of integrin functions and the mechanisms by which integrins are regulated by inside-out signaling cascades. PKC activation also results in an increase in the number of focal adhesions. Regulation of focal adhesion formation and turnover is critical for the reorganization of focal contacts during migration. Cells lacking Src family kinases or FAK have a migratory defect due to inhibition of focal adhesion turnover, while their formation is not affected; therefore, these cells exhibit an increased number of focal adhesions (Klinghoffer et al.1999, Sieg et al. 1999). In our system, we did not monitor the turnover rate of focal adhesions in PMA versus untreated cells, to determine whether PKC also affects the turnover of focal adhesions. However, it is likely that the PKC-induced focal adhesion formation is accompanied by an increased turnover of focal adhesions, since we observed an increase in cell migration following PMA treatment.

Altogether, we have found that PKC a and e play opposite roles in the regulation of integrin-mediated adhesion and migration in human glioma cells.

CHAPTER SIX

Aim 4: Mechanism of PKC-induced Integrin-Mediated Adhesion and Migration.

6.1. Introduction.

A prominent factor in the pathology of malignant gliomas is their ability to migrate from the primary tumor mass and to diffusely infiltrate the brain parenchyma, prohibiting complete resection of the tumor by surgery (Holland 2000c). A better understanding of the processes controlling glioma migration is thus required in order to improve the therapy of the disease. Several cell surface receptors are involved in promoting glioma cell migration, including the epidermal growth factor receptor (Lund-Johansen et al. 1990, El-Obeid et al. 1997), and several integrins (Friedlander et al. 1996). Downstream of these cell surface receptors, activation of the PKC pathway has been implicated in the regulation of glioma cell migration: inhibition of PLC y, an enzyme involved in PKC activation, blocked glioma cell migration and invasion *in vitro* (Khoshyomm et al. 1999). Furthermore, inhibition of PKC could inhibit glioma cell motility and invasion (Zhang et al. 1997), while PKC activation with phorbol esters promoted migration *in vitro* (Tysnes and Laerum 1993).

A means of PKC regulation is through their association with targeting proteins, providing a tight control of PKC subcellular localization and substrate specificity (Mochly-Rosen and Gordon 1998, Jaken and Parker 2000). One such protein, RACK1 (Receptor for Activated C-Kinase 1), specifically binds to activated PKC (Mochly-Rosen et al. 1991, Ron et al. 1994). RACK1 is a 36-kDa protein formed of 7 WD-40 repeats; WD-40 repeats are usually involved in protein-protein interactions. It was recently found that RACK1 interacts with the membrane proximal region of the cytoplasmic tail of integrins pi, P2, and P5 (Liliental and Chang 1998). RACK1 can also associate with other signaling proteins, such as phospholipase C yi (Disatnik et al. 1994), c-Src (Chang et al. 1998), the cAMP-specific phosphodiesterase PDE4D5 (Yarwood et al. 1999), and the p chain of the IL-5/IL-3/GM-CSF receptors, allowing the recruitment of PKC P to the receptor following IL-5 or PMA stimulation (Geijsen et al. 1999). Thus, RACK1 may act

as a scaffold or anchoring protein that regulates the localization of various signaling enzymes to specific subcellular compartments, to allow the formation of signaling complexes. Interestingly, another WD-40 repeats-containing protein, WAIT-1, was found to interact with the cytoplasmic tail of integrin (Rietzler et al. 1998), suggesting that WD-40 repeats proteins may constitute a common means of regulation of integrins. However, the functional significance of the interaction between these proteins and integrins has not been investigated.

The involvement of PKC in the activation of the ERK pathway (reviewed in Schaeffer and Weber 1999) has been well documented in other cell systems (El-Shemerly et al. 1997, Schonwasser et al. 1998, Howe and Juliano 1998, Miranti et al. 1999, Short et al. 2000, Rigot et al. 1998, Traub et al. 1997). In several cell types, PKC was required for integrin-mediated ERK activation (Howe and Juliano 1998, Miranti et al. 1999, Short et al. 2000, Rigot et al. 1998, Traub et al. 1997). In addition, activation of the ERK pathway following different stimuli at the cell surface was required for cell migration in various cell systems (Rigot et al. 1998, Klemke et al. 1997, Nguyen et al. 1999). For example, ERK activation was required for PKC-induced migration of colon carcinoma cells (Rigot et al. 1998). Also, the e isoform of PKC was found to mediate ERK1/2 activation following adhesion on fibronectin in bovine aortic endothelial cells (Traub et al. 1997).

We have previously shown that several PKC isoforms regulate different phenotypes of human glioma cells. PKC a was found to regulate cell cycle progression and proliferation (Besson and Yong 2000), and to negatively regulate adhesion and motility. On the other hand, PKC **E** activation resulted in increased focal adhesion formation, clustering of several integrins, and enhanced integrin-mediated adhesion and motility. Here we address the mechanism by which PKC regulates adhesion and migration of human glioma cells. We provide evidence that PKC £ regulates integrin-mediated adhesion and motility through its association with the anchoring protein RACK1 and with integrin (3 chains. We also found that ERK is targeted to focal adhesions following activation by PKC. Our data indicate that the targeting of activated ERKs to focal adhesions is dependent on PKC activity. Moreover, ERK activation is required for PKC-induced integrin-mediated adhesion and motility of human glioma cells.

6.2. Results.

6.2.1. RACK1 links activated PKC 8 to integrin P chains.

Despite considerable evidence showing the importance of PKC in integrin-mediated processes, the mechanism by which PKC modulates integrin activity remains unclear. Interestingly, the PKC anchoring protein RACK1 was recently shown to bind the cytoplasmic tail of several p integrins (Liliental and Chang 1998). To test whether RACK1 was involved in the integrin-mediated events induced by PKC activation in glioma cells, we performed co-immunoprecipitation experiments using PKC, RACK1, or integrin antibodies at various times following PMA treatment. Upon PMA stimulation, a rapid and stable association between PKC £ and RACK1 was detected; Pi and Ps integrins were also part of this complex (Fig. 39A). These results suggest that upon activation, PKC £ associates with RACK1 and with integrin P chains. To determine whether the association between PKC £ and RACK1 was specific, PKC a immunoprecipitations were performed, and no RACK1 could be detected in the immunoprecipitates (Fig. 39B).

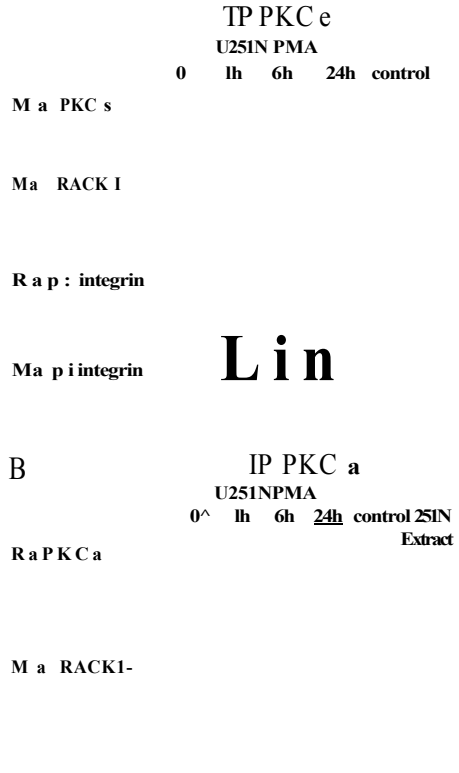


Figure 39: RACK1 and integrin pj **and** p_s **chains co-precipitate with activated PKC** e. Co-immunoprecipitation experiments on U251N cell lysates at various timepoints following PMA (100 nM) stimulation. Immunoprecipitates were run on SDS-PAGE and probed as indicated. (A) PKC £ immunoprecipitation shows the association with RACK1, and integrin pj and p_s following PMA treatment. (B) PKC a immunoprecipitation shows no interaction between RACK1 and PKC a. No PKC a was immunoprecipitated at the PMA 24 h timepoint because of the downregulation of the protein. Control refers to an JP performed with 5 pg of goat anti-IgG+M antibody.

To confirm the formation of a complex between RACK1, PKC e, and integrin P chains, reciprocal immunoprecipitations were carried out using either RACK1 or Pi integrin antibodies (Fig. 40 and 41, respectively). Following PMA treatment, PKC e (but not a), integrins Pi and Ps, and FAK could be co-precipitated with RACK1 (Fig. 40) and appear to form a complex stable over the 24 h-period studied. Similarly, after PMA stimulation, integrin pi associated with RACK1, PKC e, FAK, and vinculin (Fig. 41). Further confirmation of the interaction between integrin and RACK1 was provided by co-localization analyses of cells stained for RACK1 and integrin ps (Fig. 42) or pi (data not shown). In the absence of PMA, RACK1 is mostly cytosolic. It is translocated to the membrane following PMA treatment, mainly to the lamellipodia, as is the case for p_5 integrin (Fig. 42). Taken together, these results indicate that activated PKC e associates with RACK1, and with Pi and Ps integrins. This is accompanied by the incorporation of focal adhesion proteins such as FAK and vinculin in these complexes. In essence, our data suggest that the formation of PKC-RACK1-integrin complexes leads to integrin clustering and focal adhesion assembly and could subsequently lead to the increased adhesion and migration observed following PKC activation.

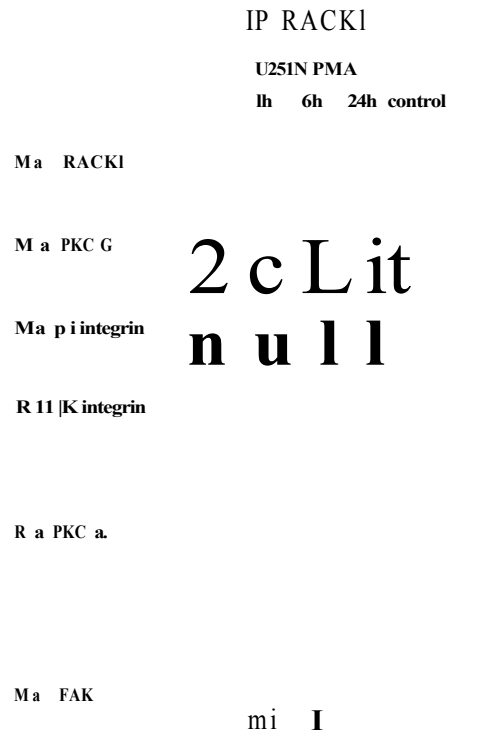


Figure 40: PKC s, Pi, and P_5 integrins, and FAK, co-precipitate with RACK1 following PKC s activation.

Co-immunoprecipitation experiments on U251N cell lysates at various timepoints following PMA (100 nM) stimulation. Immunoprecipitates were run on SDS-PAGE and probed as indicated. RACK1 immunoprecipitation shows the formation of a PMA-induced complex between RACK1, PKC e, and integrin p, or p_5; FAK was also seen as part of the complex. In contrast, no PKC a could be detected associated with FACK1 in these experiments. Control refers to an IP performed with 5 pg of goat anti-IgG+M antibody.

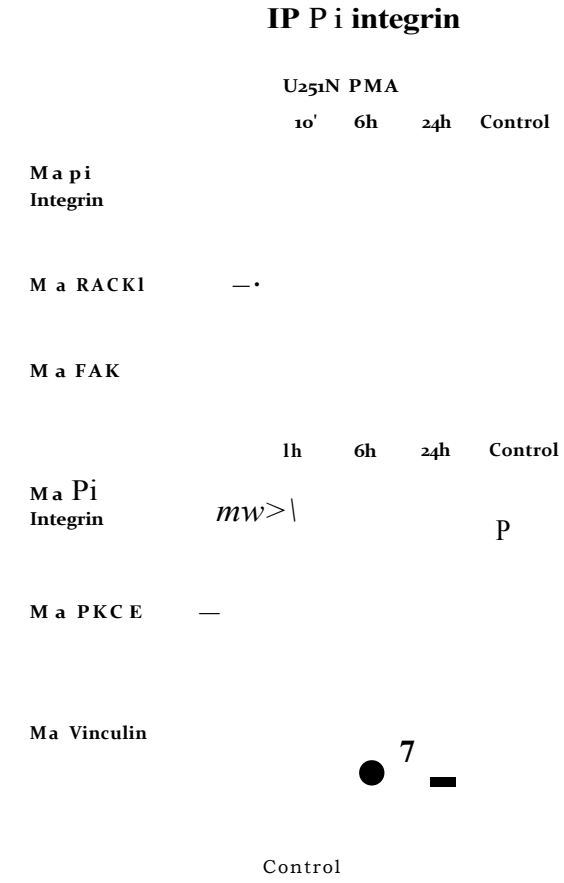


Figure 41: PKC s, RACK1, vinculin, and FAK, co-precipitate with P! integrin following PKC activation.
Co-immunoprecipitation experiments on U251N cell lysates at various timepoints following PMA (100 nM) stimulation. Immunoprecipitates were run on SDS-PAGE and probed as indicated. The immunoprecipitation of PJ integrin shows the association between the integrin chain, RACK1 and PKC e; FAK and vinculin were also part of this complex, suggestive of focal adhesion formation. Control refers to an IP performed with 5 pg of goat anti-IgG+M antibody.

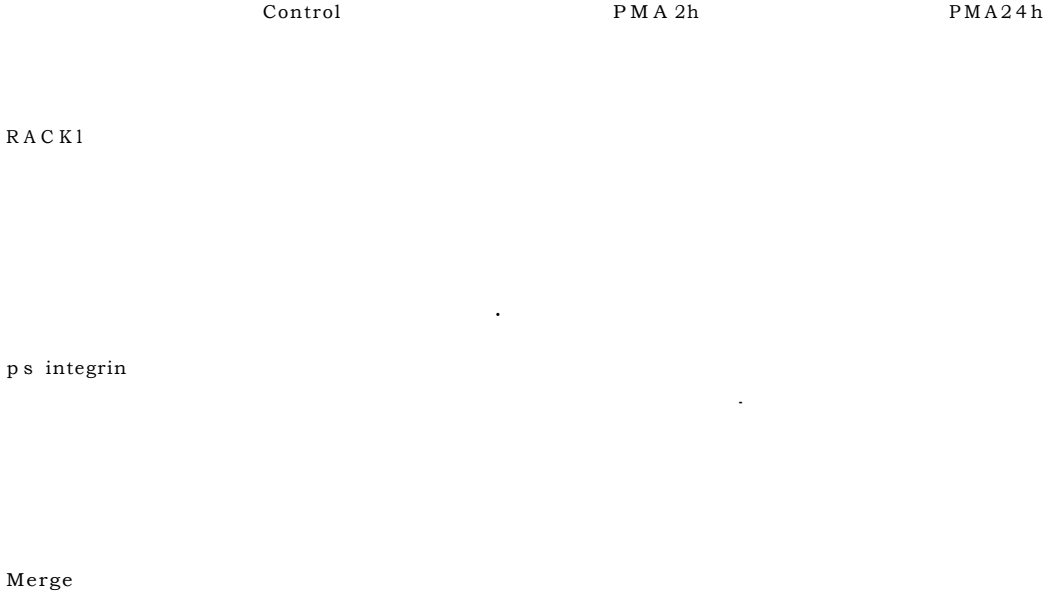


Figure 42: Co-localization of RACK1 and Ps to lamellipodia following PMA stimulation. U251N glioma cells were treated, or not (Control), with 100 nM PMA, fixed at the indicated time, and stained with a mouse anti-RACK1 (l/200)(green), or with rabbit anti-Ps integrin (l/100)(red) antibody.

6.2.2. RACK1 and PKC e are required for PMA-induced integrin-mediated adhesion and motility.

To establish the role of RACK1 and PKC £ in PMA-induced adhesion and migration, we utilized an antisense strategy to partially deplete cells of their endogenous PKC £ or RACK1. The respective expression levels of the control vector transfected cells or antisense transfected cells are displayed in Fig. 43. Transient transfection efficiency was approximately 50%, as evaluated by expression of the green fluorescent protein following transfection of the pTracer vector (data not shown).

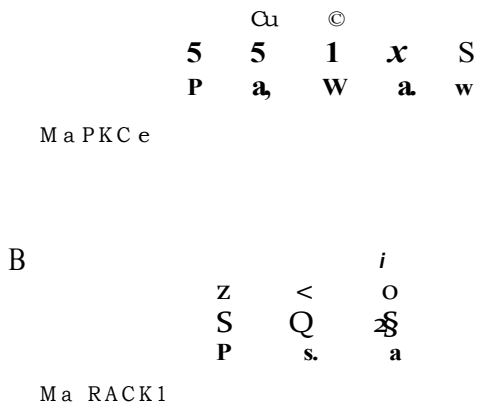


Figure 43: Depletion of endogenous PKC £ and RACK1 by antisense strategy. (A) Decreased PKC e levels in cells transiently transfected with the antisense PKC £ construct pREP-Eas (Eas) or stably transfected with pREP-Eas (Eas30). (B) Decreased RACK1 levels in cells transiently transfected with pcDNA3.1-RACKas (pcRACKas). One hundred pg of proteins were loaded per well for the PKC £ Western blot, and 10 pg of proteins were loaded per well for the RACK1 Western blot.

Depletion of the endogenous PKC £, either by transient transfection (Fig. 44A) or stable transfection (Fig. 44B), and RACK1 by transient transfection (Fig. 44C), markedly reduced both the basal and PMA-induced adhesion on all integrin substrates when compared to the respective control vector transfected cells. Similarly, in both antisense PKC £ and antisense RACK1 transfected cells, the PMA-induced and basal migration were considerably reduced when compared to wild type and control vector-transfected cells (Fig. 45). These results confirm the role of PKC £ in mediating the effects of PMA on integrin-mediated functions. Likewise, the role of RACK1 as a link between PKC £ and integrin p chains appears to be critical for the PKC-induced integrin mediated adhesion and migration.

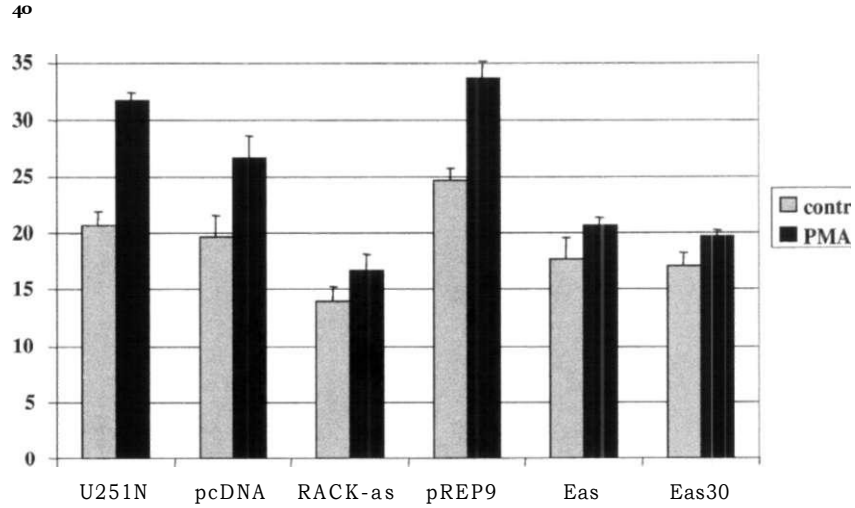


Figure 45: RACK1 and PKC e are required for PMA-induced migration. Migration assay on antisense transfected cells and corresponding control (empty) vectors. Both antisense PKC e and antisense RACK1 transfected cells exhibit a marked reduction in basal and PMA-induced migration. Cells were allowed to migrate for 48 h, and then fixed and stained.

6.2.3. ERK1/2 are activated downstream of PKC and localize to focal adhesions in human glioma cells.

To investigate whether the ERK pathway was activated downstream of PKC in human glioma cells, phospho-specific antibodies that recognize the activated form of ERK1 and ERK2 were used. PMA stimulation rapidly induced a robust and sustained activation of both p44/ERK1 and p42/ERK2, as indicated by their phosphorylation levels on Thr202/Tyr204 (Fig. 46). ERK activation was blunted by pre-treatment with PKC-specific inhibitors (Calphostin C, Bisindolylmaleimide I, and G66983) 1 h prior to PMA stimulation, indicating that PMA-induced ERK activation is a consequence of PKC activation (Fig. 46).

Immunofluorescence experiments revealed that phospho-ERK was localized to cellular structures resembling focal adhesions, and this distribution was more pronounced following PMA treatment (Fig. 47A). To confirm that activated ERKs were localized to focal adhesions in glioma cells, co-localization studies with vinculin and paxillin, two proteins known to be present at focal adhesions, were performed. Phospho-ERK

extensively co-localized with both vinculin (Fig. 47B) and paxillin (Fig. 47C), and as the number of focal adhesions increased following PMA treatment, so did the phospho-ERK distribution at focal adhesions (Fig. 47B-C). Together, the results indicate that the ERK pathway is activated downstream of PKC and that activated ERK localizes to focal adhesions.

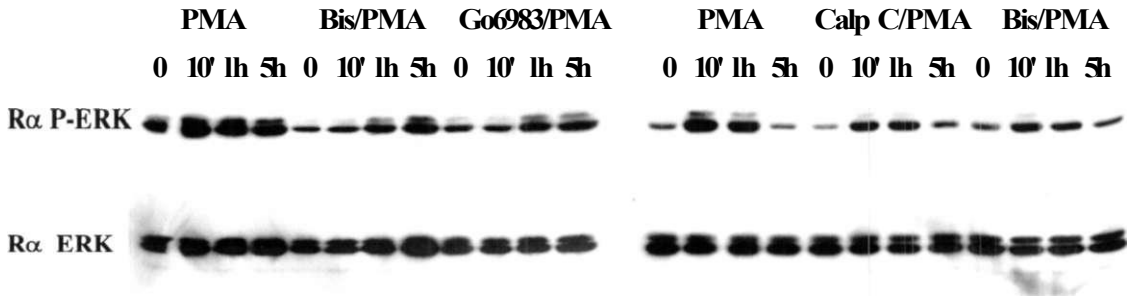


Figure 46: ERK1 and ERK2 are activated following PKC activation.
ERK activation was evaluated with a phospho-ERK specific antibody (Thr202/Tyr204). The amount of total ERK was determined by reprobing the membranes, after stripping, with a polyclonal anti-ERK antibody. ERK activation following PMA (100 nM) treatment could be prevented by PKC-specific inhibitors (Bisindolylmaleimide I, 5 pM; Go6983, 5 pM; Calphostin C, 200 nM). Fifty pg of proteins were loaded per well.

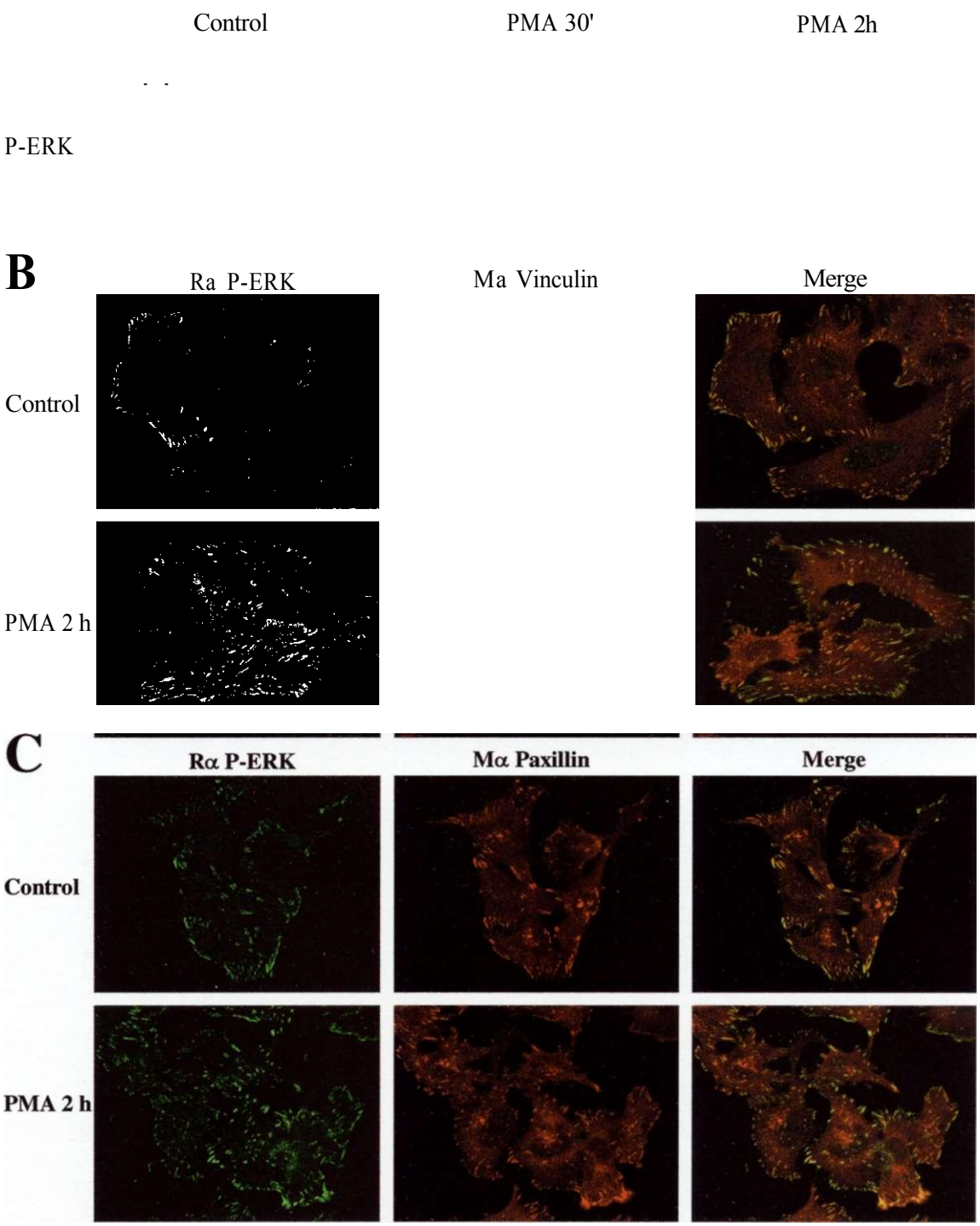


Figure 47: Activated ERKs localize to focal adhesions.
Immunofluorescence analysis of U251N glioma cells at various times following 100 nM PMA stimulation with the phospho-ERK specific antibody (Thr202/Tyr204). (A) Increased localization of activated ERK to focal adhesion-like structures and lamellipodia. (B) Co-localization of phospho-ERK with vinculin. (C) Co-localization of phospho-ERK with paxillin. Rabbit anti-phospho-ERK (1/250), Mouse anti-vinculin (1/1000), Mouse anti-paxillin (1/400).

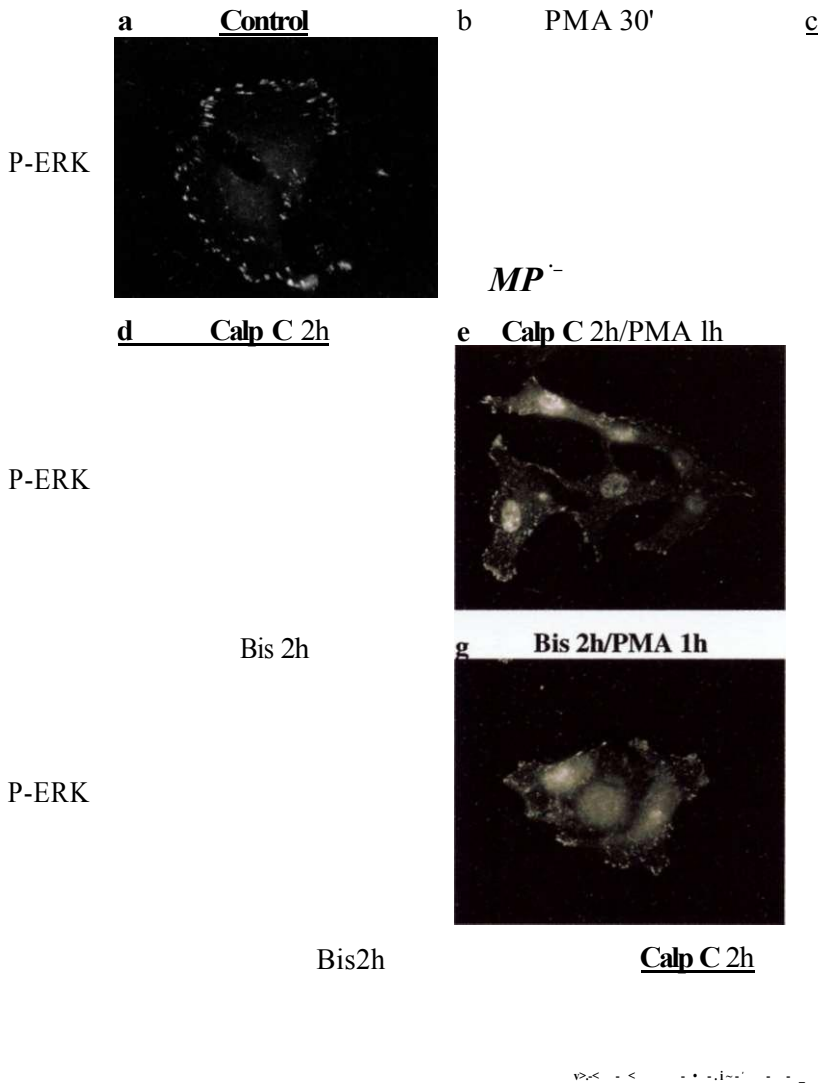


Figure 48: Localization of activated E R K to focal adhesions is abolished by P K C inhibitors.

U251N cells treated with PMA (100 nM) for 0, 30 min, and 2 h, respectively (a-c) or with Calphostin C (200 nM) (d) or Bisindolylmaleimide I (5 pM) (f) for 2 h. PKC inhibitor-treated cells were also stimulated with PMA (100 nM) an hour after addition of the inhibitor (e, g). At the given time, U251N cells were fixed and stained with the phospho-ERK specific antibody (Thr202ATyr204) (1/250) (a-g).

(h-i) The PKC inhibitors do not disrupt focal adhesions, visualized by vinculin staining (1/1000) of cells treated with Bisindolylmaleimide I (5 pM) (h) or Calphostin C (200 nM) (i) for 2 h.

6.2.4. PKC activity is required for localization of activated ERKs to focal adhesions.

We then investigated whether the localization of activated ERK to focal adhesions was dependent on PKC activity. In the presence of either Calphostin C (200 nM) or Bisindolylmaleimide I (5 pM), phospho-ERK failed to localize to focal adhesions and was found in the cytoplasm and the perinuclear region (Fig. 48d, f). The mislocalization of phospho-ERK was not due to the disruption of focal adhesions by the PKC inhibitors since these structures could still be visualized by vinculin staining (Fig. 48h-i). Addition of PMA in the presence of PKC inhibitors only weakly rescued the localization of phospho-ERK to focal adhesions (Fig. 48e, g). These results indicate that PKC activity is required for phospho-ERK localization to focal adhesions.

6.2.5. Different PKC isoforms target activated ERK to distinct subcellular locations.

The results presented in Chapters 3 and 5 indicate that PKC a and e were controlling different phenotypes of glioma cells, namely, proliferation and adhesion/motility. An attractive hypothesis is that since PKC e regulates glioma cell motility, it could be the isoform responsible for ERK activation and localization to focal adhesions. To determine whether a specific PKC isoform had an effect on ERK subcellular localization, we analyzed various clones overexpressing either PKC a or e, or transfected with an antisense PKC e construct, by immunofluorescence (Fig. 49). In wild type U251N cells and empty vector transfected cells (pBK) (Fig. 49A-B), phospho-ERK was localized mostly to focal adhesions after PMA stimulation, and some phospho-ERK was detected in the nucleus. However, in two clones overexpressing PKC *a* (aS20 and aS27) (Fig. 49C-D), a significantly increased proportion of phospho-ERK was localized in the nucleus. In contrast, in clones overexpressing PKC £ (eSl and eSlO) (Fig. 49E-F), phospho-ERK was extensively targeted to focal adhesions. Cells transfected with an antisense PKC 8 cDNA (eAS30), in which PKC s levels are markedly decreased, provided further evidence that PKC e is responsible for targeting phospho-ERK to focal adhesions, since the localization of phospho-ERK to focal adhesions was greatly

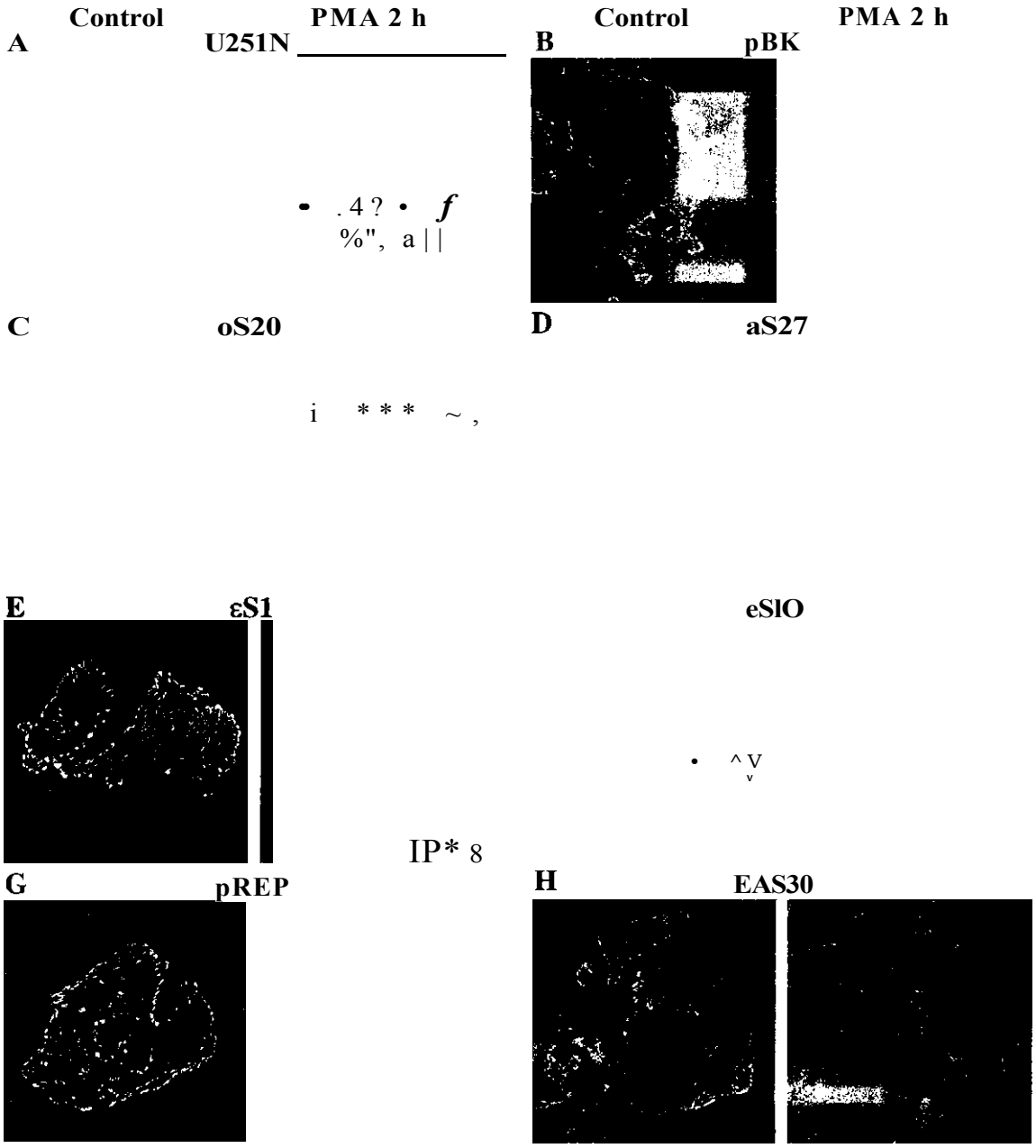


Figure 49: Different PKC isoforms target activated ERK to distinct subcellular locations. PKC e targets activated ERK to focal adhesions, while PKC a induces the nuclear translocation of phospho-ERK.

Phospho-ERK staining (Thr202/Tyr204) of several clones of U251N glioma cells in the absence (control) or presence of PMA (100 nM) for 2 h. All images in this figure were obtained from an experiment in which cells were processed identically, and analyzed with identical settings of the confocal microscope. (A) U251N wild type. (B) pBK vector transfected cells, used as control for the clones aS20 (C), aS27 (D), that overexpress PKC a; and eS1 (E), and eSlO (F), that overexpress PKC e. (G) pREP9 vector transfected cells, used as control for the clone eAS30 (H), transfected with antisense PKC e.

diminished (Fig. 49H) when compared to its empty vector transfected counterpart (pREP) (Fig. 49G). Together, the data indicate that PKC a induces the nuclear localization of phospho-ERK, while PKC £ targets activated ERK to focal adhesions.

Consistent with the differential targeting of ERK by distinct PKC isoforms, we found that PKC a and £ were localized to distinct subcellular locations following activation. It is well known that activated PKCs associate with membranes (Newton 1997); however, the subcellular localization of PKC isoforms when activated has never been investigated in glioma cells. Thus, we investigated the distribution between nuclear (including the nuclear envelope) and cytoplasmic fractions (including plasma, ER, and Golgi membranes) of PKC a and £ following activation. Interestingly, PMA stimulation rapidly translocated PKC a to the nucleus (Fig. 50A), while PKC £ was mostly retained in the cytoplasmic fraction (which contains other particulate matter, including the plasma membrane) (Fig. 50B).

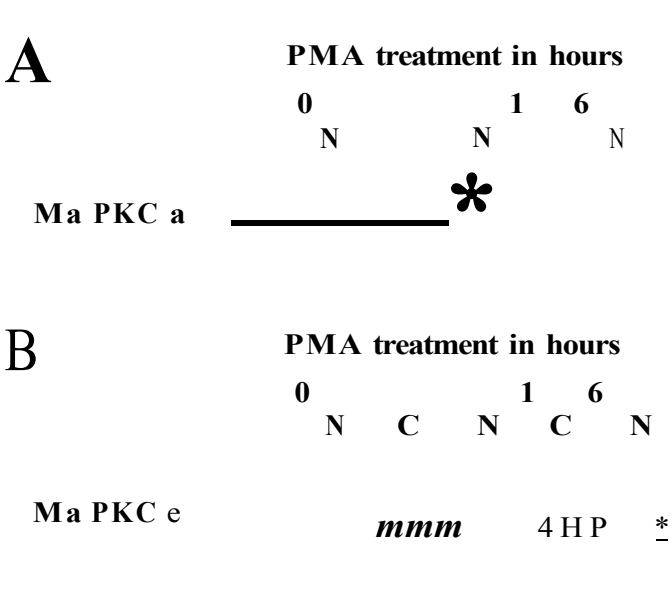


Figure 50: PKC a and £ translocate to different subcellular locations following activation.
Cells were fractionated into cytoplasmic (including the plasma membrane) and nuclear fractions (including the nuclear envelope), 50 pg of proteins were loaded per well. (A) mouse anti-PKC a (1/1000) antibody. (B) mouse anti-PKC £ (1/500) antibody. PKC a translocates to the nucleus (A) following activation with PMA, while PKC £ remains mostly cytoplasmic (B).

6.2.6. PKC-induced ERK activation occurs through a MEKl/2-dependent mechanism in human glioma cells.

We next attempted to determine by what mechanism PKC activates ERK, as several distinct pathways have been described in various model systems (El-Shemerly et al. 1997, Schonwasser et al. 1998, Howe and Juliano 1998, Miranti et al. 1999, Short et al.

2000, Rigot et al. 1998, Traub et al. 1997). Pharmacological studies showed that ERK activation by PKC occurs by a MEK1/2-dependent mechanism, since the MEK1/2 inhibitors PD98059 and U0126 efficiently blocked PMA-induced ERK activation (Fig. 51 A). Because Fincham et al. (2000) recently reported that targeting of activated ERK to focal adhesions was dependent on v-Src activity, we tested whether the Src-family kinase inhibitor PP2 could prevent ERK activation. We found that PP2 had no effect on PKC-induced ERK activation (Fig. 5IB), indicating that Src is not involved downstream of PKC in this signaling cascade.

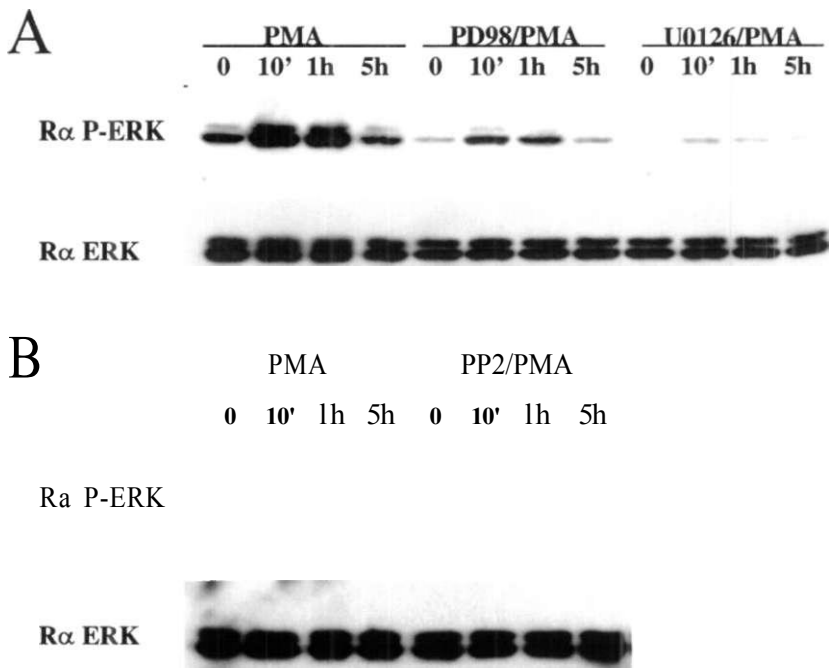


Figure 51: PMA-induced activation of ERK occurs through a MEK1/2-dependent mechanism. ERK activation was evaluated with a phospho-ERK specific antibody (Thr202/Tyr204). The amount of total ERK was determined by reprobing the membranes, after stripping, with a polyclonal anti-ERK antibody. (A) U251N cells were pre-treated with the MEK1/2 specific inhibitors PD98059 (25 pM) and U0126 (10 pM) for 1 h prior to PMA (100 nM) stimulation. (B) PKC-induced ERK activation occurs in a Src-family kinase-independent manner. U251N cells were pre-treated with the Src-family kinase inhibitor PP2 (5 pM) for 1 h prior to PMA stimulation. Fifty pg of proteins were loaded per well.

6.2.7. ERK activation downstream of PKC is required for PMA-induced integrin-mediated adhesion and migration.

We next tested the effect of ERK inhibition on PKC-induced integrin-mediated adhesion and motility. The presence of the MEK1/2 inhibitors PD98059 (25 pM) or U0126 (10 pM), that prevent PKC-induced ERK activation (Fig. 51A), completely abolished the PMA-induced increase in adhesion on laminin (Fig. 52A), vitronectin (Fig. 52B), or fibronectin (Fig. 52C) in EslO cells that overexpress PKC e. The Src family inhibitor PP2 (10 pM) could also abolish the PMA-induced increase in adhesion on these integrin substrates (Fig. 52) (see Discussion).

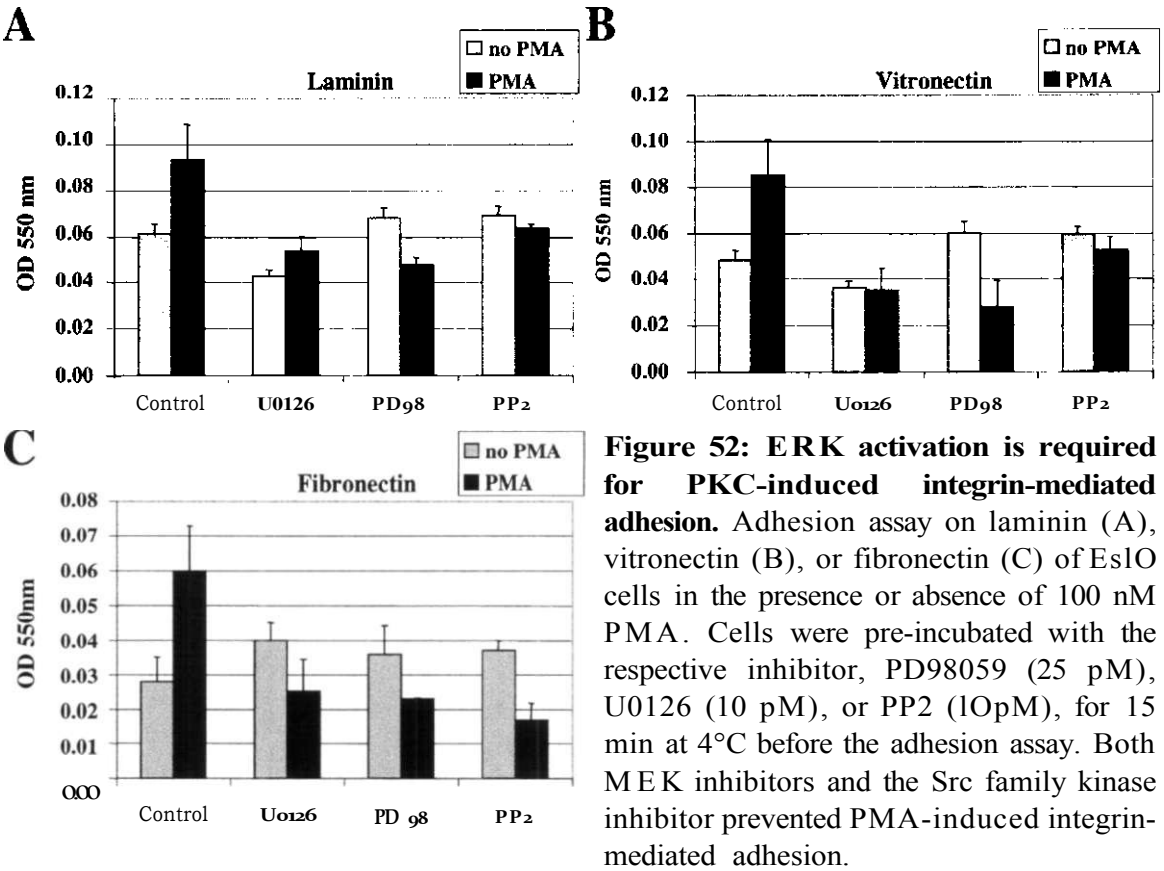


Figure 52: ERK activation is required for PKC-induced integrin-mediated adhesion. Adhesion assay on laminin (A), vitronectin (B), or fibronectin (C) of EslO cells in the presence or absence of 100 nM PMA. Cells were pre-incubated with the respective inhibitor, PD98059 (25 pM), U0126 (10 pM), or PP2 (lOpM), for 15 min at 4°C before the adhesion assay. Both MEK inhibitors and the Src family kinase inhibitor prevented PMA-induced integrin-mediated adhesion.

Similarly, both PD98059 (25 pM) and U0126 (10 pM) reduced the basal and the PMA-induced migration of pBK and EslO cells (Fig. 53). The Src family kinase inhibitor PP2 (10 pM) completely blocked both basal and PKC-induced migration (Fig. 53), while the PI 3-kinase inhibitor wortmannin had no effect on PKC-induced migration (Fig. 53).

Together, these results indicate that ERK activation downstream of PKC is required for PMA-induced integrin-mediated adhesion and migration.

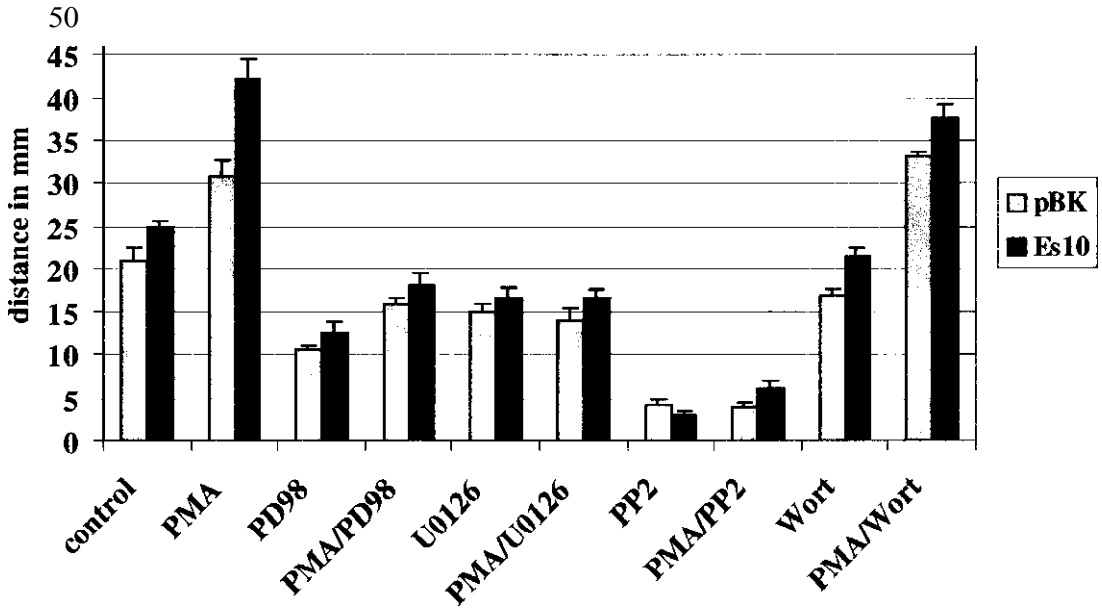


Figure 53: ERK activation is required for PKC-induced migration.
Migration assay on pBK (gray bars) and EslO (black bars) cells in the presence or absence of various inhibitors: the MEK inhibitors PD98059 (25 pM) or U0126 (10 pM), the Src family kinase inhibitor PP2 (10 pM), or the PI 3-kinase inhibitor wortmannin (200 nM). Cells were allowed to migrate for a 48 h period, fixed, and stained with hematoxylin to quantify the migration. Both MEK inhibitors decreased the basal and PMA-induced migration; the Src family kinase inhibitor completely abolished cell motility; and the PI 3-kinase inhibitor had no significant effect on basal or PMA-induced motility of glioma cells.

MLCK has been shown to be a major effector in ERK-mediated cell migration (Klemke et al. 1997, Nguyen et al. 1999). We therefore tested whether MLCK was playing a role in the regulation of adhesion and migration downstream of PKC and ERK in glioma cells. The MLCK inhibitor ML7 had no effect on PKC-induced adhesion (data not shown); however, it effectively blocked PKC-induced migration of EslO cells (Fig. 54). This observation is in agreement with the report by Gillespie et al. (1999) in which they demonstrated the efficacy of MLCK inhibitors to inhibit glioma cell migration.

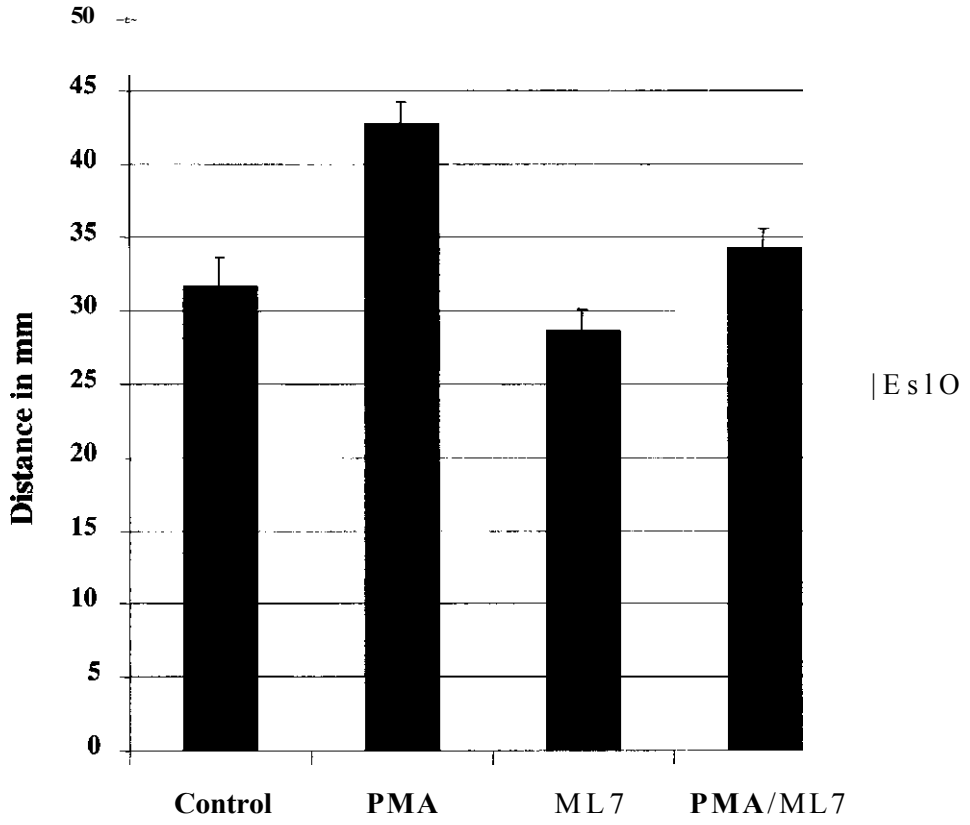


Figure 54: MLCK activation is required for PKC-induced migration.
Migration assay on Es1O cells in the presence or absence of PMA (100 nM), or ML7 (20 pM), a myosin light chain kinase inhibitor. Cells were allowed to migrate for a 48 h period, fixed, and stained with hematoxylin to quantify the migration.

6.3. Discussion.

PKC activity plays a critical role in integrin-mediated adhesion, spreading, migration, and focal adhesion assembly (Vuori and Ruoslahti 1993, Woods and Couchman 1992, Lewis et al. 1996, Disatnik and Rando 1999). However, the mechanism by which PKC regulates integrin functions remains unclear, and more particularly, how PKC is targeted to the vicinity of integrin is unknown. Here, we demonstrate that PKC e is required for integrin-mediated adhesion, migration, and focal adhesion formation in human glioma cells. We describe for the first time that the mechanism by which PKC e regulates integrin function is through its association with the scaffold protein RACK1, and with integrin (3 chains. Accordingly, the reduction of endogenous RACK1 or PKC e levels diminished the PMA-induced adhesion and motility of glioma cells. Overall, our results provide a novel mechanistic link between PKC activation and cell adhesion and motility

events. An attractive hypothesis is that upon activation, PKC £ first binds to RACK1, and that in turn this complex associates with integrin p chains, leading to integrin clustering and increased adhesion and motility.

Our finding that RACK1 is required for PKC e-induced adhesion and migration, possibly by serving as an adaptor between PKC and select integrin P chains, stresses the importance of such anchoring proteins in providing the proper subcellular localization and in regulating the substrate specificity of PKC. It also suggests that WD-40 repeat proteins, such as RACK1 and WATT-1 (Liliental and Chang 1998, Rietzler et al. 1998), could be a class of proteins that links the PKC system to integrins, bringing PKC to the close proximity of the focal adhesion machinery, which includes several PKC targets. It would be interesting to determine whether PKC can interact with WAIT-1. We found that the interaction between RACK1 and integrin was induced by the association of PKC £ with RACK1. Liliental and Chang (1998) had also reported that the association of RACK1 with integrin otLp2 *in vivo* was dependent on the presence of PMA. Others have shown a coordinated movement of RACK1 with activated PKC (Ron et al. 1999), suggesting that RACK1 acts as a shuttling protein that regulates the movement of active PKC from one subcellular location to another.

It is unclear why in human glioma cells RACK1 associated specifically with PKC 8 but not with PKC a. RACK1 was first identified as a PKC (3 binding protein (Mochly-Rosen et al. 1991, Ron et al. 1994), and although it binds to PKC p with the highest affinity, RACK1 (or RACK1-derived peptides) could also bind other PKC isoforms, including a, y, 8, and £ (Ron et al. 1995, Rotenberg and Sun 1998, Pass et al. 2001). One possible explanation is that PKC pi and **p2** being absent from glioma cells (Fig. 7) (Besson and Yong 2000), RACK1 is more readily available to bind PKC £. Another attractive hypothesis is that the various PKC isoforms expressed in these cells likely occupy distinct subcellular compartments, and/or associate with different RACK proteins. For example, we found that PKC a translocates to the nucleus upon activation, while PKC £ is mainly associated with the cytoplasmic fraction (Fig. 50).

Recently, Berrier et al. (2000) reported that an activated form of PKC £ (Myr-PKC **E)** could restore the spreading ability of CHO cells overexpressing a mutant form of pi

integrin in which the cytoplasmic domain of integrin is fused to the extracellular and transmembrane domains of Interleukin-2 Receptor. The ability of Myr-PKC e to rescue spreading was dependent upon an intact cytoplasmic domain of the integrin (Berrier et al. 2000). Interestingly, the ability of Myr-PKC e to restore pVmediated spreading required Racl activity, as it could be prevented by a dominant negative form of Racl (N17Racl), but not PI 3-kinase activity (Berrier et al. 2000), indicating that Racl is downstream of PKC **E** in (3i integrin-mediated cell spreading. Thus PKC £ appears to be an important mediator of P integrin functions in various cell systems, and it is possible that the targeting of PKC £ to integrins constitutes a general mechanism of regulation of integrin function. It remains to be determined whether, in glioma cells, Racl is required downstream of PKC £ to mediate adhesion and migration.

What activated PKC £ does in glioma cells, once it is anchored to integrin P chains by RACK1, remains uncertain. One may expect that activated PKC! £ phosphorylates a number of targets in focal adhesions, thus facilitating their assembly, and leading to integrin clustering, increased adhesion and migration. PKC has previously been reported to be able to phosphorylate several integrin chains (Valmu et al. 1991, Hogervorst et al. 1993, Gimond et al. 1995, Rabinovitz et al. 1999), paxillin (DeNichilo and Yamada 1996), talin (Litchfield and Ball 1986), and vinculin (Weekes et al. 1996, Perez-Moreno et al. 1998). These phosphorylation events are thought to participate in integrin activation and clustering and focal adhesion assembly and stability. PKC £ could also participate in the activation of Racl, leading to actin rearrangements, lamellipodia formation, and integrin clustering, as suggested by Berrier et al. (2000). Other signaling cascades could be initiated by PKC £ to modulate integrin function, such as the ERK pathway, which was required for PKC-induced integrin-mediated adhesion and migration.

This is, to our knowledge, the first time that 2 different PKC isoforms have been shown to target ERK to distinct subcellular localizations. The ability of several PKC isoforms to activate ERKs within the same cells has been reported previously (Schonwasser et al. 1998); however, the subcellular localization of ERKs was not studied. It remains unclear at this point how PKC mediates this differential targeting of ERKs. However, one may speculate that upon activation, each PKC isoform translocates

to a specific location, the nuclear envelope in the case of PKC a and the plasma membrane in the case of PKC £, and then activates different pools of ERKs that reside in these locations. Consistent with this hypothesis, staining with a total ERK antibody (Zymed) revealed a population of ERK in the nucleus, and another one at focal adhesions in human glioma cells (data not shown). It remains to be determined whether PKC a-mediated ERK activation and targeting to the nucleus play a role in the regulation of proliferation by PKC a in human glioma cells (Besson and Yong 2000). This seems likely since the nuclear translocation of ERKs is generally associated with a proliferative response.

Our finding that activated ERK translocates to focal adhesions in glioma cells contrasts with many other cell types, in which ERK usually translocates to the nucleus and mediates growth factor-induced proliferative responses. However, Fincham et al. (2000) recently reported that activated ERK was targeted to focal adhesions following integrin engagement, in rat and chick embryo fibroblasts, in a v-Src-dependent manner, and also that activation of the myosin light chain kinase (MLCK) downstream of ERKs was required for proper targeting of activated ERK to focal adhesions. We therefore tested the effect of the Src family kinase inhibitor PP2 and the MLCK inhibitors ML7 and ML9 on the targeting of phospho-ERK to focal adhesions following PKC activation. Neither of these inhibitors prevented the PKC-induced localization of activated ERK to focal adhesions in glioma cells (data not shown), suggesting that a different mechanism is involved. We also tested whether the Src inhibition with PP2 could prevent ERK activation, and consistent with Src-family kinases not being involved in PKC-induced ERK localization to focal adhesions, it was found that PP2 had no effect on PKC-induced ERK activation (Fig. 5IB), indicating that Src is not downstream of PKC in this signaling cascade. However, it is possible that Src kinases play a critical role in ERK activation downstream of integrins or receptor tyrosine kinases, and PKC could be involved downstream of Src in this process. Although Src inhibition had no effect on PKC-induced ERK activation and localization to focal adhesions, we found that Src inhibition could block PKC-induced integrin-mediated adhesion and migration. These results are not very surprising considering the critical role played by Src in focal adhesion assembly/disassembly, and in integrin-mediated signal transduction (Giancotti and

Ruoslahti 1999, Klinghoffer et al.1999, Sieg et al. 1999). Thus, inhibition of Src family kinases likely results in a general inhibition of most processes initiated by integrins (including adhesion and migration).

The exact functions of ERKs at focal adhesions remain unclear at this point. Although, as our data indicate, they are clearly involved in the regulation of adhesion and migration, their direct targets are still unknown. Likely candidates for ERK phosphorylation at focal adhesions are MLCK, known to be directly activated by ERKs (Klemke et al. 1997, Nguyen et al. 1999, Fincham et al. 2000), and other cytoskeletal or cytoskeleton-associated proteins.

In summary (Fig. 55), we have found that the scaffolding protein RACK1 targets activated PKC e to integrin P chains, leading to integrin clustering, focal adhesion formation, and increased adhesion and migration on integrin substrates. Depletion experiments revealed that RACK1 is required for PKC e-induced adhesion and migration. These findings provide a novel mechanism by which PKC regulates integrin function. We also obtained evidence indicating that PKC activation induced the activation of ERK1 and ERK2, and that activated ERK localizes to focal adhesions. Moreover, we found that PKC a overexpression induced the nuclear localization of activated ERK, while PKC e overexpression induced the targeting of activated ERK to focal adhesions. Also, ERK activation downstream of PKC appeared to be required for PMA-induced integrin mediated adhesion and migration. Future therapeutic strategies targeting the PKC pathway in gliomas should aim at inhibiting the activity of both these isoforms.

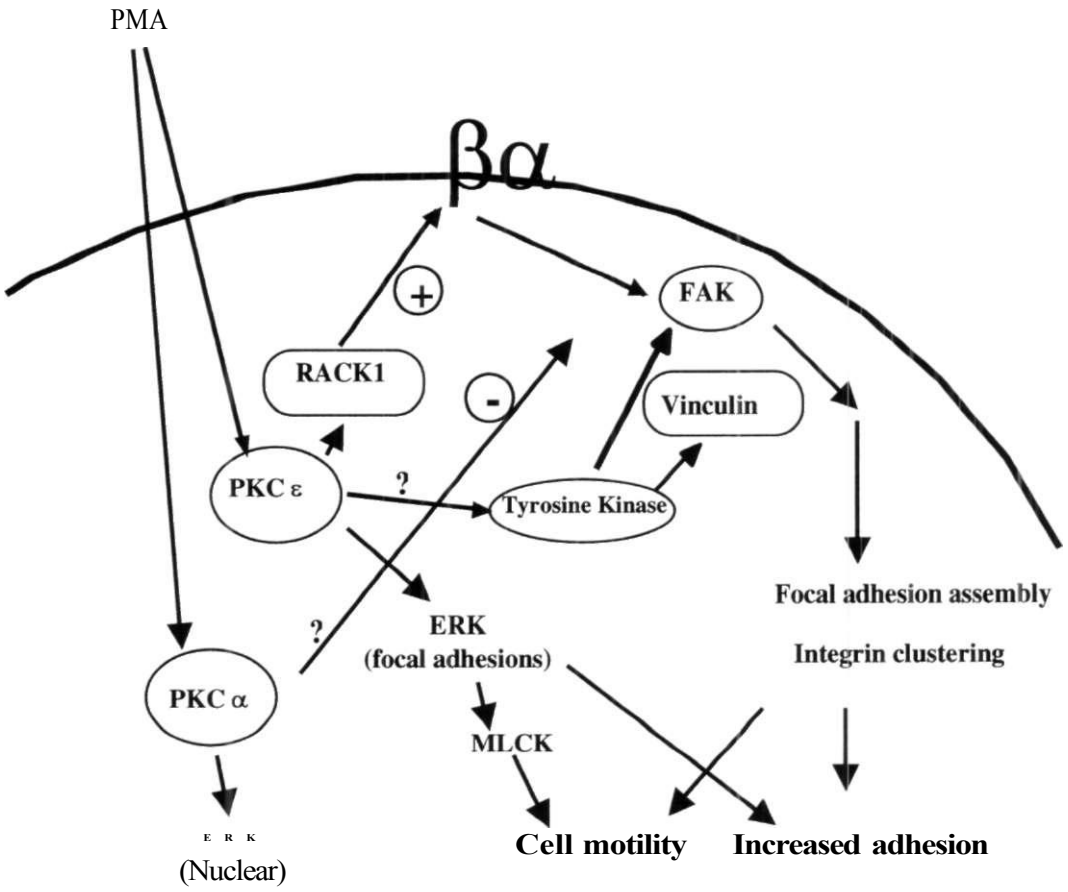


Figure 55: Summary of the data obtained in Aims 3 and 4.
Activated PKC e associates with the anchoring protein RACK1 and integrin β chains, leading to integrin clustering, tyrosine phosphorylation of FAK and vinculin, focal adhesion formation, and increased adhesion and migration on integrin substrates. PKC a has the opposite roles, possibly by gearing the cell toward proliferation.
PKC activation induced the activation of ERK1 and ERK2. PKC a induces the nuclear localization of activated ERK, while PKC e targets activated ERK to focal adhesions. ERK activity was required for PMA-induced adhesion, and migration, possibly through an MLCK-dependent mechanism.

CHAPTER SEVEN

Aim 5: Involvement of PKC Downstream of c-Met to Mediate Glioma Cell Migration.

7.1. Introduction.

There is considerable evidence to indicate a role for growth factor receptors in the malignancy of gliomas (reviewed in Besson and Yong 2001). Several growth factor receptors are overexpressed, mutated, and/or constitutively stimulated through the existence of autocrine loops. It is thought that the signaling cascades generated by these receptors initiate proliferative, migratory, and angiogenic responses.

There is increasing evidence to suggest that PKC plays an important role in the regulation of glioma cell proliferation and invasion (reviewed in Baltuch et al. 1995, Bredel and Pollack 1997, Uhm et al. 1997). PKC activity was markedly increased in astrocytoma cell lines when compared to non-transformed astrocytes and directly correlated with their growth rate (Couldwell et al. 1991). PKC activity was also elevated in astrocytoma specimens (Couldwell et al. 1992). A link between cell surface receptor stimulation and PKC activation was established in a number of reports, since the mitogenic effect of serum addition or stimulation with EGF, PDGF, or FGF on established glioma cell lines or low passage glioma cells was completely abrogated by PKC inhibition (Pollack et al. 1990b, Couldwell et al. 1992, Baltuch et al. 1993a, Baltuch and Yong 1996). These results suggest that mitogenic signaling from tyrosine kinase receptors in astrocytoma cells is mediated by a PKC-dependent pathway.

The RTK c-Met, and its ligand HGF/SF, plays a role in the regulation of cell growth, motility, and morphogenesis, and is a potent angiogenic factor. Expression of both c-Met and HGF/SF was found in the majority of GBMs, suggesting the presence of an autocrine loop (Nabeshima et al. 1997, Moriyama et al. 1998b, Koochekpoor et al. 1997). Several lines of evidence suggest a role for PKC in c-Met-mediated processes. In retinal endothelial cells, PKC was activated by HGF stimulation, and PKC inhibition partially prevented HGF-induced migration and proliferation, but did not prevent ERK activation (Cai et al. 2000). HGF induced the translocation of PKC a, y, and e in rat neocortical

cells, and ERK activation in a PKC-dependent manner; however, PKC was not activated by HGF in rat primary astrocytes (Machide et al. 1998). It was subsequently found that PLC y is constitutively associated with the tyrosine phosphatase SHP-1 at the c-Met receptor, and that in the presence of a dominant negative SHP-1, PLC y and PKC were activated, and mediated the proliferative response to HGF in rat primary astrocytes, but not the migratory response (Machide et al. 2000). HGF-induced motility was independent of PKC in hepatocellular carcinoma cells (Nakanishi et al. 1999). It would appear that, depending on the cell type studied, HGF stimulation may, or may not, elicit a PKC-dependent response. Also, the HGF-induced response in which PKC is involved may vary depending on the cell type.

The results describing the involvement of PKC in the regulation of various phenotypes of human glioma cells presented in this thesis are all based on the utilization of phorbol esters to stimulate PKC activity. PMA, because of its potency and its ability to activate other CI domain-containing proteins, may not constitute the ideal agonist. Therefore, to place the results in a more physiologically relevant setting, we wanted to determine whether stimulation of PKC by naturally occurring agonists, such as growth factors, would induce similar responses to those observed with the use of PMA.

7.2. Results.

7.2.1. Lack of proliferative response of human glioma cells to growth factors.

There are numerous reports describing the mitogenic response of human glioma cells to various growth factors (Besson and Yong 2001). We stimulated human glioma cells using various growth factors (1 to 100 ng/ml), including EGF, PDGF-BB, and HGF, to elicit a proliferative response. Despite repeated attempts, in low (0.1%) to high (10%) serum-containing medium, we were unable to induce cell proliferation or cell cycle progression following growth factor stimulation, as monitored by flow cytometry, ^{3}H-thymidine incorporation, and MTT-based proliferation assays (data not shown). It was therefore impossible to study the involvement of PKC in the growth factor-induced mitogenic response.

7.2.2. HGF induces a potent PKC-dependent migratory response in human glioma cells.

We next tested whether HGF had an effect on glioma cell migration. The migratory response induced by HGF was more potent than that induced by PMA, and could be completely inhibited by PKC inhibition with Bisindolylmaleimide I (5 pM) (Fig. 56). As previously observed, the migratory response of PKC £ overexpressing cells was clearly greater than in wild type or control vector transfected cells, while that of PKC a overexpressing cells was lower (Fig. 56). These results indicate that HGF is a potent inducer of migration in human glioma cells, and that the HGF response intersects with PKC signal transduction at some stage.

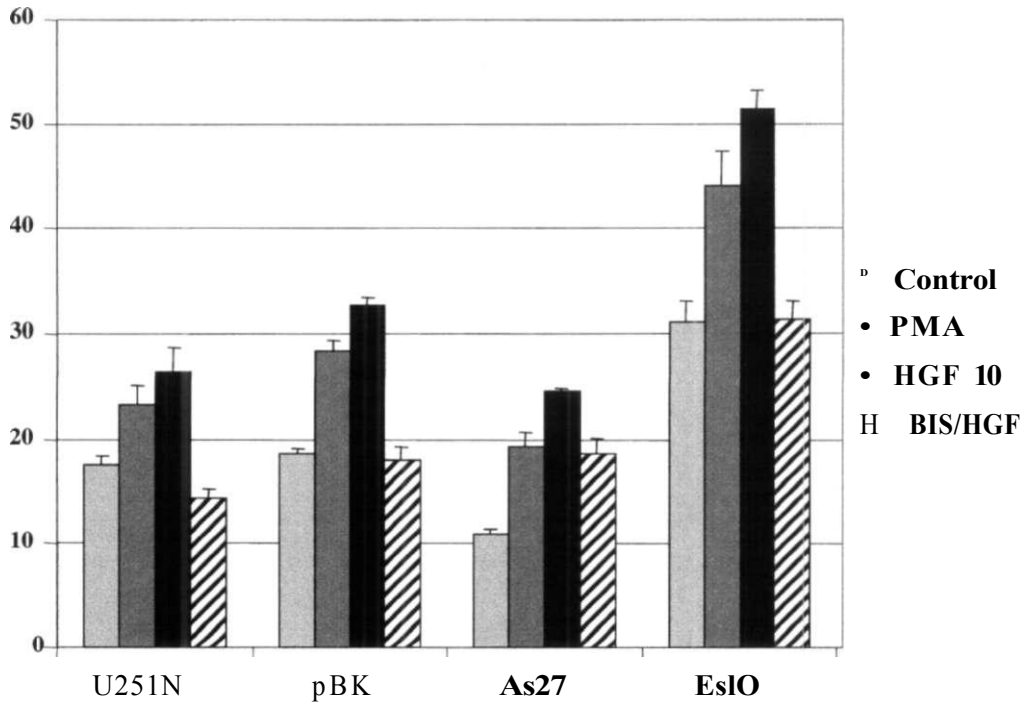


Figure 56: Hepatocyte growth factor induces PKC-dependent glioma cell motility. Cells were allowed to migrate for a 48 h period, fixed, and stained with hematoxylin to quantify the migration. Different clones of U251N were subjected to a migration assay in the presence of vehicle (control), PMA (100 nM), HGF (10 ng/ml), or HGF and Bisindolylmaleimide I (5 pM) (BIS). HGF potently induced migration, which was inhibited by the PKC inhibitor. U251N (wild type), pBK (empty vector transfected cells), As27 (cells overexpressing PKC a), EslO (cells overexpressing PKC £).

7.2.3. HGF stimulation fails to induce PKC activation.

We next monitored the effect of HGF stimulation on translocation of various PKC isoforms. HGF did not induce the translocation of PKC a, e, or r| (Fig. 57), and no change in the kinase activity of these PKC isoforms against a synthetic peptide was detected in an *in vitro* kinase assay (data not shown).

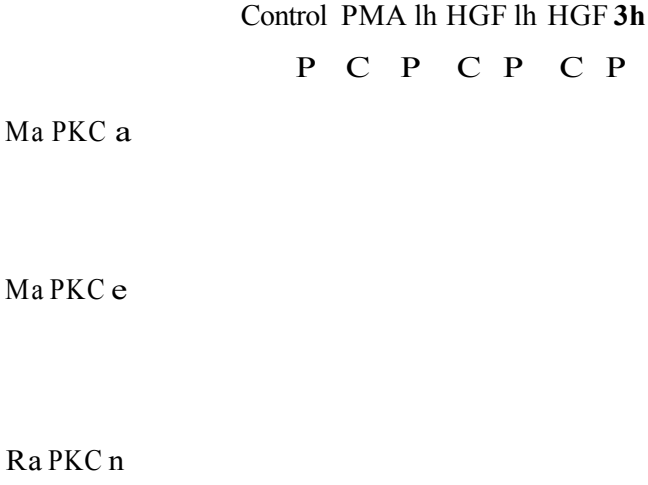


Figure 57: HGF fails to induce PKC translocation.
Western Blot analysis of the subcellular distribution between cytosolic and membrane fractions of PKC a, e, and r| following PMA (100 nM) stimulation for 1 h, or HGF (20 ng/ml) stimulation for 1 and 3 h. U251N protein extracts collected at various times following PMA treatment were fractionated into cytosolic (C) and particulate (P) fractions. Fifty pg of proteins were loaded in each well.

7.2.4. HGF induces ERK activation in a PKC-independent manner.

HGF potently stimulated ERK activation in U251N glioma cells, between 5 min and 2 h; however, ERK activation was not prevented in the presence of PKC inhibitors (Fig. 58), indicating that ERK activation downstream of HGF occurs by a PKC-independent mechanism.

Figure 58: ERK is activated downstream of HGF in a PKC-independent manner. U251N glioma cells were stimulated with PMA (100 nM) for 1 h, or with HGF (20 ng/ml) for the indicated time; Where appropriate, PKC inhibitors were added (Calphostin C, 200 nM, Go6983, 5 pM, or Bisindolylmaleimide **I,** 5 pM) 1 h prior to stimulation with HGF for 15 min. Cell lysates were collected and submitted to SDS-PAGE. ERK activation was evaluated with a phospho-ERK specific antibody (Thr202/Tyr204) (1/1000). The amount of total ERK was determined by reprobing the membranes, after stripping, with a polyclonal anti-ERK antibody (1/1000). Fifty pg of proteins were loaded per well.

7.2.5. HGF stimulation does not affect glioma cell adhesion.

To determine the effect of HGF stimulation on adhesion, we performed adhesion assays on various integrin substrates. HGF stimulation had no effect on integrin-mediated adhesion of EslO cells, while PMA increased adhesion, as previously observed (Fig. 59).

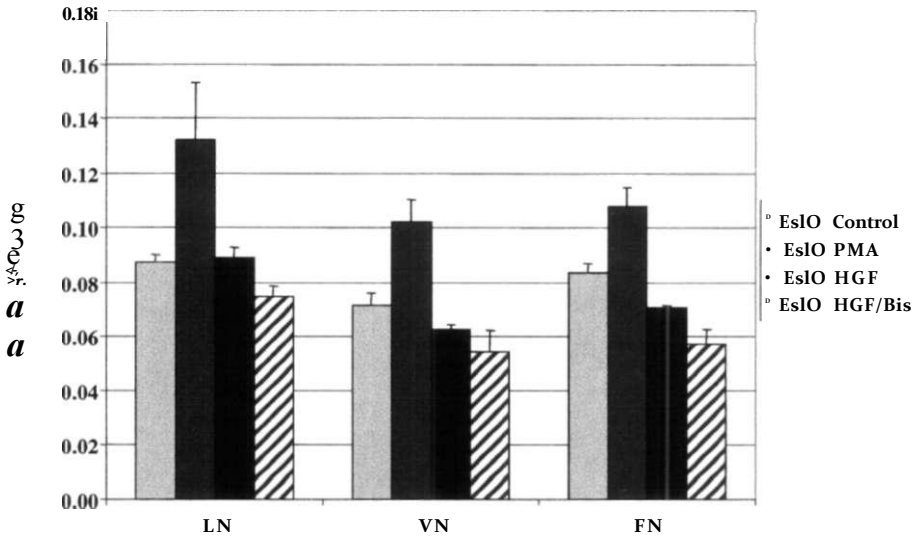


Figure 59: HGF has no effect on glioma cell adhesion.
Adhesion assay on EslO cells. EslO cells were treated for 15 min prior to the adhesion assay with PMA (100 nM), HGF (10 ng/ml), or HGF and Bisindolylmaleimide I (5 pM).

7.2.6. Inhibition of HGF-induced migration by PKC, ERK, and MLCK inhibitors.

To determine the mechanism by which HGF induces glioma cell motility, we tested the effect of various inhibitors that could prevent PMA-induced motility on HGF-induced migration. Inhibition of PKC with Bisindolylmaleimide I (5 pM) completely abolished HGF-induced migration (Fig. 60). Similarly, ERK and MLCK inhibitors (PD98059, U0126, and ML7 and ML9, respectively) abrogated HGF-induced migration of human glioma cells (Fig. 60). These results indicate the requirement for HGF-induced motility of PKC, ERK, and MLCK.

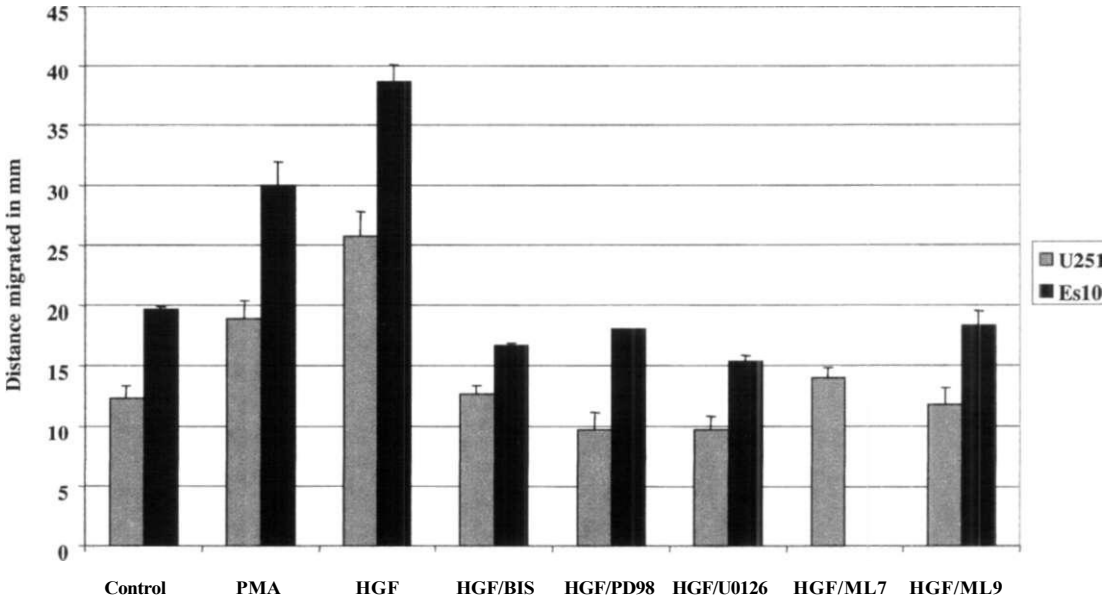


Figure 60: Inhibition of HGF-induced migration by ERK and MLCK inhibitors. Migration assay on U251N (gray bars) and Es1O (black bars) cells in the presence or absence of PMA (100 nM) or HGF (10 nM). Cells treated with HGF were also incubated in the presence of various inhibitors: Bisindolylmaleimide I (10 pM), PD98059 (25 pM), U0126 (10 pM), ML7 (20 pM), ML9 (30 pM). Cells were allowed to migrate for a 48 h period, fixed, and stained with hematoxylin to quantify the migration. The migration of Es1O cells in the presence of HGF/ML7 was not measured.

7.3. Discussion.

The lack of proliferative response to several growth factors in our cells is probably due to a problem in the experimental procedure, since many groups have reported potent mitogenic responses of glioma cells to these growth factors. Possible reasons to explain

the absence of proliferative response include medium constituents, insufficient serum starvation procedure, or accumulated mutations in cells, which made them insensitive to growth factor stimulation.

The results in this chapter indicate that HGF is a potent inducer of human glioma cell migration. Although we did not detect a significant activation of PKC downstream of c-Met, and that HGF-induced ERK activation occurred in a PKC-independent manner, we found that PKC, ERK, and MLCK are all required for HGF-induced motility.

The lack of PKC a, e, and T) activation or translocation following HGF stimulation suggests that PKC may not be activated downstream of c-Met in human glioma cells, or that it activates one of the PKC isoforms that was not tested (PKC 8, p, and Q. However, several PKC inhibitors with a specificity for all PKC isoforms did not prevent ERK activation downstream of c-Met, indicating that ERK activation occurs through a PKC-independent mechanism, as reported by others (Cai et al. 2000). ERK activation may be mediated through PI 3-kinase activation, which is strongly activated by HGF stimulation (Nakanishi et al. 1999, Cai et al. 2000).

Nonetheless, PKC inhibition totally abolished the migratory response induced by HGF. One attractive hypothesis is that HGF does not directly involve PKC in its migratory response, as Machide et al. (1998, 2000) reported that HGF-induced migration was independent of PKC in primary astrocytes. Rather, the effect observed in the presence of PKC inhibitors could be due to the inhibition of a more general mechanism of migration in which PKC plays a critical role. We found previously that PKC £ and RACK1 played an important role in promoting focal adhesion assembly and integrin clustering, and it is therefore possible that inhibition of these events may inhibit cell motility in a general way, independent of the mechanism by which migration is stimulated.

Although our results do not point to a direct role of PKC in the regulation of HGF-induced migration, PKC activity is still required for HGF-induced motility. This implies that targeting of PKC in gliomas may be extremely valuable since it could potentially block cell migration independent of the stimulus.

CHAPTER EIGHT

General Discussion

8.1. Summary and Significance.

Astrocytomas are the most common primary brain tumors. Glioblastoma multiforme (WHO grade IV) represent approximately 70% of astrocytomas and are characterized by a rapid proliferation rate and the ability to diffusely invade the surrounding normal brain tissue. The prognosis for patients with GBM is very poor and has not significantly improved in the past 30 years. Hence, there is a dire need for new therapeutic strategies to emerge. There is increasing evidence to suggest an important role for the PKC family in the regulation of glioma cell phenotypes, including cell survival, proliferation, migration, and invasion; however the identity of the PKC isoforms involved and the mechanism by which they accomplish their function remain elusive.

During the course of this work, we found that human glioma cell lines express 6 isoforms of PKC: PKC a, 8, e, **TJ**, p, and PKC activation in glioma cells increased their progression through the cell cycle. Of the 6 PKC isoforms that were present in glioma cells, PKC a was both necessary and sufficient to promote cell cycle progression. Also, decreased PKC a expression resulted in a marked decrease in cell proliferation. The only cell cycle regulatory molecule whose expression was rapidly altered and increased by PKC ***a*** activity was the cyclin-CDK inhibitor p21/Wafl/Cipl. Co-immunoprecipitation studies revealed that p21/Wafl/Cipl upregulation was accompanied by the incorporation of p21/Wafl/Cipl into various cyclin-CDK complexes, and that the kinase activity of these complexes was increased, thus resulting in cell cycle progression. Furthermore, depletion of p21/Wafl/Cipl by an antisense strategy attenuated the PKC-induced cell cycle progression. These results indicate that PKC a activity controls glioma cell cycle progression through the upregulation of p21AVafl/Cipl, which facilitates active cyclin/CDK complex formation.

In the study of the regulation of glioma cell migration by PKC, we found that PKC e positively regulated integrin-dependent adhesion, spreading, and motility; in contrast, PKC a had an opposite role. PKC e activation was associated with increased focal adhesion and lamellipodia formation, and integrin clustering; and was required for PMA-

induced adhesion and motility. Also, it appeared that the scaffolding protein RACK1 mediated the interaction between integrin [3 chain and activated PKC £; this was accompanied by the association of FAK and vinculin with integrin. Depletion of RACK1 by an antisense strategy decreased PKC e-induced adhesion and migration. These results provide a novel mechanistic link between PKC activation and integrin-mediated adhesion and motility.

The extracellular-signal regulated kinase (ERK) pathway was activated following PKC stimulation. It appeared that specific PKC isoforms were able to differentially target activated ERK to distinct cellular locations: while PKC a induced the nuclear translocation of phospho-ERK, PKC £ targeted activated ERK to focal adhesions. Inhibition of the ERK pathway completely abolished the PKC-induced integrin-mediated adhesion and migration. These results provide novel evidence that PKC £ is able to target activated ERK to focal adhesions to mediate glioma adhesion and motility; and also provide a potential molecular mechanism to explain the different biological functions of PKC a and £ in glioma cells.

Finally, HGF was a potent inducer of glioma cell motility. Although HGF stimulation did not activate PKC, its activity was required for HGF-induced migration, as inhibition of PKC completely abolished HGF-induced migration.

In conclusion, we have found that specific isoforms of PKC regulate distinct phenotypes of glioma cells: PKC a activation stimulates cell cycle progression and proliferation, while PKC £ activation induces integrin-mediated adhesion and motility (Fig. 61).

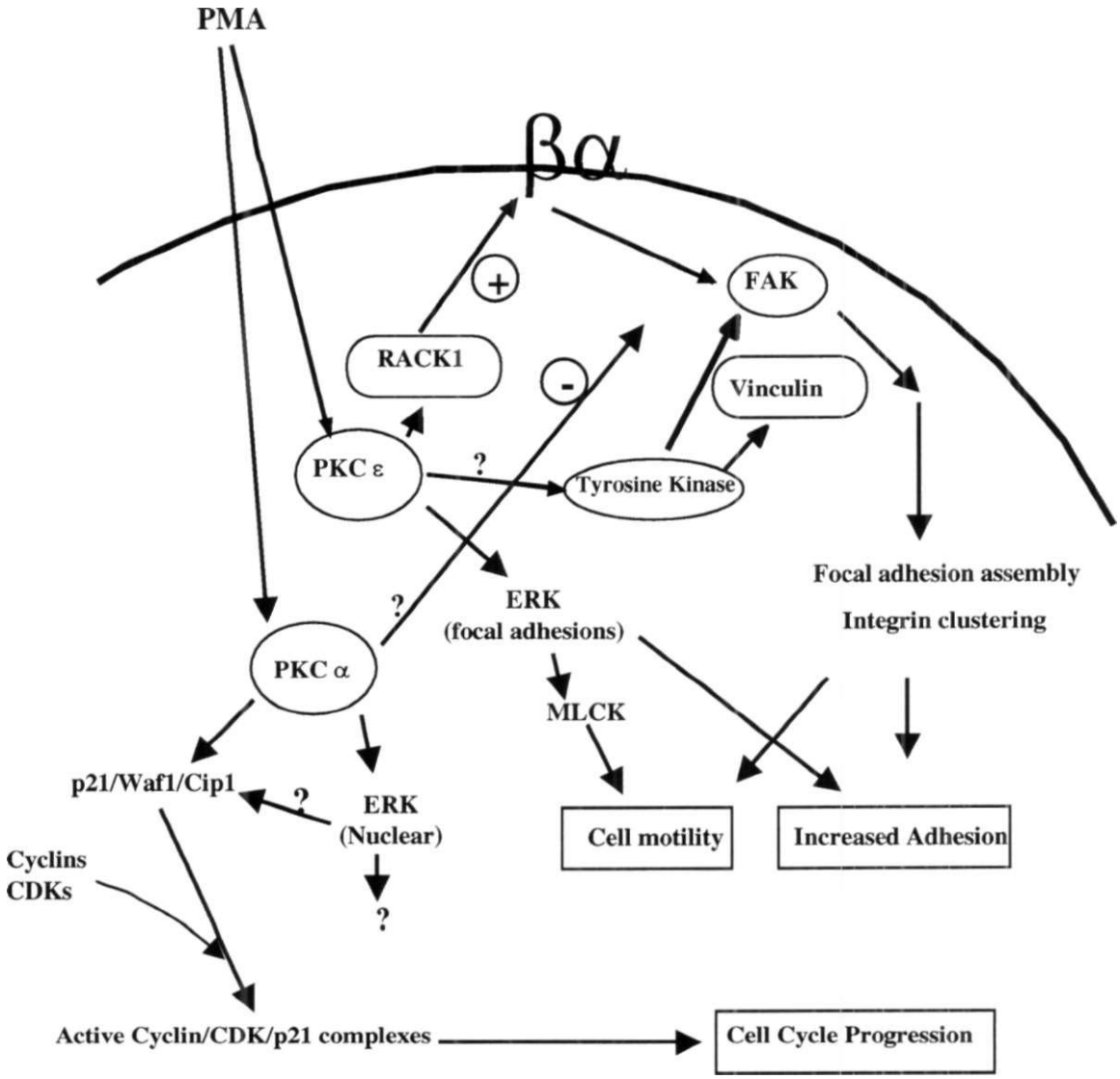


Figure 61: Specific isoforms of PKC control distinct phenotypes **of human glioma cells.**

PKC a activation induces p21AVafl/Cipl upregulation. p21/Wafl/Cipl facilitates the assembly of active ternary cyclin/CDK/p21 complexes, which increase cell cycle progression. PKC a induces the nuclear localization of activated ERK, which may be involved in p21/wafl/Cipl upregulation. PKC a negatively regulates glioma cell adhesion and migration, possibly by gearing the cell toward proliferation.

Activated PKC £ associates with the anchoring protein RACK1 and integrin (3 chains, leading to integrin clustering, tyrosine phosphorylation of FAK and vinculin, focal adhesion formation, and increased adhesion and migration on integrin substrates. PKC e activates and targets activated ERK to focal adhesions. ERK activity is required for PMA-induced adhesion and migration, possibly through an MLCK-dependent mechanism.

Our findings provide novel evidence for the role of PKC in the malignant phenotype of gliomas, and describe some of the mechanisms PKC isoforms utilize to regulate these phenotypes. We have shown that distinct PKC isoforms may have distinct functions in glioma cells, and that a single isoform may regulate several phenotypes. For example, PKC a played a critical role in the regulation of cell cycle progression and proliferation, but also negatively regulated cell adhesion and motility. These aspects of PKC functions will have to be kept in mind when designing novel therapeutic strategies targeting the PKC family. For example, specific targeting of PKC ***a*** in gliomas may not prove completely beneficial since it will likely decrease cell proliferation and induce apoptosis, as found by others (Ahmad et al. 1994, Dean et al. 1996, Yazaki et al. 1996, Sioud and Sorensen 1998, Leirdal and Sioud 1999, Shen et al. 1999), but may also increase cell migration, as we found that cells overexpressing PKC a were less motile than wild type counterparts. Thus, the specific inhibition of a single PKC isoform may inhibit one phenotype, but promote another that could be detrimental to the patient. One may hypothesize that a broad-spectrum inhibitor that targets multiple PKC isoforms would be more beneficial.

PKC a-induced cell cycle progression was mediated through the induction of p21/Wafl/Cipl, which participated in the assembly of active cyclin/CDK/p21 complexes. This finding is surprising considering that p21/Wafl/Cipl is a candidate tumor suppressor. However, there is increasing evidence to indicate a dual role for this protein in the regulation of cell cycle progression (Michieli et al. 1994, Nourse et al. 1994, Zhang et al. 1994, Harper et al. 1995, McLeod et al. 1995, Mantel et al. 1996, LaBaer et al. 1997, Hiyama et al. 1998, Cheng et al. 1999, Weiss et al. 2000, Stern et al. 2000). Our results indicate that using p21/Wafl/Cipl as an anti-tumor agent may not constitute a good choice to inhibit glioma cell proliferation. Moreover, high p21/Wafl/Cipl levels were associated with resistance to chemotherapeutic agents (Ruan et al. 1998, 1999). It could even be hypothesized that targeting of p21/Wafl/Cipl could decrease glioma cell proliferation and increase their sensitivity to chemotherapeutic agents.

Our finding that PKC e regulates integrin-mediated functions through its association with RACK1 may open new avenues of treatment. The function of PKC e seems to be fairly general for all integrin-mediated processes and is conserved in different cell types,

and across species, since Berrier et al. (2000) found that PKC ε could rescue integrin signaling and cell spreading in Chinese hamster ovary cells. Moreover, the fact that HGF-induced migration could be inhibited by PKC inhibitors, although PKC was not activated downstream of the c-Met receptor, points toward a very general role of PKC (possibly ε) in the regulation of cell migration. Hence, targeting of PKC ε may prove useful to inhibit glioma cell migration and invasion independent of the stimulus that induced cell migration in the first place. This may be due to a critical role for PKC ε in promoting focal adhesion assembly (and/or turnover), or in the activation of additional signaling pathways (such as ERK) downstream of integrins. Interestingly, RACK1 overexpression was detected in various carcinomas, and in angiogenically active endothelial cells *in vitro* and *in vivo,* suggesting an important role for RACK1 during angiogenesis (Berns et al. 2000). Our glioma cell lines had very high RACK1 levels; it would be interesting to test whether RACK1 is overexpressed in glioma specimens, particularly at sites of angiogenesis in the tumors. Considering the critical role of RACK1 in mediating integrin functions, and its possible role in angiogenesis, this anchoring protein could constitute a target of choice for tumor therapies.

8.2. Limitations.

One has to keep in mind that our results were obtained in an *in vitro* context, on cell lines grown in monolayer cultures. This experimental paradigm presents considerable advantages to perform biochemical assays, but the relevance of the results obtained may be questioned, as it is uncertain that they would still be valid in a 3-dimensional context, such as a tissue or organ. For example, our finding that PKC ε promotes cell migration may not be as relevant within a tissue in which, in order to migrate, a cell would have to break down ECM, and make its way between cells in an environment that probably offers considerable resistance to cell migration. In a 3-dimensional setting, other aspects than just cell motility have to be taken into account, such as the capacity of the cell to proteolyse ECM; different cues provided by ECM proteins and other soluble factors that may promote or inhibit cell migration also have to be considered. On the other hand, working in such a 3-dimensional setting presents technical challenges that may not be solvable with the current tools available to us, and the results obtained may not be as

readily interpretable because of the complexity of the multiple molecules, signals, and factors involved in a complex process such as cell migration/invasion. Thus, our work, by dissecting the molecular mechanism of cell migration or cell cycle progression induced by PKC isoforms, sets the basis for testing these results in a more physiologically relevant context.

All this work is based on the use of established cell lines that may be very different from the original tumors. These cells may have lost most of their original characteristics simply because they are grown in an *in vitro* environment that lacks a number of cues provided by the surrounding ECM, soluble factors, and interactions with other cell types (reviewed in Boudreau and Bissell 1998). These cells may also have accumulated a number of additional mutations during their growth *in vitro,* adding differences to the original tumor. For example, the truncated form of EGFR commonly found in glioblastomas is not stable in *in vitro* conditions, and cell lines invariably lose this mutant EGFR in tissue culture (Ekstrand et al. 1994, 1995).

Another limitation is the use of the phorbol ester PMA, or phorbol ester analogs such as thymeleatoxin, as PKC activators. One may question the specificity of these agonists, as other proteins have been described that can be activated by phorbol esters (see Introduction for more details). To verify that the effects observed were indeed due to PKC activation, in most experiments, we tried to block PMA-induced responses by the addition of PKC-specific inhibitors. Another concern is the potency of phorbol esters, as these compounds induce a massive activation of PKC that may not reflect the intensity of activation one would obtain using physiological stimuli. The use of activated forms of PKC may solve the problem of specificity encountered when using PMA. However, since constitutively activated PKC does not require co-factors for full activation, due to point mutations in the kinase domain, one could fear that overexpression of an activated PKC isoform would induce responses that may be non-specific due to absence of targeting (through translocation, or association with anchoring proteins) to the proper subcellular localization. The use of PKC isoforms constitutively anchored to the membrane (through myristoylation at the N-terminus of the protein) may help circumvent part of that problem. An alternative to the use of PKC inhibitors is overexpression of dominant-negative forms of PKC (kinase-dead mutants), that would still translocate in response to

agonists, thus occupying the binding sites on anchoring proteins and preventing further association with the native PKC. In our migration study, the use of a dominant-negative RACK1, that can bind to activated PKC but not to the integrin P chain would be an asset to clearly establish the importance of RACK1 in mediating the interaction between PKC and integrins.

During the investigation of the mechanism of induction of cell cycle progression by PKC a, not all the potential roles of PKC were tested. For example, we did not test whether PKC a activation was affecting the phosphorylation of lamin-B, as PKC P2 activity was shown to be required for the G2/M transition and nuclear envelope breakdown in HL60 cells, by acting as a lamin B kinase (Thompson and Fields 1996). Also, the expression levels of CDK7 and cyclin H were not assessed during our analysis of expression of various cell cycle regulatory proteins, as Hamada et al. (1993) had shown that PMA induced the downregulation of CDK7 and cyclin H in LMR-90 cells, resulting in Gl arrest. CDK7 and cyclin H assemble to form the cyclin-CDK activating kinase (CAK), whose activity is required for activation of CDKs.

The above limitations would have to be taken into account when considering the significance of the results obtained.

8.3. Future directions.

In the results of aims 1 and 2, we found that PKC a was controlling cell cycle progression through the upregulation of p21/Wafl/Cipl, which was incorporated into active ternary cyclin/CDK/p21 complexes. The mechanism by which PKC a induces p21/Wafl/Cipl upregulation remains unclear at this point. It may involve the ERK pathway, as found by other groups (Akashi et al. 1999, Das et al. 2000). Also, the identity of the transcription factors responsible for increasing p21 mRNA levels remains to be determined. An attractive hypothesis is that PKC phosphorylates and inactivates the transcriptional repressor Cut, which in turn would derepress the p21AVafl/Cipl gene promoter (Coqueret et al. 1996, 1998). Other candidates known to be regulated in a PKC dependent manner and to control the activity of the p21/Wafl/Cipl gene promoter include Spl and AP-1 (Biggs et al. 1996, Agadir et al. 1999). Another appealing possibility is that PKC a, which translocates to the nucleus in glioma cells, could

associate with and phosphorylate p21AVafl/Cipl, thus modulating its cyclin/CDK inhibitory activity; in other cell types PKC was found to bind and phosphorylate p21/Wafl/Cipl and, by doing so, altered p21/Wafl/Cipl inhibitory activities toward PCNA and CDK2 (Kashiwagi et al. 2000, Scott et al. 2000). Finally, PKC a may modulate the ability of known or yet-to-be identified p21/Wafl/Cipl-interacting proteins to associate with p21/Wafl/Cipl. This could also play an important role in the regulation of p21/Waf 1/Cip 1 functions.

In aims 3 and 4, we found that PKC e was increasing integrin-mediated adhesion and migration, and inducing focal adhesion assembly and integrin clustering, through its association with RACK1 and integrin p chains. PKC a, on the other hand, negatively regulated focal adhesion assembly, adhesion, and migration. The depletion of endogenous RACK1 by an antisense strategy abolished the PKC e-induced adhesion and migration. Another proof of the importance of RACK1 in this process could be provided with the use of a dominant-negative RACK1 that could still associate with PKC £ but lacks the integrin binding domain. This would clearly establish the requirement for RACK1 to target PKC £ to integrin p chain. The role of PKC £ once it is anchored to integrin P chains remains to be investigated. One may expect that activated PKC £ phosphorylates a number of targets in focal adhesions, thus facilitating their assembly, leading to integrin clustering, and increased adhesion and migration. PKC has previously been reported to be able to phosphorylate several integrin chains (Valmu et al. 1991, Hogervorst et al. 1993, Gimond et al. 1995, Rabinovitz et al. 1999), paxillin (DeNichilo and Yamada 1996), talin (Litchfield and Ball 1986), and vinculin (Weekes et al. 1996, Perez-Moreno et al. 1998). These phosphorylation events may participate in integrin activation and clustering and focal adhesion assembly and stability. Thus, the identification of the targets of PKC £ at focal adhesions would provide valuable information about the mechanism of these events. It is unclear how PKC a antagonizes the PMA-induced adhesion and migration. It may be an indirect consequence of the role of PKC a in promoting growth and cell cycle progression (go or grow hypothesis), or it may be due to another function of PKC a that remains to be uncovered.

We also found that PKC activation induced the activation of the ERK pathway, and that PKC a induced the nuclear translocation of ERK, while PKC e targeted activated ERK to focal adhesions. The exact mechanism of ERK activation remains unclear; although we have shown that PKC-induced ERK activation occurs through a MEK1/2-dependent mechanism, the upstream events remain to be determined, as several mechanisms have been identified in other cell types. PKC-induced ERK activation downstream of integrins could involve the serine phosphorylation of She, and subsequent association with Grb2/Sos, and Ras/Raf/MEK/ERK activation (El-Shemerly et al. 1997, Miranti et al. 1999). ERK activation could also occur through direct activation of Raf by PKC (Howe and Juliano 1998, Schonwasser et al. 1998). The mechanisms by which PKC e and a induce the localization of ERK to focal adhesions and to the nucleus, respectively, remain unknown, and will be of critical importance in the understanding of ERK function in the cell. The exact functions of ERKs at focal adhesions remain unclear at this point. Although, as our data indicate, they are clearly involved in the regulation of adhesion and migration, their direct targets are still unknown. Possible candidates for ERK phosphorylation at focal adhesions include MLCK and other cytoskeletal or cytoskeleton-associated proteins (Klemke et al. 1997, Nguyen et al. 1999, Fincham et al. 2000).

Our study has focused on two isoforms of PKC, ***a*** and £, and the functions of the four other PKC isoforms expressed in glioma cells (8, ***r\,*** p, and ***Q*** remain to be investigated. Moreover, the functions of these isoforms may add another level of regulation to the functions we found for PKC a and £. For example, PKC a and PKC £ were found to mediate VEGF upregulation in U373 glioma cells (Shih et al. 1999). Also, inhibition of PKC £ decreased the proliferation of U373MG cells in vitro (Sharif et al. 2001), although in our experiments, neither overexpression nor depletion of PKC £ had a noticeable effect on U251N cell proliferation.

The mechanism underlying the overactivation of PKC remains poorly understood. Although some evidence points toward the activation of PKC downstream of the various RTKs that are constitutively activated in gliomas (reviewed in Baltuch et al. 1995, Bredel and Pollack 1997), it is mainly based on the ability of PKC inhibitors to prevent the mitogenic effect of growth factor stimulation (Pollack et al. 1990a, 1990b, Couldwell et

al. 1992, Baltuch et al. 1993a, Baltuch and Yong 1996). The signaling cascades that may link EGFR, PDGFR, or FGFR to PKC activation remain to be investigated. A likely candidate to mediate PKC activation downstream of RTKs is PLC y, which can be directly activated by the receptors. However, other pathways may also be of importance. It will be very interesting to determine whether the newly identified PLC e, which is directly activated by Ras, is a major player in the activation of PKC downstream of RTKs (Kelley et al. 2001). This hypothesis is very tempting since both inhibition of PKC and inhibition of Ras had a comparable inhibitory effect (>90% inhibition) on growth factor induced proliferation (Guha et al. 1997, Couldwell et al. 1992, Baltuch et al. 1993a, Baltuch and Yong 1996). These results suggest that Ras and PKC are involved either in the same signaling cascade (possibly involving PLC e) or in parallel signaling pathways that are both required for growth factor-induced proliferation. To differentiate between these two possibilities, one could test whether the proliferation of activated Ras transformed cells is dependent on PKC activity.

Another appealing hypothesis that remains to be tested is that PKC overactivation could be due (at least in part) to the presence of inactivating mutations in the PTEN gene, which are common in high-grade gliomas. By decreasing the availability of PIP_3 for binding to the PH domain of PLC *y,* which allows its translocation to the membrane, PTEN was shown to suppress the activation of PLC *y* (Morimoto et al. 2000, Shan et al. 2000). One can hypothesize that PTEN could also regulate the activation of PDK-1, which also has a PH domain, in a similar manner. Thus, PTEN could be a major regulator of two enzymes whose activities lead to PKC activation. PDK-1 is a crucial player in the maturation process of newly synthesized PKC, and overactivation of PDK-1 could lead to the accumulation of catalytically competent PKCs. Overactivation of PLC y may be the cause of the PKC overactivation reported in glioma cells and glioma specimens (Couldwell et al. 1991, 1992). These hypotheses could be tested by re-introducing a wild type PTEN protein into glioma cells, and monitoring PDK-1, PLC *y,* and PKC activities and their relative association with membranes, and the amounts of PKCs phosphorylated at the activation loop (the PDK-1 phosphorylation site).

CHAPTER NINE

References

Aaltomaa S., Lipponen P., Eskelinen M., Ala-Opas M., Kosma V.M. 1999. Prognostic value and expression of p21AVafl/Cipl protein in prostate cancer. Prostate 39:8-15.

Adkins J.N., Lumb K.J. 2000. Stoichiometry of cyclin A-cyclin-dependent kinase 2 inhibition by p21/CiplAVafl. Biochemistry 39: 13925-13930.

Agadir A., Chen G.Q., Bost F., Li Y., Mercola D., Zhang X. 1999. Differential effect of retinoic acid on growth regulation by phorbol ester in human cancer cell lines. J. Biol. Chem. 274: 29779-29785.

Aguirre Ghiso J.A., Alonzo D.F., Farias E.F., Gomez D.E., Bal de Kier Joffe E. 1999. Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. Eur. J. Biochem. 263: 295-304.

Ahmad S., Mineta T., Martuza R.L., Glazer R.I. 1994. Antisense expression of protein kinase C alpha inhibits the growth and tumorigenicity of human glioblastoma cells. Neurosurgery 35: 904-909.

Ahmed S., Lee J., Kozma R., Best A., Montfries C, Lim L. 1993. A novel functional target for tumor promoting phorbol esters and lysophosphatidic acid. The p21/Rac GTPase activating protein n-chimaerin. J. Biol. Chem. 268: 10709-10712.

Akashi M., Osawa Y., Koeffler H.P., Hachiya M. 1999. p21WAF1 expression by an activator of protein kinase C is regulated mainly at the post-transcriptional level in cells lacking p53: important role of RNA stabilization. Biochem. J. 337: 607-616.

Aplin A.E., Howe A.K., Juliano R.L. 1999. Cell adhesion receptors, signal transduction and cell growth. Curr. Opin. Cell Biol. 11: 737-744.

Arita Y., Buffolino P., Coppock D.L. 1998. Regulation of the cell cycle at the G2/M boundary in metastatic melanoma cells by 12-O-tetradecanoyl phorbol-13-acetate (TPA) by blocking p34cdc2 kinase activity. Exp. Cell Res. 242: 381-390.

Baltuch G.H., Couldwell W.T., Villemure J.G., Yong V.W. 1993a. Protein kinase C inhibitors suppress cell growth in established and low passage glioma cell lines. A comparison between staurosporine and tamoxifen. Neurosurgery 33: 495-501.

Baltuch G.H., Shenouda G, Langleben A., Villemure J.H, 1993b. High dose tamoxifen in the treatment of recurrent high-grade glioma: a report of clinical stabilization and tumor regression. Can. J. Neurol. Sci. 20: 168-170.

Baltuch G.H., Dooley N.P., Villemure J.G., Yong V.W. 1995. Protein kinase C and growth regulation of malignant gliomas. Can. J. Neurol. Sci. 22: 264-271.

Baltuch G.H., Yong V.W. 1996. Signal transduction for proliferation of glioma cells in vitro occurs predominantly through a protein kinase C-mediated pathway. Brain Res. 710: 143-149.

Bao J., Alroy I., Waterman H., Scheiter E.D., Brodie C. Gruenberg J., Yarden Y. 2000. Threonine phosphorylation diverts internalized epidermal growth factor receptors from a degradative pathway to the recycling endosome. J. Biol. Chem. 275: 26178-26186.

Begemann M., Kashimawo S.A., Choi Y.J.A., Kim S., Christiansen K.M., Duigou G., Mueller M., Schieren I., Ghosh S., Fabbro D., Lampen N.M., Heitjan D.F., Schiff P.B., Bruce J.N., Weinstein I.B. 1996. Inhibition of the growth of glioblastomas by CGP 41251, an inhibitor of protein kinase C, and by a phorbol ester tumor promoter. Clin. Cancer Res. 2: 1017-1030.

Begemann M., Kashimawo S.A., Heitjan D.F., Schiff P.B., Bruce I.N., Weinstein I.B. 1998a. Treatment of human glioblastoma cells with the staurosporine derivative CGP 41251 inhibits CDC2 and CDK2 kinase activity and increases radiation sensitivity. Anticancer Res. 18: 2275-228.

Begemann M., Kashimawo S.A., Lunn R.M., Delohery T., Choi Y.J.A., Kim S., Heitjan D.F., Santella R.M., Schiff P.B., Bruce J.N., Weinstein I.B. 1998b. Growth inhibition induced by Ro 31-8220 and calphostin C in human glioblastoma cell lines is associated with apoptosis and inhibition of CDC2 kinase. Anticancer Res. 18: 3139-3152.

Belien A.T., Paganetti P.A., Schwab M.E. 1999. Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter. J. Cell Biol. 144: 373-384.

Berens M.E., Rief M.D., Shapiro J.R., Haskett D., Giese A., Joy A., Coons S.W. 1996. Proliferation and motility responses of primary and recurrent gliomas related to changes in epidermal growth factor receptor expression. J. Neuro-Oncol. 27: 11-22.

Berns H., Humar R., Hengerer B., Kiefer F.N., Battegay E.J. 2000. RACK1 is upregulated in angiogenesis and in human carcinomas. FASEB J. 14: 2549-2558.

Berrier A.L., Mastrangelo A.M., Downward J., Ginsberg M., LaFlamme S.E. 2000. Activated R-Ras, Rac1, PI 3-Kinase and PKC e can each restore cell spreading inhibited by isolated integrin Pi cytoplasmic domains. J. Cell Biol. 151: 1549-1560.

Besson A., Robbins S.M., Yong V.W. 1999. PTEN/MMAC1/TEP1 in signal transduction and tumorigenesis. Eur. J. Biochem. 263: 605-611.

Besson A., Yong V.W. 2000. Involvement of p21AVafl/Cipl in protein kinase C alpha-induced cell cycle progression. Mol. Cell. Biol. 20: 4580-4590.

Besson A., V.W. Yong. 2001. Mitogenic signaling and the relationship to cell cycle regulation in astrocytomas. J. Neuro-Oncol. In press.

Biernat W., Tohma Y., Yonekawa Y., Kleihues P., Ohgaki H. 1997. Alterations of cell cycle regulatory genes in primary (de novo) and secondary glioblastomas. Acta Neuropathol. 94: 303-309.

Biggs J.R., Kudlow J.E., Kraft A.S. 1996. The role of the transcription factor Sp1 in regulating the expression of the WAF1/CIP1 gene in U937 leukemic cells. J. Biol. Chem. 271: 901-906.

Bigner S.H., Burger P.C., Wong A.J., Werner M.H., Hamilton S.R., Muhlbaier L.H., Vogelstein B., Bigner D.D. 1988. Gene amplification in malignant human gliomas: Clinical and histopathological aspects. J. Neuropathol. Exp. Neurol. 47: 191-205.

Black J.D. 2000. Protein kinase C-mediated regulation of the cell cycle. Front. Biosci. 5: D406-D423.

Blackshear P.J., Stumpo D.J., Huang J.K., Nemenoff R.A., Spach D.H. 1987. Protein kinase C dependent and independent pathways of proto-oncogene induction in human astrocytoma cells. J. Biol. Chem. 262: 7774-7781.

Bornancin F., Parker P.J. 1996. Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase C alpha. Curr. Biol. 6: 1114-1123.

Bornancin F., Parker P.J. 1997. Phosphorylation of protein kinase C alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase resistant state. J. Biol. Chem. 272: 3544-3549.

Bostrom J., Cobbers J.M.J.L., Wolter M., Tabatabai G, Weber R.G., Lichter P., Collins V.P., Reifenberger G. 1998. Mutations of the PTEN/MMAC1 tumor suppressor gene in a subset of glioblastomas but not in meningiomas with loss of chromosome lOq. Cancer Res. 58: 29-33.

Boudreau N., Bissell M.J. 1998. Extracellular matrix signaling: integration of form and function in normal and malignant cells. Curr. Opin. Cell Biol. 10: 640-646.

Bredel M., Pollack I.F. 1997. The role of protein kinase C (PKC) in the evolution and proliferation of malignant gliomas, and the application of PKC inhibition as a novel approach to anti-glioma therapy. Acta Neurochir. 139: 1000-1013.

Burns K.L., Ueki K, Jhung S.L., Koh J., Louis D.N. 1998. Molecular genetics correlates of p16, CDK4, and pRb immunochemistry in glioblastomas. J. Neuropathol. Exp. Neurol. 57: 122-130.

Cai K, Dynlacht B.D. 1998. Activity and nature of p21AVafl complexes during the cell cycle. Proc Natl. Acad. Sci. USA 95: 12254-12259.

Cai W., Rook S.L., Jiang Z.Y., Takahara N., Aiello L.P. 2000. Mechanisms of hepatocyte growth factor-induced retinal endothelial cell migration and growth. Invest. Ophthalmol. Vis. Sci. 41: 1885-1893.

Caskey L.S., Fuller G.N., Bruner J.M., Yung W.K.A., Sawaya R.E., Holland E.C., Zhang W. 2000. Toward a molecular classification of the gliomas: histopathology, molecular genetics, and gene expression profiling. Histol. Histopathol. 15: 971-981.

Chang B Y., Conroy K.B., Machleder E.M., Cartwright C.A. 1998. RACK1, a receptor for activated C kinase and a homolog of the p subunit of G proteins, inhibits activity of Src tyrosine kinase and growth of NIH 3T3 cells. Mol. Cell. Biol. 18: 3245-3256.

Chen C, Okayama H. 1987. High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7: 2745-2752.

Cheng M., Olivier P., Dielh J.A., Fero M., Roussel M.F., Roberts J.M., Sherr C.J. 1999. The p21(Cipl) and p27(Kipl) CDK Inhibitors'are essential activators of cyclin D-dependent kinases in murine fibroblasts. EMBO J. 18: 1571-1583.

Cho K.K., Mikkelsen T, Lee Y.J., Jiang F., Chopp M., Rosenblum M.L. 1999. The role of protein kinase C a in U87 glioma invasion. Int. J. Devi. Neuroscience 17: 447-461.

Collins V.P. 1982. Cultured human glia and glioma cells. Int. Rev. Exp. Pathol. 24: 135-192.

Collins V.P. 1995. Gene amplification in human gliomas. Glia 15: 289-296.

Coqueret O., Berube G., Nepveu A. 1996. DNA binding by cut homeodomain proteins is down-modulated by protein kinase C. J. Biol. Chem. 271: 24862-24868.

Coqueret O., Berube G., Nepveu A. 1998. The mammalian Cut homeodomain protein functions as a cell-cycle-dependent transcriptional repressor which downmodulates p21WAFl/CIPl/SDIl in S phase. EMBO J. 17: 4680-4694.

Couldwell W.T., Antel J.P., Apuzzo M.L, Yong V.W. 1990. Inhibition of growth of established human glioma cell lines by modulators of the protein kinase C system. J. Neurosurg. 73: 594-600.

Couldwell W.T., Uhm J.H., Antel J.P., Yong V.W. 1991. Enhanced protein kinase C activity correlates with the growth rate of malignant gliomas in vitro. Neurosurgery 29: 880-887.

Couldwell W.T., Antel J.P., Yong V.W. 1992. Protein kinase C activity correlates with the growth rate of malignant gliomas: part II. Effects of glioma mitogens and modulators of protein kinase C. Neurosurgery 31: 717-724.

Couldwell W.T., Hinton D.R., Surnock A.A., DeGiorgio C M., Weiner L.P., Apuzzo M.L.J., Masri L., Law R.E., Weiss M.H. 1996. Treatment of recurrent malignant gliomas with chronic oral high-dose tamoxifen. Clin. Cancer Res. 2: 619-622.

Croze E., Usacheva A., Asarnow D., Minshall R.D., Perez H.D., Colamonici O. 2000. Receptor for activated C-kinase (RACK-1), a WD motif-containing protein, specifically associates with the human type IIFN receptor. J. Immunol. 165: 5127-5132.

Csukai M., Chen C.H., De Matteis M.A., Mochly-Rosen D. 1997. The coatomer protein beta COP, a selective binding protein (RACK) for protein kinase C epsilon. J. Biol. Chem. 272: 29200-29206.

Current Protocols in Cell Biology. 2000. Edited by Bonifacino J.S., Dasso M., Harford J.B., Lippincott-Schwartz J., Yamada K.M.

Da Rocha A.B., Mans D.R., Lenz G., Fernandez A.K., De Lima C, Monteiro V.F., Goncalves D., Moreira J.C., Brunetto A.L., Rodnight R., Schwartsmann G. 2000. Protein kinase C mediated in vitro invasion of human glioma cells through extracellular signal regulated kinase and ornithine decarboxylase. Pathobiology 68: 113-123.

Das D., Pintucci G., Stern A. 2000. MAPK-dependent expression of p21(WAF) and p27(kipl) in PMA-induced differentiation of HL60 cells. FEBS Lett. 472: 50-52.

Dean N., McKay R., Miraglia L, Howard R., Cooper S., Giddings J., Nicklin P., Meister L., Ziel R., Geiger T., Muller M., Fabbro D. 1996. Inhibition of growth of human tumor cell lines in nude mice by an antisense oligonucleotide inhibitor of protein kinase C alpha expression. Cancer Res. 56: 3499-3507.

DeFilippi P., Venturino M., Gulino D., Duperray A., Boquet P., Fiorentini C, Volpe G, Palmieri M., Silengo L., Tarone G. 1997. Dissection of pathways implicated in integrin mediated actin cytoskeleton assembly. Involvement of protein kinase C, Rho GTPase, and tyrosine phosphorylation. J. Biol. Chem. 272: 21726-21734.

Delphin C, Baudier J. 1994. The protein kinase C activator, phorbol ester, cooperates with wild type p53 species in ras transformed embryo fibroblasts growth arrest. J. Biol. Chem. 269: 29579-29587.

DeNichilo M.O., Yamada K.M. 1996. Integrin apVdependent serine phosphorylation of paxillin in cultured human macrophages adherent to vitronectin. J. Biol. Chem. 271: 11016-11022.

Dirks P.B., Rutka J.T. 1997. Current concepts in neuro-oncology: The cell cycle, a review. Neurosurgery 40: 1000-1015.

Dirks P.B., Hubbard S.L., Murakami M., Rutka J.T. 1997. Cyclin and cyclin-dependent kinase expression in human astrocytoma cell lines. J. Neuropathol. Exp. Neurol. 56: 291-300.

Disatnik M.H., Hernandez-Sotomayor S.M.T., Jones G., Carpenter G, Mochly-Rosen D. 1994. Phospholipase C-yi binding to intracellular receptors for activated protein kinase C. Proc. Natl. Acad. Sci. U.S.A. 91: 559-563.

Disatnik M.H., Rando T.A. 1999. Integrin-mediated muscle cell spreading. The role of protein kinase C in outside-in and inside-out signaling and evidence of integrin cross talk. J. Biol. Chem. 274: 32486-32492.

Dooley N.P., Baltuch G.H., Groome N., Villemure J.G., Yong V.W. 1998. Apoptosis is induced in glioma cells by antisense oligonucleotides to protein kinase C alpha and is enhanced by cycloheximide. Neuroreport 9: 1727-1733.

Dutil E.M., Toker A., Newton A.C. 1998. Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase-1 (PDK-1). Curr. Biol. 8: 1366-1375.

Ebinu J.O., Bottorff D.A., Chan E.Y., Stang S.L., Dunn R.J., Stone J.C. 1998. RasGRP, a Ras guanyl nucleotide release protein with calcium- and diacylglycerol-binding motifs. Science 280: 1082-1086.

Ekstrand A.J., James C D., Cavenee W.K., Seliger B., Pettersson R.F., Collins V.P. 1991. Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. Cancer Res. 51: 2164-2172.

Ekstrand A.J., Longo N., Hamid M.L., Olson J.J., Liu L, Collins V.P., James C D. 1994. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. Oncogene 9: 2313-2320.

Ekstrand A.J., Liu L, He L, Hamid M.L., Longo N., Collins V.P., James C D. 1995. Altered subcellular location of an activated and tumour associated epidermal growth factor receptor. Oncogene 10: 1455-1460.

El-Obeid A., Bongcam-Rudloff E., Sorby M., Ostman A., Nister M., Westermark B. 1997. Cell scattering and migration induced by autocrine transforming growth factor *a.* in human glioma cells *in vitro.* Cancer Res. 57: 5598-5604.

El-Shemerly M.Y.M., Besser D., Nagasawa M., Nagamine Y. 1997. 12-0-tetradecanoylphorpbol-13-acetate activates the Ras/extracellular signal-regulated kinase (ERK) signaling pathway upstream of Sos involving serine phosphorylation of She in NIH 3T3 cells. J. Biol. Chem. 272: 30599-30602.

Estanyol J.M., Jaumont M., Casanovas O., Rodriguez-Vilarrupla A., Agell N., Bachs O. 1999. The protein SET regulates the inhibitory effect of p21/Cipl on cyclin-E-cyclin dependent kinase-2 activity. J. Biol. Chem. 274: 33161-33165.

Fahraeus R., Lane D.P. 1999. The $p16^{INK4a}$ tumour suppressor protein inhibits a_vP3 integrin-mediated cell spreading on vitronectin by blocking PKC-dependent localization of $a_v p_3$ to focal adhesion. EMBO J. 18: 2106-2118.

Fathallah-Shaykh H. 1999. New molecular strategies to cure brain tumors. Arch. Neuropathol. 56: 449-453.

Feldkamp M.M., Lala P., Lau N., Roncarci L., Guha A. 1999. Expression of activated epidermal growth factor receptors, ras-guanosine triphosphate, and mitogen activated protein kinase in human glioblastoma multiforme specimens. Neurosurgery 45: 1442-1453.

Fincham V.J., James M., Frame M.C., Winder S.J. 2000. Active ERK7MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. EMBO J. 19:2911-2923.

Fishman D.D., S. Segal, E. Livneh. 1998. The role of protein kinase C in Gl and G2/M phases of the cell cycle. Int. J. Oncol. 12: 181-186.

Fleming T.P., Saxena A., Clark W.C., Robertson J.T., Oldfield E.H., Aaronson S.A., Ali I.U. 1992. Amplification and/or overexpression of platelet-derived growth factor receptors and epidermal growth factor receptor in human glial tumors. Cancer Res. 52: 4550-4553.

Friedlander D.R., Zagzag D., Shiff B., Cohen PL, Allen J.C., Kelly P.J., Grumet M. 1996. Migration of brain tumor cells on extracellular matrix proteins *in vitro* correlates with tumor type and grade and involves oc, and pi integrins. Cancer Res. 56: 1939-1947.

Frey M.R., Saxon M.L., Zhao X., Rollins A., Evans S.S., Black J.D. 1997. Protein kinase C mediated cell cycle arrest involves induction of p21 Wafl/Cipl and p27Kipl and hypophosphorylation of the retinoblastoma protein in intestinal epithelial cells. J. Biol. Chem. 272: 9424- 9435.

Funk J.O., Waga S., Harry J.B., Espling E., Stillman B., Galloway D.A. 1997. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. Genes Dev. 11: 2090-2100.

Garcia-Paramio P., Cabrerizo Y., Bornancin F., Parker PJ. 1998. The broad specificity of dominant inhibitory protein kinase C mutants infers a common step in phosphorylation. Biochem. J. 333: 631-636.

Geijsen N., Spaargaren M., Raaijmakers J.A.M., Lammers J.W.J., Koenderman L., Coffer P.J. 1999. Association of RACK1 and PKC p with the common P-chain of the IL-5/IL-3/GM-CSF receptor. Oncogene 18: 5126-5130.

Giancotti F.G., Ruoslahti E. 1999. Integrin signaling. Science 285: 1028-1032.

Giese A., Loo M.A., Norman S.A., Treasurywala S., Berens M.E. 1996. Contrasting migratory response of astrocytoma cells to tenascin mediated by different integrins. J. CellSci. 109: 2161-2168.

Gimond C, De Melker A., Aumailley M., Sonnenberg A. 1995. The cytoplasmic domain of alpha 6A integrin subunit is an in vitro substrate for protein kinase C. Exp. Cell Res. 216:232-235.

Gomez D.E., Skilton G., Alonso D.F., Kazanietz M.G. 1999. The role of protein kinase C and novel phorbol ester receptors in tumor cell invasion and metastasis. Oncol. Reports 6: 1363-1370.

Grossman S.A., Alavi K, Carson K, Priet R., Dorr A., Holmlund J. 1999. The efficacy of an antisense oligonucleotide directed against protein kinase C alpha (ISIS 3521) delivered as a 21-day continuous intravenous infusion in patients with recurrent high-grade astrocytomas. Abstract #84 for the Society of Neuro-Oncology 1999. Neuro-Oncology 1: 313.

Gu J., Masahito T., Yamada K.M. 1998. Tumor suppressor PTEN inhibits integrin and growth factor mediated mitogen activated protein (MAP) kinase signaling pathways. J. Cell Biol. 143: 1375-1383.

Gu J., Tamura M., Pankov R., Danen E.H., Takino T., Matsumoto K, Yamada K.M. 1999. She and FAK differentially regulate cell motility and directionality modulated by PTEN. J. Cell Biol. 146: 389-403.

Guha A., Feldkamp M.M., Lau N., Boss G., Pawson A. 1997. Proliferation of human malignant astrocytomas is dependent on ras activation. Oncogene 15: 2755-2765.

Guizzetti M., Costa L.G. 2000. Possible role of protein kinase C zeta in muscarinic receptor induced proliferation of astrocytoma cells. Biochem. Pharmacol. 60: 1457-1466.

Gulliford T., Ouyang X., Epstein R.J. 1999. Intensification of growth factor receptor signaling by phorbol treatment of ligand-primed cells implies a dimer-stabilizing effect of protein kinase C-dependent juxtamembrane domain phosphorylation. Cell. Signal. 11: 245-252.

Haas-Kogan D., Shalev N., Wong M., Mills G, Yount G, Stokoe D. 1998. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the rumor suppressor PTEN/MMAC. Curr. Biol. 8: 1195-1198.

Hall M., Peters G. 1996. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. Adv. Cancer Res. 68: 67-108.

Haller H, Lindschau C, Maasch C, Olthoff H, Kurscheid D., Luft F.C. 1998. Integrin-induced protein kinase C a and e translocation to focal adhesions mediates vascular smooth muscle cell spreading. Circ. Res. 82: 157-165.

Harper J.W., Elledge S.J., Keyomarsi K, Dynlacht B. Tsai L.H., Zhang P., Dobrowolski S., Bai C, Connell-Crowley L., Swindell E., Fox M.P., Wei N. 1995. Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell 6: 387-400.

Hass R., Gunji H., Hirano M., Weichselbaum R., Kufe D. 1993. Phorbol ester induced monocytic differentiation is associated with G2 delay and down regulation of cdc25 expression. Cell Growth Diff. 4: 159-166.

Hamada K, Takuwa N., Zhou W., Kumada M., Takuwa Y. 1993. Protein kinase C inhibits the CAK-CDK2 cyclin dependent kinase cascade and G1/S cell cycle progression in human diploid fibroblasts. Biochim. Biophys. Acta 1310: 149-156.

Harrington E.O., Loffler J., Nelson P.R., Kent K.C., Simons M., Ware J.A. 1997. Enhancement of migration by protein kinase C a and inhibition of proliferation and cell cycle progression by protein kinase C 8 in capillary endothelial cells. J. Biol. Chem. 272: 7390-7397.

He J., Olson J.J., James C D. 1995. Lack of p16INK4 or retinoblastoma protein (pRb), or amplification-associated overexpression of CDK4 is observed in distinct subsets of malignant glial tumors and cell lines. Cancer Res. 55: 4833-4836.

Hemler M.E. 1998. Integrin associated proteins. Curr. Opin. Cell Biol. 10: 578-585.

Hengst L., Gopfert U., Lashuel H.A., Reed S.I. 1998. Complete inhibition of CDK/cyclin by one molecule of p21/Cipl. Genes Dev. 12: 3882-3888.

Herman J.G., Jen J., Merlo A., Baylin S.B. 1996. Hypermethylation associated inactivation indicates a tumor suppressor role for p15/INK4bl. Cancer Res. 56: 722-727.

Hermanson M., Funa K., Hartman M., Claesson-Welsh L., Heldin C.H., Westermark B., Nister M. 1992. Platelet-derived growth factor (PDGF) and its receptors in human glioma tissue: expression of mRNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res. 52: 3213-3219.

Hiraga S., Ohnishi T., Izumoto S., Miyahara E., Kanemura Y., Matsumura H., Arita N. 1998. Telomerase activity and alterations in telomere length in human brain tumors. Cancer Res. 58: 2117-2125.

Hiyama H., Iavarone A., Reeves S.A. 1998. Regulation of the CDK inhibitor p21 gene during cell cycle progression is under the control of the transcription factor E2F. Oncogene 16: 1513-1523.

Hogervorst F, Kuikman I., Noteboom E., Sonnenberg A. 1993. The role of phosphorylation in activation of the O^API laminin receptor. J. Biol. Chem. 268: 18427-18430.

Holland E.C., Hively W.P., DePinho R.A., Varmus H.E. 1998. A constitutively active epidermal growth factor receptor cooperates with disruption of Gl cell cycle arrest pathways to induce glioma like lesions in mice. Genes Dev. 12: 3675-3685.

Holland E.C. 2000a. A mouse model for glioma: Biology, pathology, and therapeutic opportunities. Toxicologic. Pathol. 28: 171-177.

Holland E.C, Celestino J., Dai C, Schaefer L., Sawaya R.E., Fuller G.N. 2000b. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. Nat. Genetics 25: 55-57.

Holland E.C. 2000c. Glioblastoma multiforme: the terminator. Proc. Natl. Acad. Sci. USA. 97: 6242-6244.

Horwitz A.R., Parsons J. 1999. Cell migration-movin'on. Science 286: 1102-1103.

Howe A.K., Juliano R.L. 1998. Distinct mechanisms mediate the initial and sustained phases of integrin-mediated activation of the Ras/MEK/mitogen-activated protein kinase cascade. J. Biol. Chem. 273: 27268-27274.

Huang H.J.S., Nagane M., Klingbeil C.K., Lin H., Nishikawa R., Ji X.D., Huang CM., Gill G.N., Wiley H.S., Cavenee W.K. 1997. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. J. Biol. Chem. 272: 2927-2935.

Hughes P.E., Pfaff M. 1998. Integrin affinity modulation. Trends Cell Biol. 8: 359-364.

Hundle B., McMahon T, Dadgar J., Chen C.H., Mochly-Rosen D., Messing R.O. 1997. An inhibitory fragment derived from protein kinase C epsilon prevents enhancement of nerve growth factor response by ethanol and phorbol esters. J. Biol. Chem. 272: 15028-15035.

Hussaini I.M., Karns L.R., Vinton G., Carpenter J.E., Redpath G.T., Sando J.J., VandenBerg S.R. 2000. Phorbol 12-myristate 13-acetate induces protein kinase C *r*\-specific proliferative response in astrocytic tumor cells. J. Biol. Chem. 275: 22348-22354.

Hynes R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69: 11-25.

Ichimura K., Schmidt E.E., Goike H.M., Collins V.P. 1996. Human glioblastomas with no alterations of the CDK2A(p16/INK4A/MTS1) and CDK4 genes have frequent mutations of the retinoblastoma gene. Oncogene 13: 1065-1072.

Ichimura K., Bondesson Bolin M., Goike H.M., Schmidt E.E., Moshref A., Collins V.P. 2000. Deregulation of the p14-ARF/MDM2/p53 pathway is a prerequisite for human astrocytic gliomas with G1/S transition control gene abnormalities. Cancer Res. 60: 417-424.

Jaken S. 1996. Protein kinase C isozymes and substrates. Curr. Opin. Cell. Biol. 8: 168-173.

Jaken S., Parker P.J. 2000. Protein kinase C binding proteins. Bioessays 22: 245-254.

Jimenez-deAsua L., Goin M. 1992. Prostaglandin F2 alpha decreases the affinity of epidermal growth factor receptors in Swiss mouse 3T3 cells via protein kinase C activation. FEBS Lett. 299: 235-238.

Johnson J., Grey M., Mochly-Rosen D. 1996. A protein kinase C translocation inhibitor as an isozyme selective antagonist of cardiac function. J. Biol. Chem. 271: 24962-24966.

Jones D.L., Alani R.M., Munger K. 1997. The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cipl-mediated inhibition of cdk2. Genes Dev. 11: 2101-2111.

Jordan M., Schallhorn A., Wurm F.M. 1996. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucl. Acids Res. 24: 596-601.

Jung J.M., Bruner J.M., Ruan S., Langford L.A., Kyritsis A.P., Kobayashi T., Levine V.A., Zhang W. 1995. Increased levels of p21/Wafl/Cipl in human brain tumors. Oncogene 11: 2021-2028.

Kashiwagi M., Ohba M., Watanabe H., Ishino K., Kasahara K., Sanai Y., Taya Y., Kuroki T. 2000. PKC eta associates with cyclin E/CDK2/p21 complex, phosphorylates p21, and inhibits CDK2 kinase in keratinocytes. Oncogene 19: 6334-6341.

Kazanietz M.G., Areces L.B., Bahador A., Mischak H., Goodnight J., Mushinski J.R, Blumberg P.M. 1993. Characterization of ligand and substrate specificity for the calcium-dependent and calcium independent protein kinase C isozymes. Mol. Pharmacol. 44: 298-307.

Kazanietz M.G. 2000. Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. Mol. Carcinogenesis 28: 5-11.

Kelley G.G., Reks S.E., Ondrako J.M., Smrcka A.V. 2001. Phospholipase C e: a novel Ras effector. EMBO J. 20: 743-754.

Khoshyomm S., Penar P.L., Rossi J., Wells A., Abramson D.L., Bhushan A. 1999. Inhibition of phospholipase C yi activation blocks glioma cell motility and invasion of fetal rat brain aggregates. Neurosurgery 44: 568-578.

Khosravi-Far R., Campbell S., Rossman K.L., Der C.J. 1998. Increasing complexity of Ras signal transduction: involvement of Rho family proteins. Adv. Cancer Res. 72: 57-107.

Kleihues P., Soylemezoglu F., Schauble B., Scheithauer B.W., Burger P.C. 1995. Histopathology, classification, and grading of gliomas. Glia 15: 211-221.

Klemke R.L., Yebra M., Bayna E.M., Cheresh D.A. 1994. Receptor tyrosine kinase signaling required for integrin a,Ps-directed cell motility but not adhesion on vitronectin. J. Cell Biol. 127: 859-866.

Klemke R.L., Cai S., Giannini A.L., Gallagher P.J., De Lanerolle P., Cheresh, D.A. 1997. Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. 137: 481-492.

Klinghoffer R.A., Sachsenmaier C, Cooper J.A., Soriano P. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J. 18: 2459-2471.

Kolanus W., Seed B. 1997. Integrins and inside-out signal transduction: converging signals from PKC and PIP3. Curr. Opin. Cell. Biol. 9: 725-731.

Kolch W., Heldecker G., Kochs G., Hummel R., Vahidi H., Mischak H., Finkenzeller G., Marme D., Rapp U.R. 1993. Protein kinase Coc activates Raf-1 by direct phosphorylation. Nature 364: 249-252.

Koochekpour S., Jeffers M., Rulong S., Taylor G., Klineberg E., Hudson E.A., Resau J.H., Vande Woude G.F. 1997. Met and hepatocyte growth factor expression in human gliomas. Cancer Res. 57: 5391-5398.

Korkolopoulou P., Kouzelis K., Christodoulou P., Papanikolaou A., Thomas-Tsagli E. 1998. Expression of retinoblastoma gene product and p21AVafl/Cipl protein in gliomas: correlations with proliferation markers, p53 expression and survival. Acta Neuropathol. 95: 617-624.

Kosaka C, Sasaguri T., Ishida A., Ogata J. 1996. Cell cycle arrest in G2 phase induced by phorbol ester and diacylglycerol in vascular endothelial cells. Am. J. Physiol. 270: C170-C178.

Kumar Bagul T., Jackson R.J., Agrawal D., Pledger W.J. 2000. Analysis of cyclin D3-CDK4 complexes in fibroblasts expressing and lacking p27/Kipl and p21/Cipl. Mol. Cell. Biol. 20: 8748-8757.

LaBaer J., Garrett M.D., Stevenson L.F., Slingerland J.M., Sandhu C, Chou H.S., Fattaey A., Harlow E. 1997. New functional activities for the p21 family of CDK inhibitors. Genes Dev. 11: 847-862.

Lang F.F., Miller D.C., Koslow M., Newcomb E.W. 1994. Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. J. Neurosurg. 81: 427-436.

Laudanna C, Mochly-Rosen D., Liron T., Constantin G, Butcher E.C. 1998 Evidence of ^ protein kinase C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. J. Biol. Chem. 273: 30306-30315.

Lauffenburger D.A., Horwitz A.F. 1996. Cell migration: a physically integrated molecular process. Cell 84: 359-369.

Laws E.R., Goldberg W.J., Bernstein J.J. 1993. Migration of human malignant astrocytoma cells in the mammalian brain: Scherer revisited. Int. J. Dev. Neurosci. 11: 691-697.

Le Good J.A., Ziegler W.H., Parekh D.B., Alessi D.R., Cohen P., Parker P.J. 1998. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein PDK-1. Science 281: 2042-2045.

Leirdal M., Sioud M. 1999. Ribozyme inhibition of the protein kinase C a triggers apoptosis in glioma cells. Br. J. Cancer 80: 1558-1564.

Lewis J.M., Cheresh D.A., Schwartz M A . 1996. Protein kinase C regulates a,**pV** dependent cytoskeletal associations and focal adhesion kinase phosphorylation. J. Cell Biol. 134: 1323-1332.

Li S., MacLachlan T.K., De Luca A., Claudio P.P., Condorelli G, Giordano A. 1995. The cdc-2 related kinase, PISSLRE, is essential for cell growth and acts in G2 phase of the cell cycle. Cancer Res. 55: 3992-3995.

Libermann T.A., Razon N., Bantal A.D., Yarden Y., Schlessinger J., Soreq H. 1984. Expression of epidermal growth factor receptors in human brain tumors. Cancer Res. 44: 753-760.

Liliental J., Chang D.D. 1998. Rackl, a receptor for activated protein kinase C, interacts with integrin p subunit. J. Biol. Chem. 273: 2379-2383.

Lipponen P., Aaltomaa S., Eskelinen M., Ala-Opas M., Kosma V.M. 1998. Expression of p21AVafl/Cipl protein in transitional cell bladder tumors and its prognostic value. Eur. Urol. 34: 237-243.

Litchfield D.W., Ball E.H. 1986. Phosphorylation of the cytoskeletal protein talin by protein kinase C. Biochem. Biophys. Res. Commun. 134: 1276-1283.

Litvak V., Tian D., Shaul Y.D., Lev S. 2000. Targeting of PYK2 to focal adhesions as a cellular mechanism for convergence between integrins and GPCR signaling cascades. J. Biol. Chem. 275: 32736-32746.

Liu W., James C D., Frederick L, Alderete B.E., Jenkins R.B. 1997. PTEN/MMAC1 mutations and EGFR amplification in glioblastomas. Cancer Res. 57: 4997-5000.

Livneh E., Shimon T., Bechor E., Doki Y., Schieren I., Weinstein I.B. 1996. Linking protein kinase C to the cell cycle: ectopic expression of PKC eta in NIH-3T3 cells alters the expression of cyclins and CDK inhibitors and induces adipogenesis. Oncogene 12: 1545-1555.

Livneh E., D.D. Fishman. 1997. Linking protein kinase C to cell cycle control. Eur. J. Biochem. 248: 1-9.

Lorenzo P.S., Kung J.W., Bottorff D.A., Garfield S.H., Stone J.C, Blumberg P.M. 2001. Phorbol esters modulate the ras exchange factor RasGRP. Cancer Res. 61: 943-949.

Lu Z., Liu D., Hornia A., Devonish W., Pagano M., Foster D.A. 1998. Activation of protein kinase C triggers its ubiquitination and degradation. Mol. Cell. Biol. 18: 839-845.

Lund K.A., Lazar C.S., Chen W.S., Walsh B.J., Welsh J.B., Herbst J.J., Walton G.M., Rosenfeld M.G., Gill G.N., Wiley H.S. 1990. Phosphorylation of the epidermal growth factor receptor at threonine 654 inhibits ligand-induced internalization and down-regulation. J. Biol. Chem. 265: 20517-20523.

Lund-Johansen M., Bjerkvig R., Humphrey P.A., Bigner S.H., Bigner D.D., Laerum, O.D. 1990. Effect of epidermal growth factor on glioma cell growth, migration, and invasion *in vitro.* Cancer Res. 50: 6039-6044.

Lund-Johansen M., Forsberg K., Bjerkvig R., Laerum O.D. 1992. Effects of growth factors on a human glioma cell line during invasion into rat brain aggregates in culture. Acta Neuropathol. 84: 190-197.

Luo W., Sharif T.R., Houghton P.J., Sharif M. 1997. CGP 41251 and tamoxifen selectively inhibit mitogen-activated protein kinase activation and c-Fos phosphorylation induction by substance-P in human astrocytoma cells. Cell Growth Differ. 8: 1225-1240.

Macleod K.F., Sherry N., Hannon G., Beach D., Tokino T., Kinzler K., Vogelstein B., Jacks T. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev. 9: 935-944.

Machide M., Kamitori K., Nakamura Y., Kohsaka S. 1998. Selective activation of phospholipase C gammal and distinct protein kinase C subspecies in intracellular signaling by hepatocyte growth factor/scatter factor in primary cultured rat neocortical cells. J. Neurochem. 71: 592-602.

Machide M., Kamitori K., Kohsaka S. 2000. Hepatocyte growth factor-induced differential activation of phospholipase c gamma 1 and phosphatidylinositol 3-kinase is regulated by tyrosine phosphatase SHP-1 in astrocytes. J. Biol. Chem. 275: 31392-31398.

Maity A., Pore N., Lee J., Solomon D., O'Rourke D.M. 2000. Epidermal growth factor receptor transcriptionally up-regulates vascular endothelial growth factor expression in human glioblastoma cells via a pathway involving phosphatidylinositol 3'-kinase and distinct from that induced by hypoxia. Cancer Res. 60: 5879-5886.

Mann D.J., Higgins T., Jones N.C., Rozengurt E. 1997. Differential control of cyclins Dl and D3 and the CDK inhibitor p27/Kipl by diverse signaling pathways in Swiss 3T3 cells. Oncogene 14: 1759-1766.

Mantel C, Luo Z., Canfield J., Braun S., Deng C, Broxmeyer H.E. 1996. Involvement of p21Cipl and p27Kipl in the molecular mechanisms of Steel Factor induced proliferative synergy in vitro and of p21Cipl in the maintenance of stem/progenitor cells in vivo. Blood 88: 3710-3719.

Marais R., Light Y., Mason C, Paterson H, Olson M.F., Marshall C.J. 1998. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science 280: 109-112.

Marshall C. 1999. How do small GTPase signal transduction pathways regulate cell cycle entry. Curr. Opin. Cell Biol. 11: 732-736.

Maruno M., Kovach J.S., Kelly P.J., Yanagihara T. 1991. Transforming growth factor-alpha, epidermal growth factor receptor, and proliferating potential in benign and malignant gliomas. J. Neurosurg. 75: 97-102.

Mastronardi L., Puzzilli F., Couldwell W.T., Osman Farah J., Lunardi P. 1998. Tamoxifen and carboplatin combinational treatment of high-grade gliomas. J. Neuro-Oncol. 38: 59-68.

McDonough W.S., Johansson A., Joffee H., Giese A., Berens M.E. 1999. Gap junction intercellular communication in gliomas is inversely related to cell motility. Int. J. Dev. Neurosci. 17:601-611.

McShea A., Samuel T., Eppel J.T., Galloway D.A., Funk J.O. 2000. Identification of CIP-1 -associated regulator of cyclin B (CARB), a novel p21-binding protein acting in the G2 phase of the cell cycle. J. Biol. Chem. 275: 23181-23186.

Mellor H., Parker P.J. 1998. The extended protein kinase C superfamily. Biochem. J. 332: 281-292.

Michieli P., Chedid M., Lin D., Pierce J.H., Mercer W.E., Givol D. 1994. Induction of WAF1/CIP1 by a p53 independent pathway. Cancer Res. 54: 3391-3395.

Minden A., Lin A., McMahon M., Lange-Carter C, Derijard B., Davis R.J., Johnson G.L., Karin M. 1994. Differential activation of ERK and JNK mitogen activated protein kinases by Raf-1 and MEKK. Science 266: 1719-1723.

Miranti C.K., Ohno S., Brugge J.S. 1999. Protein kinase C regulates integrin-induced activation of the extracellular regulated kinase pathway upstream of She. J. Biol. Chem. 274: 10571-10581.

Mishima K, Ohno S., Shitara N., Yamaoka K, Suzuki K. 1994. Opposite effects of the overexpression of protein kinase C gamma and delta on the growth properties of human glioma cell line U251 MG. Biochem. Biophys. Res. Commun. 201: 363-372.

Mishima K., Higashiyama S., Asai A., Yamaoka K., Nagashima Y., Taniguchi N., Kitanaka C, Kirino T., Kuchino Y. 1998. Heparin binding epidermal growth factor-like growth factor stimulates mitogenic signaling and is highly expressed in human malignant gliomas. Acta Neuropathol. 96: 322-328.

Mishra-Press A., Fields A.P., Samols D., Goldthwait D.A. 1992. Protein kinase C isoforms in glioblastoma cells. Glia 6: 188-197.

Mitsui K, Matsumoto A., Ohtsuka S., Ohtsubo M., Yoshimura A. 1999. Cloning and characterization of a novel p21/CiplAVafl interacting zing finger protein, Ciz-1. Biochem. Biophys. Res. Commun. 264: 457-464.

Mochly-Rosen D., Khaner H., Lopez J. 1991. Identification of intracellular receptor proteins for activated protein kinase C. Proc. Natl. Acad. Sci. U.S.A. 88: 3997-4000.

Mochly-Rosen D., Miller K.G., Scheller R.H., Khaner H., Lopez J., Smith B.L. 1992. p65 fragments, homologous to the C2 region of protein kinase C, bind to the intracellular receptors for protein kinase C. Biochemistry 31: 8120-8124.

Mochly-Rosen D., Gordon A.S. 1998. Anchoring proteins for protein kinase C: a means for isozyme selectivity. FASEB J. 12: 35-42.

Montgomery R.B., Moscatello D.K., Wong A.J., Cooper J.A., Stahl W.L. 1995. Differential modulation of mitogen-activated protein (MAP) kinase/extracellular signal regulated kinase kinase and MAP kinase activities by a mutant epidermal growth factor receptor. J. Biol. Chem. 270: 30562-30566.

Morimoto A.M., Tomlinson M.G., Nakatani K, Bolen J.B., Roth R.A., Herbst R. 2000. The MMAC1 tumor suppressor phosphatase inhibits phospholipase C and integrin linked kinase activity. Oncogene 19: 200-209.

Moriyama T., Kataoka H., Hamasuna R., Yokogami K., Uehara H., Kawano H., Goya T., Tsubouchi H., Koono M., Wakisaka S. 1998a. Up-regulation of vascular endothelial growth factor induced by hepatocyte growth factor stimulation in human glioma cells. Biochem. Biophys. Res. Commun. 249: 73-77.

Moriyama T., Kataoka H., Kawano H., Yokogami K., Nakano S., Goya T., Uchino H., Koono M., Wakisaka S. 1998b. Comparative analysis of expression of hepatocyte growth factor and its receptor, c-met, in gliomas, meningiomas and schwannomas in human. Cancer Lett. 124: 149-155.

Moriyama T., Kataoka H., Hamasuna R., Yokogami K, Uehara H., Kawano H., Goya T., Tsuboushi H., Koono M., Wakisaka S. 1998c. Up-regulation of vascular endothelial growth factor induced by hepatocyte growth factor/scatter factor stimulation in human glioma cells. Biochem. Biophys. Res. Commun. 249: 73-77.

Morrison P., Saltiel A.R., Rich Rosner M. 1996. Role of mitogen-activated protein kinase in regulation of the epidermal growth factor receptor by protein kinase C. J. Biol. Chem. 271: 12891-12896.

Murphy G., Gavrilovic J. 1999. Proteolysis and cell migration: creating a path? Curr. Opin. Cell Biol. 11: 614-621.

Murphy P.R., Sato Y., Sato R., Friesen H.G. 1988. Regulation of multiple basic fibroblast growth factor messenger ribonucleic acid transcripts by protein kinase C activators. Mol. Endocrinol. 2: 1196-1201.

Nabeshima K, Shimao Y., Sato S., Kataoka H., Moriyama T., Kawano H., Wakisaka S., Koono M. 1997. Expression of c-Met correlates with grade of malignancy in human astrocytic tumors: an immunohistochemical study. Histopathol. 31: 436-443.

Nakanishi K., Fujimoto J., Ueki T., Kishimoto K, Hashimoto-Tamaoki T., Furuyama J., Itoh T., Sasaki Y., Okamoto E. 1999. Hepatocyte growth factor promotes migration of human hepatocellular carcinoma via phosphatidylinositol 3-kinase. Clin. Exp. Metastasis 17:507-514.

Newton A.C. 1995. Protein kinase C, structure, function, and regulation. J. Biol. Chem. 270: 28495-28498.

Newton A.C. 1997. Regulation of protein kinase C. Curr. Opin. Cell Biol. 9: 161-167.

Ng T., Shima D., Squire A., Bastiaens P.I.H., Gschmeissner S., Humphries M.J., Parker P.J. 1999. PKC a regulates p\integrin-dependent cell motility through association and control of integrin traffic. EMBO J. 18: 3909-3923.

Nguyen D.H.D., Catling A.D., Webb D.J., Sankovic M., Walker L.A., Somlyo A.V., Weber M.J., Gonias S.L. 1999. Myosin light chain kinase functions downstream of Ras/ERK to promote migration of urokinase-type plasminogen activator-stimulated cells in an integrin-selective manner. J. Cell Biol. 146: 149-164.

Nister M., Claesson-Welsh L., Eriksson A., Heldin C.H., Westermark B. 1991. Differential expression of platelet-derived growth factor receptors in human malignant cell lines. J. Biol. Chem. 266: 16755-16763.

Nourse J., Firpo E., Flanagan W.M., Coats S., Polyak K., Lee M.H., Massague J., Crabtree G.R., Roberts J.M. 1994. Interleukin-2 mediated elimination of the p27Kipl cyclin dependent kinase inhibitor prevented by rapamycin. Nature 372: 570-573.

Oerhing R.D., Miletic M., Valter M.M., Pietsch T., Neumann J., Fimmers R., Schlegel U. 1999. Vascular endothelial growth factor (VEGF) in astrocytic gliomas--a prognostic factor? J. Neuro-Oncol. 45: 117-125.

Ono T., Kitaura H., Ugai H., Murata T., Yokoyama K.K., Iguchi-Ariga S.M.M., Ariga H. 2000. TOK-1, novel p21/Cipl-binding protein that cooperatively enhances p21-dependent inhibitory activity toward CDK2 kinase. J. Biol. Chem. 275: 31145-31154.

Owens G.C., Orr E.A., DeMasters B.K., Muschel R.J., Berens M.E., Kruse C.A. 1998. Overexpression of a transmembrane isoform of neural cell adhesion molecule alters the invasiveness of rat CNS-1 glioma. Cancer Res. 58: 2020-2028.

Park M.J., Park I.C., Hur J.H., Rhee C.H., Choe T.B., Yi D.H., Hong S.I., Lee S.I. 2000. Protein kinase C activation by phorbol ester increases in vitro invasion through regulation of matrix metalloproteinases/tissue inhibitors of metalloproteinases system in D54 human glioblastoma cells. Neurosci. Lett. 290: 201-204.

Pass J.M., Zheng Y., Wead W.B., Zhang J., Li R.C.X., Bolli R., Ping P. 2001. PKC e activation induces dichotomous cardiac phenotypes and modulates PKC e-RACK interactions and expression. Am. J. Physiol. Circ. Physiol. 280: H946-H955.

Paulus W., Tonn J.C. 1994. Basement membrane invasion of glioma cells mediated by integrin receptors. J. Neurosurg. 80: 515-519.

Paulus W., Baur I., Beutler A.S., Reeves S.A. Diffuse brain invasion of glioma cells requires Pi integrins. Lab. Invest. 75: 819-826.

Perez-Moreno M., Avila A., Islas S., Sanchez S., Gonzales-Mariscal L. 1998. Vinculin but not a-actinin is a target of PKC phosphorylation during junctional assembly induced by calcium. J. Cell Sci. I11: 3563-3571.

Plow E.F., Haast T.A., Zang L., Loftus J., Smith J.W. 2000. Ligand binding to integrins. J. Biol. Chem. 275: 21785-21788.

Pollack I.F., Randall M.S., Kristofik M.P., Kelly R.H., Selker R.G., Vertosick F.T. 1990a. Effect of tamoxifen on DNA synthesis and proliferation of human malignant glioma cell lines. Cancer Res. 50: 7134-7138.

Pollack I.F., Randall M.S., Kristofik M.P., Kelly R.H., Selker R.G., Vertosick F.T. 1990b. Response of malignant glioma cell lines to activation and inhibition of protein kinase C-mediated pathways. J. Neurosurg. 73: 98-105.

Pollack I.F., Kawecki S., Lazo J.S. 1996. Blocking of glioma proliferation in vitro and in vivo and potentiating the effect of BCNU and cisplatin; UCN-01, a selective protein kinase C inhibitor. J. Neurosurg. 84: 1024-1032.

Pollack I.F., Kawecki S. 1997. The effect of Calphostin C, a potent photodependent protein kinase C inhibitor, on the proliferation of glioma cells in vitro. J. Neuro-Oncol. 31: 255-266.

Ponting CP., Parker P.J. 1996. Extending the C2 domain family: C2s in PKCs 8, e, rj, 0, phospholipases, GAPs, and perforin. Protein Sci. 5: 162-166.

Press R.D., Misra A., Gillaspy G., Samols D., Goldthwait D.A. 1989. Control of the expression of c-sis mRNA in human glioblastoma cells by phorbol ester and transforming growth factor beta-1. Cancer Res. 49: 2914-2920.

Prigent S.A., Nagane M., Lin H., Huvar I., Boss G.R., Feramisco J.R., Cavenee W.K., Huang H.J.S. 1996. Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway. J. Biol. Chem. 271: 25639-25645.

Rabinovitz I., Toker A., Mercurio A.M. 1999. Protein kinase C-dependent mobilization of the ***a$4*** integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. J. Cell Biol. 146: 1147-1159.

Reifenberger G, Liu L., Ichimura K, Schmidt E.E., Collins V.P. 1993. Amplification and overexpression of the Mdm2 gene in a subset of human malignant gliomas without p53 mutations. Cancer Res. 53: 2736-2739.

Ren X.D., Kiosses W.B., Sieg D.J., Otey C.A., Schlaepfer D.D., Schwartz M.A. 2000. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. J. Cell Sci. 113:3673-3678.

Rietzler M., Bittner M., Kolanus W., Schuster A., Holzmann B. 1998. The human WD repeat protein WAJT-1 specifically interacts with the cytoplasmic tails of p*7 integrins. J. Biol. Chem. 273: 27459-27466.

Rigot V., Lehmann M., Andre F., Daemi N., Marvaldi J., Luis J. 1998. Integrin ligation and PKC activation are required for migration of colon carcinoma cells. J. Cell Sci. 111:3119-3127.

Roberts J.M. 1999. Evolving ideas about cyclins. Cell 98: 129-132.

Rodrigues G.A., Falasca M., Zhang Z., Ong S.H., Schlessinger J. 2000. A novel positive feedback loop mediated by the docking protein Gabl and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. Mol. Cell. Biol. 20: 1448-1459.

Ron D., Chen C.H., Caldwell J., Jamieson L., Orr E., Mochly-Rosen D. 1994. Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. Proc. Natl. Acad. Sci. USA 91: 839-843.

Ron D., Mochly-Rosen D. 1994. Agonists and antagonists of protein kinase C function, derived from its binding proteins. J. Biol. Chem. 269: 21395-21398.

Ron D., Luo J., Mochly-Rosen D. 1995. C2 region derived peptides inhibit translocation and function of beta protein kinase C in vivo. J. Biol. Chem. 270: 24180-24187.

Ron D., Mochly-Rosen D. 1995. An autoregulatory region in PKC: the pseudoanchoring site. Proc. Natl. Acad. Sci. USA 92: 492-496.

Ron, D., Jiang Z., Yao L., Vagts A., Diamond I., Gordon A. 1999. Coordinated movement of RACK1 with activated pTlPKC. J. Biol. Chem. 274: 27039-27046.

Ron D., Kazanietz M.G. 1999. New insights into the regulation of protein kinase C and novel phorbol ester receptors. FASEB J. 13: 1658-1676.

Rooprai H.K., Vanmeter T., Panou C, Schnull S., Trillo G., Davies P.D., Pilkington G.J. 1999. The role of integrin receptors in aspects of glioma invasion in vitro. Int. J. Devi. Neuroscience 17: 613-623.

Rotenberg S.A., Sun X.G. 1998. Photoinduced inactivation of protein kinase C by dequalinium identifies the RACK1 binding domain as a recognition site. J. Biol. Chem. 273: 2390-2395.

Ruan S., Okcu M.F., Ren J.P., Chiao P., Andreeff M., Levin V., Zhang W. 1998. Overexpressed Wafl/Cipl renders glioblastoma cells resistant to chemotherapy agents 1,3-bis(2-chloroethyl)-l-nitrosourea and cisplatin. Cancer Res. 58: 1538-1543.

Ruan S., Okcu M.F., Pong R.C., Andreeff M., Levin V., Hsieh J.T., Zhang W. 1999. Attenuation of Wafl/Cipl expression by an antisense adenovirus expression vector sensitizes glioblastoma cells to apoptosis induced by chemotherapeutic agents 1,3-bis(2-chloroethyl)-l-nitrosourea and cisplatin. Clin. Cancer Res. 5: 197-202.

Ryves W.J., Evans A.T., Olivier A.R., Parker P.J., Evans F.J. 1991. Activation of the PKC isotypes alpha, beta 1, gamma, delta, and epsilon by phorbol esters of different biological activities. FEBS Lett. 288: 5-9.

Schaeffer H.J., Weber M.J. 1999. Mitogen activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol. 19: 2435-2444.

Schmidt E.E., Ichimura K, Reifenberger G., Collins V.P. 1994. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. Cancer Res. 54: 6321-6324.

Schonwasser D.C., Marais R.M., Marshall C.J., Parker P.J. 1998. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol. Cell. Biol. 18: 790-798.

Scott M.T., Morrice N., Ball K.L. 2000. Reversible phosphorylation at the C-terminal regulatory domain of p21/Wafl/Cipl modulates proliferating nuclear antigen binding. J. Biol. Chem. 275: 11529-11537.

Shan X., Czar M.J., Bunnell S.C., Liu P., Liu Y., Schwartzberg P.L., Wange R.L. 2000. Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. Mol. Cell. Biol. 20: 6945-6957.

Sharif T.R., Sharif M. 1999. Overexpression of protein kinase C epsilon in astroglial brain tumor derived cell lines and primary tumor samples. Int. J. Oncol. 15: 237-243.

Sharif T.R., Sasakawa N., Sharif M. 2001. Regulated expression of a dominant-negative protein kinase C epsilon mutant inhibits the proliferation of U373MG human astrocytoma cells. Int. J. Mol. Med. 7: 373-380.

Shen L., Dean N.M., Glazer R.I. 1999. Induction of p53-dependent, insulin-like growth factor binding protein-3-mediated apoptosis in glioblastoma multiforme cells by a protein kinase C alpha antisense nucleotide. Mol. Pharmacol. 55: 396-402.

Sherr C.J. 1998. Tumor surveillance via the ARF-p53 pathway. Genes Dev. 12: 2984-2991.

Sherr C.J., Roberts J.M. 1999. CDK inhibitors: positive and negative regulators of GI-phase progression. Genes Dev. 13: 1501-1512.

Short S.M., Boyer J.L., Juliano R.L. 2000. Integrins regulate the linkage between upstream and downstream events in G-protein-coupled receptor signaling to mitogen-activated protein kinase. J. Biol. Chem. 275: 12970-12977.

Sieg D.J., Hauck C.R., Schlaepfer D.D. 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. J. Cell Sci. 112: 2677-2691.

Sioud M., Sorensen D.R. 1998. A nuclease-resistant protein kinase C a ribozyme blocks glioma cell growth. Nature Biotech. 16: 556-561.

Shih S.C., Mullen A., Abrams K, Mukhopadhyay D., Claffey K.P. 1999. Role of protein kinase C isoforms in phorbol ester-induced vascular endothelial growth factor expression in human glioblastoma cells. J. Biol. Chem. 274: 15407-15414.

Sonoda Y., Yoshimoto T., Sekiya T. 1995. Homozygous deletion of the MTS1/p16 and MTS2/p15 genes and amplification of the CDK4 gene in glioma. Oncogene 11: 2145-2149.

Srivenugopal K.S., Ali-Osman F. 1996. Deletions and rearrangements inactivate the p16/TNK4 gene in human glioma cells. Oncogene 12: 2029-2034.

Stern L.E., Falcone R.A., Kemp C.J., Erwin C.R., Warner B.W. 2000. p21/Wafl/Cipl is required for the mitogenic response to intestinal resection. J. Surgical Res. 90: 45-50.

Tamura M., Gu J., Matsumoto K, Aota S., Parsons R., Yamada K.M. 1998. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 280: 1614-1617.

Tamura M., Gu J., Takino T., Yamada K.M. 1999. Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and pl30Cas. Cancer Res. 59: 442-449.

Tang P., Steck P.A., Yung W.K.A. 1997a. The autocrine loop of TGF alpha/EGFR and brain tumors. J. Neuro-Oncol. 35: 303-314.

Tang S., Morgan K.G., Parker C., Ware J.A. 1997b. Requirement for protein kinase C 9 for cell cycle progression and formation of actin stress fibers and filopodia in vascular endothelial cells. J. Biol. Chem. 272: 28704-28711.

Taules M., Rodrigues-Vilarrupla A., Rius E., Estanyol J.M., Casanovas O., Sacks D.B., Perez-Paya E., Bachs O., Agell N. 1999. Calmodulin binds to p21/Cipl and is involved in the regulation of its nuclear localization. J. Biol. Chem. 274: 24445-24448.

Thompson L.J., Fields A.P. 1996. Beta2 protein kinase C is required for the G2/M transition of cell cycle. J. Biol. Chem. 271: 15045-15053.

Tognon C.E., Kirk H.E., Passmore L.A., Whitehead LP., Der C.J., Kay R.J. 1998. Regulation of RasGRP via a phorbol ester responsive CI domain. Mol. Cell. Biol. 18: 6995-7008.

Tohma Y., Gratas C, Biernat W., Peraud A., Fukuda M., Yonekawa Y., Kleihues P., Ohgaki H. 1998. PTEN/MMAC1 mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas. J. Neuropathol. Exp. Neurol. 57: 684-689.

Traub O., Monia B.P., Dean N.M., Berk B.C. 1997. PKC-e is required for mechano-sensitive activation of ERK1/2 in endothelial cells. J. Biol. Chem. 272: 31251-31257.

Treasurywala S., Berens M.E. 1998. Migration arrest in glioma cells is dependent on the alpha v integrin subunit. Glia 24: 236-243.

Tysnes O.B., Laerum O.D. 1993. Differential effects of 12-0-tetradecanoyl-13-phorbol acetate (TPA) on growth, migration, and invasion of a human glioma cell line. Anticancer Res. 13: 1325-1330.

Uhm J.H., Dooley N.P., Villemure J.G., Yong V.W. 1996. Glioma invasion in vitro: regulation by matrix metalloproteinases-2 and protein kinase C. Clin. Exp. Metastasis 14: 421-433.

Uhm J.H., Dooley N.P., Villemure J.G., Yong V.W. 1997. Mechanisms of glioma invasion: role of matrix-metalloproteinases. Can. J. Neurol. Sci. 24: 3-15.

Valmu L., Autero M., Siljander P., Patarroyo M., Gahmberg C.G. 1991. Phosphorylation of the beta subunit of CD11/CD18 integrins by protein kinase C correlates with leukocyte adhesion. Eur. J. Immunol. 21: 2857-2862.

Vecil G.G., Larsen P.H., Corley S.M., Herx L.M., Besson A., Goodyer C.G., Yong V.W. 2000. Interleukin-1 is a key regulator of matrix metalloproteinase-9 expression in human neurons in culture and following mouse brain trauma in vivo. J. Neurosci. Res. 61:212-224.

Von Deimling A., Louis D.N., Wiestler O.D. 1995. Molecular pathways in the formation of gliomas. Glia 15: 328-338.

Vuori K., Ruoslahti E. 1993. Activation of protein kinase C precedes *(Xsfii* integrin-mediated cell spreading on fibronectin. J. Biol. Chem. 268: 21459-21462.

Wang S.I., Puc J., Li J., Bruce J.N., Cairns P., Sidranski D., Parsons R. 1997. Somatic mutations of PTEN in glioblastoma multiforme. Cancer Res. 57: 4183-4186.

Weekes J., Barry S.T., Critchley D.R. 1996. Acidic phospholipids inhibit the intramolecular association between N- and C-terminal regions of vinculin, exposing actin-binding and protein kinase C phosphorylation sites. Biochem. J. 314: 827-832.

Weinberg R.A. 1995. The retinoblastoma protein and cell cycle control. Cell 81: 323-330.

Weiss R.H., Joo A., Randour C. 2000. p21/Wafl/Cipl is an assembly factor required for platelet-derived growth factor-induced vascular smooth muscle cell proliferation. J. Biol. Chem. 275: 10285-10290.

Wellner M., Maasch C, Kupprion C, Lindschau C, Luft F.C., Haller H. 1999. The proliferative effect of vascular endothelial growth factor requires protein kinase C-**CC** and protein kinase C-C Arterioscler. Thromb. Vase. Biol. 19: 178-185.

Westermark B., Heldin C.H., Nister M. 1995. Platelet-derived growth factor in human glioma. Glia 15: 257-263.

Wikstrand C.J., Hale LP., Batra S.K., Hill M.L, Humphrey PA., Kurpad S.N., McLendon R.E., Moscatello D., Pegram C.N., Reist C.J., Traweek ST., Wong A.J., Zalutski M.R., Bigner D.D. 1995. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. Cancer Res. 55:3140-3148.

Woods A., Couchman J.R. 1992. Protein kinase C involvement in focal adhesion formation. J. Cell Sci. 101: 277-290.

Worm K., Dabbagh P., Schwechhmeimer K. 1999. Reverse transcriptase polymerase chain reaction as a reliable method to detect epidermal growth factor receptor exon 2-7 gene deletion in human glioblastomas. Hum. Pathol. 30: 222-227.

Xiao H., Goldthwait D.A., Mapstone T. 1994. The identification of four protein kinase C isoforms in human glioblastoma cell lines: PKC alpha, gamma, epsilon, and zeta. J. Neurosurg. 81: 734-740.

Yamamoto M., Sawaya R., Mohanam S., Rao V.H., Bruner J.M., Nicolson G.L., Rao J.S. 1994. Expression and localization of urokinase-type plasminogen activator receptor in human gliomas. Cancer Res. 54: 5016-5020.

Yarwood S.J., Steele M.R., Scotland G, Houslay M.D., Bolger G.B. 1999. The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. J. Biol. Chem. 274: 14909-14917.

Yazaki T., Ahmad S., Chahlavi A., Zylber-Katz E., Dean N. M., Rabkin S.D., Martuza R.L., Glazer R.I. 1996. Treatment of glioblastoma U87 by systemic administration of an antisense protein kinase C alpha phosphorothioate oligonucleotide. Mol. Pharmacol. 50: 236-242.

Yong V.W., Krekoski C.A., Forsyth P.A., Bell R., Edwards D.R. 1998. Matrix metalloproteinases and diseases of the CNS. Trends Neurosci. 21: 75-80.

Zeng Y.X., El-Deiry W.S. 1996. Regulation of p21AVafl/Cipl expression by p53 independent mechanisms. Oncogene 12: 1557-1564.

Zezula J., Sexl V., Hutter C, Karel A., Schutz W., Freissmuth M. 1997. The cyclin-dependent kinase inhibitor p21cipl mediates the growth inhibitory effect of phorbol esters in human venous endothelial cells. J. Biol. Chem. 272: 29967-29974.

Zhang W., Law R.E., Hinton D.R., Couldwell W.T. 1997. Inhibition of human malignant glioma cell motility and invasion in vitro by hypericin, a potent protein kinase C inhibitor. Cancer Lett. 120: 31-38.

Zhang H., Hannon G.H., Beach D. 1994. p21-containing cyclin kinases exist in both active and inactive states. Genes Dev. 8: 1750-1758.

CHAPTER 1. BASIC PROPERTIES OF BIOLOGICAL MEMBRANE CHANNELS

Ion channels are large protein molecules incorporated into the cellular membrane [Hiller, 1992], which consists of a bimolecular layer (bilayer) of lipid molecules (Fig.1.1, [Doyle et al., 1998]).

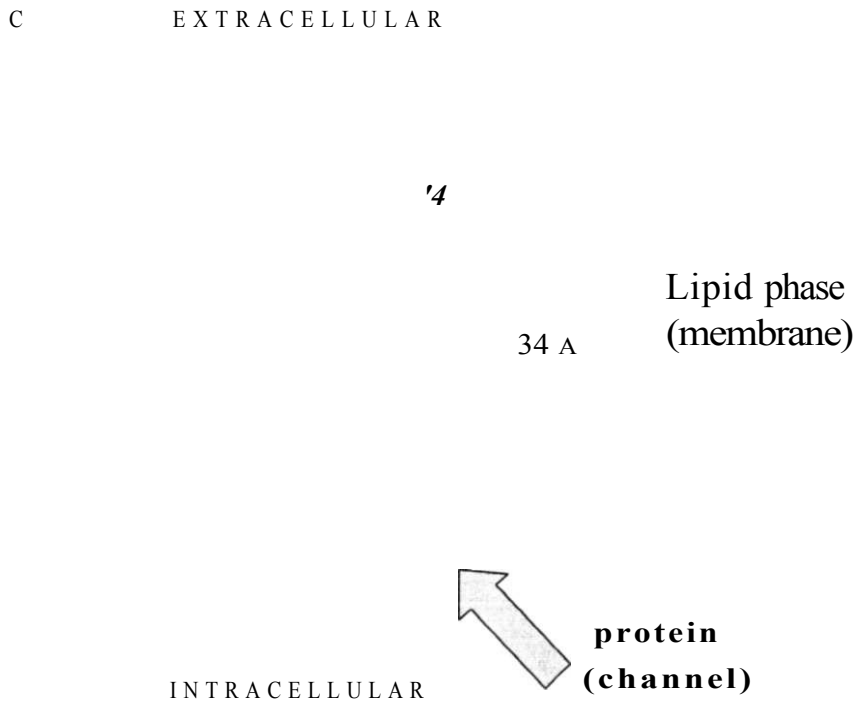


Fig. 1.1. Structure of a bacterial potassium channel (ribbon representation of protein backbone).

The membrane is surrounded by a water solution of different ions, Na^+, K^+, Ca^f, Mg^+ etc. Ion channels are highly permeable to some but not to all ions. Thus sodium channels are very permeable to Na^+ ions and less permeable to K^+, while potassium channels are very permeable to K^+ ions but not to Na^+. Ions from the surrounding solutions (intracellular or extracellular) flow through the central narrow part of the protein, or pore, carrying an electric current through the channel, the magnitude and the direction of which depends on the applied external voltage across the membrane and the concentrations of the ions in the solution. By measuring the channel current in different