Regulation of murine melanoma metastasis by Protein Kinase C (PKC) Delta

By

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Protein kinase C (PKC) delta has been shown to regulate many cellular activities involved in metastastatic process. Studies have shown that over-expression of PKC delta increases the metastatic potential of a highly metastatic murine melanoma cell line, and a mammary carcinoma cell line *(La Porta et al 2000 and Kiley et al 1999).*

In this study, we examined the effects of transfection with wild-type or mutant PKC delta genes on metastasis of B16F1 melanoma cells. Cells are transfected with either wild-type delta or delta with a substitution of phenylalanine for tyrosine 155 (Y155F). We examined the effects on 1) metastasis 2) tumor cell (TC) retention in the lung, 3) cell size, 4) invasion and adhesion to biological matrices, 5) proliferation and/or viability under adverse conditions including a) limiting serum concentrations with or without cell crowding, and b) exposure to cytotoxic agents and 6) patterns of subcellular localization. Results of this study show that over-expression of wild-type delta in B16F1 cells increases: the number and size of viable tumor cells/clumps 48 hrs after intravenous inoculation, number and size of metastatic pulmonary nodules several weeks later, in vitro adhesion of TC to matrigel, and consistently increases proliferation when compared to the control or Y155F mutant, but has only a slight or

negligible effect on cell viability. However, over-expression of Y155F mutant delta had little or no effect on TC retention, metastasis, and adhesion but markedly increased TC survival in the presence of all cytotoxic agents and exhibited striking alterations in subcellular localization. Interestingly, the effect of mutant Y155F on proliferation was highly dependent upon serum concentration and cell crowding, but often resulted in less proliferation than wildtype delta and showed diminished viability.

In summary, the collective results in this thesis demonstrates that PKC delta regulates B16F1 metastasis and suggests that phosphorylation of intact tyrosine 155 can markedly and differentially effect many of the key regulatory events essential to the metastatic cascade

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Introduction:

PKC Background:

Protein kinase C (PKC) is a family of structurally related isoforms that phosphorylates cytoplasmic and nuclear substrates on serine and threonine residues. There are 11 known isozymes that are classified into 3 groups: the classical including alpha, beta I and II and gamma, the novel including delta, epsilon, eta, theta, and the atypical including zeta, lambda, and iota. All PKC isoforms require phosphatidyl serine for activation, and classification into groups is based on their need for calcium and or responsiveness to diacylglycerol (DAG). The classical isozymes are Ca^{2+} and DAG dependent, the novel isozymes are Ca^{2+} independent but responsive to DAG or phorbol esters, and the atypical isozymes are both Ca^{2+} independent and unresponsive to DAG *(Nishizuka 1995).*

Structurally, the PKC isoforms consist of two functional domains ; the N-terminal domain and the C-terminal domain. In the classical and novel isoforms, the N-terminal or regulatory region contains a C1 domain consisting of 2 non-equivalent cysteine rich zinc fingers, C1a and C1b, and serves as a binding site for phorbol esters and DAG. The atypical PKC's are unresponsive to phorbol esters, but contain a single zinc finger motif with homology to the C1a motif. The other domains of the N-terminal include the C2, V1 and V2 domains. The C2 domain is implicated in binding to phospholipids in a Ca ++ dependent manner, and is missing in the Ca++-independent isoforms. *(Rotenberg and Weinstein 1991, Sibley and Houslay 1994, Mellor and Parker 1998).* The C-terminal or

catalytic region consists of the C3, C4, V4, V5 domains and an ATP binding site *(Rotenberg and Weinstein 1991, Sibley and Houslay 1994, Mellor and Parker 1998).* The regulatory region is separated from the catalytic region by the V3 variable region *(Sibley and Houslay 1994).*

PKC can exist in an inactive or active state. Activation occurs via stimulation by growth factors of a G protein with the activation of phospholipase C and the liberation of DAG plus IP3, and/or by a receptor tyrosine kinase mediated pathway. The activation of PKC by TPA is correlated with the phosphorylation of Thr-505 at the activation loop of PKC *(Kitamura et al 2003).*Tumor promoting phorbol esters such as 12-O-tetradecanoyl-phorbal-13 acetate (TPA) and phorbol-12-myristate-13-acetate (PMA) are potent activators that can regulate expression and activation of the classical and novel PKC's. Short term treatment with phorbol esters activates PKC whereas prolonged treatment leads to decreased expression and inactivation of PKC *(Lu et al 1997).* Although it has been shown that activated PKC appears to be translocated to the plasma membrane, recent reports have shown that in many cell lines activation leads to localization to various cellular compartments *(Goodnight et al 1995).* Activation of PKC by phorbol esters in fibroblasts over-expressing *particular* isoforms, showed that various isoforms translocated not only to the plasma membrane, but to distinct intracellular sites *(Goodnight et al 1995).* These are presumed to be areas where the isozymes' substrates are localized. Several investigators have shown that the cellular localization of different isozymes *varies*

with different cell types *(Goodnight et al 1995, Jaken et al 1989, Mischak et al 1991).*

Metastasis

Metastasis is the primary cause of death of cancer patients. Metastasis of solid tumors is an intricate process which involves many steps that are regulated by interactions between the tumor cells and the host microenvironment. It is a highly selective process in which only a small number of cells survive to establish metastatic colonies *(Fidler and Hart 1982).* Studies have shown that most blood-borne TC are killed, and cleared from the circulation within 24hrs of intravenous injection *(Fidler et al 1970, 1976 1977, 1978, Luzzi etal 1998).* Survival of tumor cells is dependent on several host factors including adequate blood supply, growth factors, oxygen supply and the hosts immune response. According to Paget's classical 'seed and soil' hypothesis, unique properties of tumor cells ('seeds') and different characteristics of an organs microenvironment ('soil'), determine organ preference during metastasis *(Aurbach 1988, Nicholson 1988).* This hypothesis suggests that metastasis is not a random process. Evidence in support of this hypothesis was obtained using B16 cells *(Fidler and Nicholson 1976).* These studies demonstrate that the organ microenvironment can influence the phenotypes of tumor cells. Further support for this hypothesis comes from several different sources. Differences in angiogenesis and microcirculation were observed for human colon adenocarcinoma at different

metastatic sites *(Fukumura et al 1997)* and TC response to chemotherapy agents varied with the organ microenvironment *(Fidler et al 1994)*

There are two viable pathways by which metastasis of solid tumors may occur. The first pathway involves a tumor cell detaching from neighboring cells, digesting the basal lamina and invading through the extracellular matrix, entering into the bloodstream and aggregating with platelets, evading the immune system while in transit through the circulatory system, attaching to the endothelium, and extravasating across the wall of blood vessels into the target organ. In order to colonize this new environment the TC must then proliferate, continue to evade immune reactions, and induce neovascularization *(Rouslahti 1996, Keller 2002, Beebe et al 1990, Chopra et al 1990)*. In a postulated alternative pathway, colonization of tumor cells at the secondary site is attributed more to the intravascular proliferation of cells attached to the endothelium in the target organ with the eventual rupture of the vasculature and release of cells into the target site *(Wong et al 2001, Al-Mehdi et al 2000)* .

Many mutations are required in a cell before it becomes capable of successfully completing the metastatic process. Some mutations alter a cell's ability to carry out proliferation and progress through the cell cycle, to synthesize or release digestive enzymes, to undergo apoptosis, or to remodel the cytoskeleton thereby affecting cell migration, adhesion, and/or invasion.

PKC and Metastasis

Many of the PKC isoforms have been shown to regulate several of the cellular processes involved in metastasis. The specific function(s) affected are often

dependent upon cell type. Stimulation of PKC is correlated with increased metastatic potential of human melanomas *(Dumont and Bitonti 1994).* Activation of PKC also enhances metastasis of B16 TC and results in translocation of PKC from the cytosol to the particulate fraction *(Gopalakrishna and Barsky 1988).* These results were obtained by treating cancer cells with tumor promoting phorbol esters. Although phorbol esters activate PKC, prolonged treatment leads to inactivation and a decrease in metastasis. Short term treatment of B16F1 melanoma cells with PMA (1hr). leads to translocation of PKC from the cytosol to the plasma membrane and also results in an increase in metastasis (Dumont et al 1992) .

Studies show that an increase in metastasis may be correlated with elevated PKC expression *(Ryves et al 1991, Brooks et al 1991)* and suggest that in tumor lines many of the PKC isoforms may be elevated when compared with the corresponding normal cell type. In fact, several studies have shown elevated expression of some PKC iosforms in human melanomas. Increased levels of PKC alpha and epsilon have been observed when compared with expression in normal melanocytes *(Arita et al 1996, Beebe et al 1990).* PKC delta, epsilon, and zeta have also been detected in normal melanocytes and in human melanoma cell lines. In addition, studies have shown that in some melanomas there is a lack of one or more isoforms. While PKC alpha and beta are both expressed in normal melanocytes *(Arita et al 1992),* in some melanoma cell lines, one of these isoforms is expressed but the other is not. Beta was not detected in cells that were freshly isolated from primary or metatastatic tumors Treatment

with TPA inhibited the growth of all the melanoma lines but the line that lacked alpha and had beta was more effectively inhibited than the other lines. These results suggest that PKC alpha or beta may be altered in the transformation of normal melanocytes to melanoma cells. Loss of alpha or beta may contribute to the inhibitory effect of TPA on growth (Oka M et al). The B16 murine melanoma cell lines are commonly used as models for studying melanoma metastasis. The original (parental) cell line was the weakly metastatic B16F1, obtained by chemical induction with DMBA (Fidler et al 1977). Subsequently numerous cell lines with varying metastatic potential and organ preference have been derived from this parental line. During metastasis, melanoma can target the lung, liver, brain, heart, testis and ovaries. The B16F10 and B16F1 cell lines have been shown to preferentially localize to the lung after tail vein injections, with little or no metastases elsewhere*(Fidler 1976, Fidler et al 1976 1977, Netland and Zetter 1984, Haming & Szalay).* Additionally, studies have shown that after intravenous inoculation B16F10 cells tend to arrest in the superficial microvasculature at or near the surface of the lung *(Paulus et al 1982),* making subsequent quantification of macroscopic metastases feasible.

Many of the PKC isoforms have been detected in several of the B16 cell lines. Our lab has previously detected at least 7 of the 11 known isoforms in B16F10, one of the more highly metastatic cell lines *(Szalay et al 2001).* The B16F1 cell line is weakly metastatic and also expresses several of the PKC isoforms. Although activation of PKC has been shown to increase metastasis of murine melanoma, the role played by individual isoforms is largely unknown.

Others have shown that PKC stimulation is capable of regulating many cellular activities considered to be necessary for successful metastasis. These properties are discussed below.

Adhesion

Adhesion to the vascular endothelium is primary step in the colonization of target organs by blood borne tumor cells. The vascular endothelium serves as a barrier between blood borne tumor cells and the target tissue. Clinical and experimental studies have demonstrated that TC preferentially metastasize to specific organs *(Fidler and Nicholson 1976, Netland and Zetter 1984).* The arrest of tumors cells in distinct blood vessels of the target organ has been shown to be regulated by organ specific adhesion molecules. For example, B16 melanoma has been shown to adhere to endothelial cells by binding to Lu-ECAM-1, a lung specific endothelial cell adhesion molecule *(Zhu et al 1991, 1993).* Binding to Lu-ECAM-1 facilitates gap junctional communication between the TC and endothelium and regulates extravasation *(El-Sabban and Pauli 1991,1994).* Other studies have shown that expression of $\alpha_{IIb}\beta 3$ integrins facilitates tumor cell induced platelet aggregation and adhesion to the endothelium by melanoma cells *(Tang et al 1993, Chang et al 1992)*. Additionally, activation of the endothelium by cytokines such as IL-1 and TNF profoundly increased human melanoma adhesion (Rice et al 1988). Activation of PKC increases attachment, spreading and migration of human endothelial cells *(Yamamura et al 1996)* and human glioma cells (*Besson et al 2002).*

Invasion and Secretion:

After tumor cells have detached from the primary tumor mass, cells intravasate into the blood circulation where they may adhere to each other and/or aggregate with platelets to form emboli. TC can also bind to the endothelial cells and to components of the underlying basement membrane such as fibronectin or laminin *(McCarthy and Furcht 1984).* TC may then secrete proteolytic enzymes to degrade the basement membrane and underlying tissue allowing cells to penetrate into tissue and complete the process of invasion. Studies have shown that there are several chemotactic factors which may promote the migration and invasion metastatic tumor cells. Some of these factors include: factors derived from reabsorbed bone *(Orr et al 1979),* C5a complement fragments (*Romualdez and Ward 1975),* collagen and related factors *(Mundy et al 1981)* laminin and fibronectin *(McCarthy and Furcht 1984)* , serum spreading factor (*Basara et al 1985)* and chemotactic factors from target tissue *(Hujanen and Terranova 1985).*The penetration of the basement membrane may occur by different mechanisms. These include tumor cell activities such as: proliferation in the vasculature with subsequent tearing or rupturing of the endothelium, or secretion of proteases and digestion of components of the basement membrane allowing metastatic cells to penetrate into the target tissue *(Liotta et al 1980, Kramer and Vogel 1984).* Activation of PKC results in increased invasion of several cell lines including melanoma cells over-expressing wnt5a *(Weeraratna et al 2002)*, and

lung carcinoma *cells (Gopalakrishna et a 1994)* .

Death and Survival

Several studies have shown that PKC can have varying effects on cell death and survival. Activation of PKC has been shown to block apoptotic cell death in rat thymocytes *(McConkey et al 1989)* ,suppress radiation-induced apoptosis in aortic endothelial cells *(Haimovitz-Friedman et al 1994)* , and prevent ceramide-induced programmed cell death in U937 monoblastic leukemic cells *(Obeid et al 1993).* Over-expression of PKC epsilon suppresses apoptosis and induces bcl-2 expression in human IL-3-dependent cells *(Gubina et al 1998),* while over-expression of atypical PKC iota protects human leukemia cells against drug-induced apoptosis *(Murray and Field 1997).* Conversely, PKC delta can induce apoptosis in several cell types in response to various apoptotic stimuli such as ionizing radiation, DNA damaging drugs, anti-fas antibody, H2O2 and cytotoxic agents such as etoposide ,camptothecin and mitomycin *(Reyland 1999, Emoto et al 1995, Denning et al 1998, Fujii etal 2000, Konishi et al 1999 Shao et al 1997*). Additionally, down regulation of delta in fibroblasts provides a survival signal and overcomes apoptotic signals induced by over-expression of c-Src *(Zhong et al 2002).* Recent studies show that PKC delta is involved in the regulation of the stress activated protein kinase/ c-Jun N-terminal kinase (SAPK/JNK) pathway in response to DNA damage *(Yoshida et al 2002)*

PKC DELTA

Protein kinase C (PKC) delta is a widely expressed member of the PKC family. PKC delta was first cloned from the rat brain and is classified as a member of the novel group of isoforms *(Kikkawa et al 2002).* Like most members of the PKC family, PKC delta has been shown to regulate many processes in varying cell types including cell growth, tumorgenicity, gene expression and apoptosis. In addition, over-expression of PKC delta has been shown to increase the metastatic potential of a highly metastatic murine melanoma cell line, and a mammary carcinoma cell line *(Kiley et al 1999, La Porta et al 2000).*

PKC delta is unusual in that it is autophosphorylated at higher levels than the other isoforms, and was the first isoform reported to be tyrosine phosphorylated in-vitro and in-vivo *(Gschwendt et al 1999)* . Although various PKC isoforms can also be phosphorylated on tyrosine residues in-vitro, PKC delta is the only isoform that has been shown to be tyrosine phosphorylated in vivo. Phosphorylation of specific tyrosine residues has been shown to have varying effects on PKC delta activity and/or function. Specifically, PMA and PDGF induces tyrosine phosphorylation of tyrosines 52, 155, 187, which are all located in the regulatory domain, and Src induces phorphrylation of tyrosine 311. Tyrosine 155 has been implicated in the regulation of tumorgenicity and cell growth in fibroblasts. Upon activation by DAG and phospholipids PKCs can undergo auto- and trans-phosphorylation on serine and threonine residues. The

results of tyrosine phosphorylation are still unclear and may depend upon the cell type involved. In MCF-7 breast cancer cells, phorbol ester treatment leads to tyrosine phosphorylation and activation of PKC delta *(Shanmugam et al 1998).*

Differential localization of tyrosine phosphorylated and catalytically activated delta was observed in-vitro *(Shanmugam et al 1998).* Tyrosine phosphorylated delta localized to the cytosolic fraction and was inactivated whereas catalytically active PKC delta was found to be localized in triton-x solubilized membrane *(Shanmugam et al 1998.)*

PKC delta and metastasis

PKC can have varying effects on cell growth. PKC delta has been shown to have inhibitory effects on tumor growth in some systems including CHO cells *(Watanbe et al 1992*), NIH3T3 fibroblasts *(Mischak et al 1993, Acs et al 2000),* smooth muscle cells *(Fukumoto et al 1997),*capillary endothelial cells *(Harrington et al 1997),* glial cells *(Brodie et al 1998, Kronfield et al 2000)* and B16BL6 melanoma cells *(LaPorta et al 2001),* whereas PKC epsilon and beta have been shown to stimulate tumor growth. In a study by *Zhimin et al (1997)* , prolonged exposure of PKC to TPA lead to a depletion of PKC delta in 3Y1 cells that over-expressed c-Src . These cells had anchorage independent growth and a transformed phenotype. The phenotype of these cells is attributed to the down regulation of PKC delta *(Lu et al 1997).* When the same cells were treated with an inhibitor of PKC delta or transfected with a dominant negative gene for PKC delta, a similar transformation phenotype was seen as with prolonged treatment of TPA . This study shows that transformation can occur in rat fibroblasts that

over-express c-Src, when PKC delta is depleted The results of this study suggest a tumor suppressive role of PKC delta *(Lu et al 1997).* In a similar study using 3Y1 cells that were V-src transformed stimulation with TPA resulted in tyrosine phosphorylation of PKC delta and the formation of a Src- PKC delta complex (Zang et al 1997) . Tyrosine phosphorylated delta became membrane associated and had reduced kinase activity. These two studies suggest, that in the fibroblast, altered Src and PKC delta are involved in a signaling system capable of causing transformation and facilitating tumor promotion.

The role of the regulatory domain and the catalytic domain of delta in cell proliferation and metastasis have been investigated. Studies have shown that both the regulatory and catalytic domains may regulate isoform function. Chimeric studies of proliferation in which the regulatory domain of PKC delta was combined with the catalytic domains of PKC alpha and epsilon and vice versa, revealed the importance of regulatory domain of delta in cell proliferation and glutamine synthetase expression in glial cells*(Brodie et al 1998, Kronfield et al 2000).* Over expression of delta containing a mutation of tyrosine 155 or 187 to phenylalanine in glial cells, revealed that tyrosine 155 is important in the regulation of cell proliferation and tyrosine 187 is important in glutamine synthetase expression *(Kronfield et al 2000).*

PKC delta has also been implicated in mammary tumor cell metastasis *(Kiley et al 1999).* In one study, a regulatory domain fragment of PKC delta (RD delta) inhibited metastasis possibly by blocking delta phosphorylation of cytoskeletal proteins necessary for adhesion, migration etc *(Kiley et al 1999).*

This study suggests that phosphorylation of cytoskeletal proteins by PKC delta may be important in mammary tumor cell metastasis.

PKC delta and apoptosis:

Protein kinase C can have varying effects on apoptosis. PKC beta I and epsilon have positive effects on cell growth and transformation of fibroblasts *(Borner et al 1995, Mischak et al 1993)* whereas PKC alpha and epsilon inhibit apoptosis *(Lee et al 1996, Gubiana et al 1998).*

PKC delta has also been shown to have a positive effect on apoptosis and can mediate apoptosis by at least two distinct mechanisms: 1) proteolytic cleavage and activation after DNA damage or 2) direct activation without cleavage *(Fujii et al 2000).* Either mechanism may involve several signaling pathways. In cells induced to undergo apoptosis in response to DNA damaging agents such as tumor necrosis factor and anti-fas antibody, delta is proteolytically cleaved by caspase 3 into a 40 kDa C-terminal fragment at the DMQD/N site in the V3 region , which separates the C1 regulatory domain from the catalytic domain *(Pra et al 1999, Bharti et al 1998).* Proteolytically cleaved PKC delta can phoshporylate several substrates including major basic protein, histone H1, myristoylated alanine rich-c (MARCKS) and ribosomal S6 protein (*Emoto et al 1996 and Musashi et al 2000).* Cleavage of delta and initiation of apoptosis in response to DNA damaging agents have been observed in several cell lines including U937 cells *(Emoto et al 1995)* and keratinocytes *(Denning et al 1998).* The apoptotic response induced by the catalytic fragment include nuclear condensation and DNA fragmentation *(Ghayur et al 1996).* Direct activation of

apoptosis by delta without cleavage has been observed in LNCaP cells *(Fuji et al 2000)* and in CHO cells where delta is tyrosine phosphorylated and activated, but is not proteolytically cleaved *(Konishi et al 1999).* The mechanisms by which PKC delta can induce apoptosis without the activation of caspase 3 remain unclear. However, studies have shown that alternative mechanisms may include delta's phoshorylation and subsequent inactivation of DNA –dependent kinases *(Bharti et al 1998)* or alteration of mitochondrial function *(Li et al 1999).*

In the present thesis, we examine the effect of over-expression of wild-type PKC delta, and of transfection with PKC delta mutants on metastasis, retention in the lung of intravenously inoculated B16F1 melanoma cells, adhesion, cell size, and invasion (Chapter 1). We also examine the ability of transfected delta to affect key cellular activities such as proliferation, and susceptibility to cell death (Chapter 2), and on the cellular localization of PKC delta in B16F1 TC (Chapter 3).

Chapter 1

Regulation of murine melanoma metastasis by PKC delta requires an intact tyrosine 155

Abstract:

In the present paper, we examine the effect of over-expression of wild-type or mutant protein kinase C (PKC) delta genes on the metastasis of murine melanoma using B16F1 melanoma cells transfected with either wild-type delta (Dtag), delta containing a substitution of phenylalanine for tyrosine 155 (Y155F), and control cells (mock) transfected with an empty vector. All transfected genes contain a 12 amino acid epsilon tag on the C-terminus of the PKC delta gene. Results show that transfection with wild-type PKC delta potentiates experimental metastasis, resulting in an increase in both the number and size of metastatic nodules in the lung ($P < 0.05$), while transfection with Y155F has little or no effect on metastasis. Quantitative confocal microscopy of lung whole mounts prepared from mice inoculated with transfected and fluorescently stained TC is used to examine the effect of wild-type and mutant delta on the very early stages of TC colonization of the lung. Results show that while all groups contain similar numbers of fluorescent loci in the lung 2 hrs after intravenous TC inoculation, by 48 hrs increased numbers of fluorescent loci are present in lungs from animals inoculated with Dtag cells ($P=0.001$) and these foci are significantly larger than those observed in lungs from control mice or from mice receiving cells transfected with Y155F ($P< 0.05$). In vitro examination of transfected cells shows that over-expression of wild-type delta has little or no effect on cell size or invasion, but increases adhesion of B16F1 cells to matrigel. Combined, the in vivo and in vitro results reported above necessitate the presence of an intact

Y155 in transfected delta, and suggest the possible importance of phosphorylation of tyrosine 155 in the PKC delta regulation of metastasis.

Introduction

Protein Kinase C (PKC) delta is a widely expressed novel member of the PKC superfamily. This isoform has been shown to regulate cellular processes such as growth, tumorgenicity, gene expression, and apoptosis (*Watanbe et al 1992, Acs et al 2000 , Reyland 1999).* Stimulation of PKC delta can inhibit *in-vitro* proliferation of several cell types (*Mischak et al 1993, Acs et al 2000, Fukumoto et al 1997 Harrington et al 1997 Brodie et al 1998, Kronfield et al 2000, LaPorta et al 2001)* and induce differentiation of keratinocytes *(Szallasi et al 1994).* Over-expression of PKC delta increases melanin synthesis and the metastatic potential of the highly metastatic BL6 murine melanoma cell line *(LaPorta et al 2000).* PKC Delta has also been shown to regulate metastasis in mammary tumor cells, a function attributed to the regulatory portion of the molecule *(Kiley et al 1999).*

PKC delta is one the isoforms reported to be tyrosine phosphorylated both in-vitro and in-vivo. At least 19 different tyrosine phosphorylation sites have been identified for PKC delta, in both the regulatory and catalytic domains, *(Brodie et al 1998).* Phosphorylation of specific tyrosine residues has been shown to have varying affects on delta activity and function *(Kronfield et al 2000).* In NIH3T3 fibroblasts, tyrosine 155 is an in-vivo phosphorylation site for the Src kinase Lyn *(Szallasi et al 1995)*, and has been implicated in the regulation of tumorgenicity, and anchorage independent growth *(Acs et al 2000),* whereas phosphorylation of tyrosine 187 is important in the inhibition of glutamine synthetase expression in glial cells *(Kronfield et al 2000).* PKC delta can also associate with various

tyrosine kinases such as such Src (*Shanmugam et al 1998, Song et al 1998 Zang et al 1997, Denning et al 1996),* Lyn *(Szallasi et al 1995, Song et al 1998)*, Fyn *(Denning et al 1996, Gschwendt et al 1994)*, and Abl *(Yuan et al 1998, Sun et al 2000)* in either a tyrosine dependent or independent manner and in response to divergent stimuli including PDGF (*Brodie et al 1998, Li et al 1994)*, EGF, H2O2 *(Konishi et al 2001),* IgE receptor *(Song et al 1998),* TPA and PMA *(Kronfield et al 2000, Brodie et al 1998).*

Although a regulatory effect of PKC delta on metastasis has been reported for highly metastatic BL6 melanoma cells and in mammary adenocarcinoma cells *(La Porta et al 2000 and Kiley et al 1999),* much remains unknown concerning the mechanism(s) involved in this regulation, or about the possible role played by delta tyrosine residues. In the present study, we use weakly metastatic B16F1 murine melanoma cell lines transfected with either wild-type delta, or delta containing a point mutation of tyrosine 155 (Y155F delta), and examine the in vivo effect of these mutations on the ability of delta to regulate both early and late events during metastasis. In vitro studies examine the ability of transfected cell lines to affect the critical properties of TC adhesion and invasion.

Materials and Methods

Cell Constructs:

Non-transfected B16F1 cells were obtained from the National Cancer Institute (NCI). The cells were expanded and transfected with either wild type PKCδ, Y155Fδ, or the empty vector in the lab of Dr. P. Blumberg at the NCI. The generation of these constructs is described in Acs et al (1997). All constructs contain vectors with the heavy metal inducible methallothionine promoter, express a geneticin resistant gene, and have a 12 amino acid epsilon tag on the C-terminus of the PKC delta gene. Transfected cells containing a vector with the promoter and epsilon tag but lacking a delta gene are identified as Mock, and are used as controls. Cells identified as Dtag over-express wild type delta. Y155F cells over-express delta that has a substitution of phenylalanine for tyrosine 155 (a known tyrosine phosphorylation site) (*Szallasi et al 1995, Kronfeld et al 2000)*. Acs et al have illustrated that mutated Y155 is stably expressed in NIH3T3 fibroblasts, and had similar localization patterns and kinase activity as wildtype delta *(Acs et al 2000).*

Cell culture

All cell lines were grown in MEMα media (Gibco BRL -Life Technologies) , supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100ug/ml streptomycin, 0.250ug/ml Amphotericin and 200ug/ml Geneticin (Gibco BRL-Life Technologies) at 37° C in humidified incubator with 5% CO^2. Cells were detached by trypsinization. Cell numbers were determined with a hemocytometer and the Trypan blue exclusion test.

Cell Lysis and Western blot analysis

To detect PKC delta in cells grown in tissue culture, sub-confluent flasks of cells were rinsed with PBS and trypsinized. The cells were then resuspended in 1ml of lysis buffer (20mM Tris pH 7.4, 5mM EGTA, 20uM Leupeptin, 1mM AEBSF, 30ul/ml Aprotinin and 1mM Sodium Orthovanadate) and homogenized with a dounce homogenizer on ice for 30 strokes. 4x laemmli buffer was added to the solutions to bring the final concentration to 1x laemmli. Two (2%) beta mercaptoethanol was then added, the samples quickly vortexed and boiled for 2 minutes, and purified isozyme or whole cell extracts were subjected to SDS-Page. Samples were electrophoresed on a 8% polyacrylimide tris-glycine SDS gel and transferred to a 0.45uM Nitrocellulose membrane. Membranes were blocked in 10% powdered milk and probed with PKC affinity purified rabbit IgG primary antibodies (Santa Cruz Biotech, CA) using 2.2ng/ml of PKC-δ antibody or 0.8ng/ml of PKC-ε antibody targeting the C-terminal epsilon tag of the vector) and with an anti-PKC goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotech). Reactive products were visualized using the ECL Plus (Amersham Biotech, NJ) chemiluminescence assay. Specificity of the antibody was determined by incubating the PKC delta antibody with blocking peptide (Santa Cruz) at 1:5 concentration for 1hr prior to the western immunoblotting.

Experimental Metastasis

10^5 parental B16F1 cells, DTag, Mock or Y155F cells in a 0.2ml saline solution were intravenously injected into the tail veins of un-anesthetized female C57BL6 mice (Charles River, CT). The animals were sacrificed after 3 weeks, and the lungs were removed, rinsed in phosphate buffer, and fixed in 37% formaldehyde. The total number of nodules on the lung's surface was counted under a dissecting scope at a magnification of 6x. Nodules were characterized as being either less than 0.5mm, 0.5-1 mm, or greater than 1mm in diameter.

Retention of viable tumor cells in the lung

10^5 cells of parental B16F1, Dtag, Mock, or Y155F cells were incubated with 25uM of Cell Tracker (Molecular Probes). Cell Tracker is a cytoplasmic fluorescent dye that will only tag cells living cells, and is detectable in live cells for up to 72 hrs or 4 cell divisions. Cells were incubated with Cell Tracker in media that lacked serum, for 1hr at 37 C. Fluorescent cells were then rinsed 3X with warm media, resuspended in sterile phosphate buffer (PBS), and inoculated intravenously (IV) into a tail vein of C57BL6 mice (Charles River). Animals were sacrificed at 2 or 48hrs and lungs were removed and fixed in 3.7% formaldehyde for 10 minutes. Lobes of the lungs were separated, and whole mounts were prepared on clean glass slides. The Meridian Ultima laser scanning confocal microscope equipped with an argon laser and a 530/30 bandpass filter for FITC fluorescence. The Meridian quantitative analysis software program was used to quantify the numbers of fluorescent cells/cell clumps present after 2 and 48 hours (10x mag, pinhole 1600uM, step size of 1uM). Fluorescent cells/cell clumps

greater than 10um^2 in diameter were quantified and an analysis of cell/clump size was determined.

Adhesion assay

Clean sterile coverslips were coated with 5ug/ml of fibronectin (Invitrogen, CA) or growth factor reduced Matrigel (BD Biosciences, MA), and dried for 1hr. at room temperature under a laminar flow hood. The matrix-coated coverslips were then blocked with 0.1% BSA in sterile PBS for 30 min. 10^5Dtag, Mock, or Y155F cells were incubated with 12.5uM Cell Tracker dye in media lacking serum for 1hr at 37°C, rinsed 3X with warm media and resuspended in sterile phosphate buffer (PBS). Fluorescently tagged TC were plated on coated coverslips in complete media and incubated for 6hrs at 37°C. Coverslips were then treated with 3.7% formaldehyde for 10 minutes, and rinsed in PBS. Quantitative confocal microscopy was used to evaluate the number of adherent fluorescent cells (10x mag, pinhole :1600uM, step size: 1um/ slice). Analysis of cell area and shape was achieved using the Meridian size analysis software program.

Invasion Assay:

A two compartment Boyden chemotaxis chamber used similar to the work described in Terranova et al (1986). The bottom of uncoated Boyden chambers with a 0.8um pore size were coated with either fibronectin or growth serum reduced matrigel. The chambers were allowed to dry overnight in a in humidified incubator at 37°C. The chambers were then placed in micro-titer plates, where the coated portion of the chamber would be in contact with media that had 10% FBS as the chemoattractant. 5x10^4 tumor cells were then placed in the upper

chamber in media which lacked FBS. The chambers were then incubated for 14hrs in a humidified incubator at 37°C. Cells that penetrated the matrix and attached to the underlying layer of fibronectin or matrigel were stained with crystal violet and counted. Cells which did not penetrate the matrix were removed before staining. The results reported are the medians of triplicate assays.

Results:

Detection of PKC delta expression in cultured cells using western blot analysis

To examine the levels of delta expression in transfected and control cells, cell lines were subjected to western blot analysis and probed with an anti-PKC delta antibody that recognized a single band with molecular weight of 85 KD. Comparable levels of delta product were detected in the mock and parent cell lines. Stronger expression of delta was observed in the Dtag and Y155F cell lines (Figure1A). Incubation of the anti-PKC delta antibody with blocking peptide completely eliminated detection of an 85kD band showing that protein detection by the primary antibody is very specific (Figure 1B).

Figure 1A: Detection of PKC delta expression in cultured cells using western blot analysis

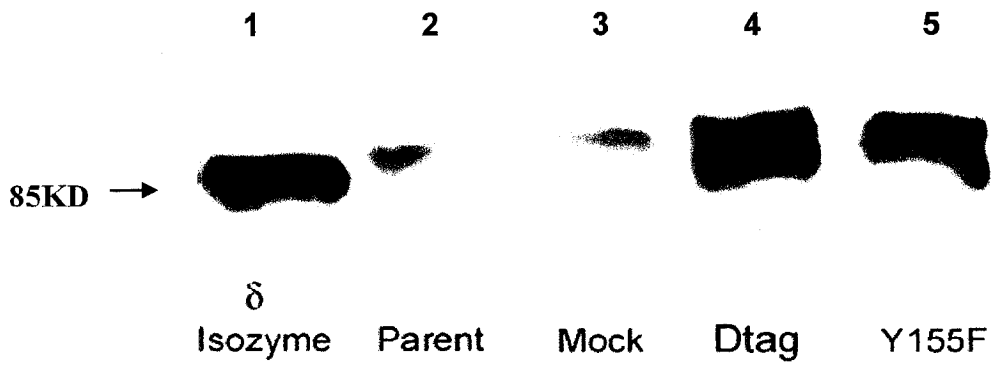

Figure 1A Legend: Western blot analysis of PKC delta in whole cell extracts. Lane 1 was loaded with purified PKC delta isozyme, 50ng/lane . Lanes 2-5 were loaded with 42ug of whole cell extracts. The primary antibody was rabbit anti-PKC delta at a dilution of 1:300, and the secondary antibody was diluted 1:5000.

Figure 1B: Western blot analysis using PKC delta antibody pre-incubated with a blocking peptide

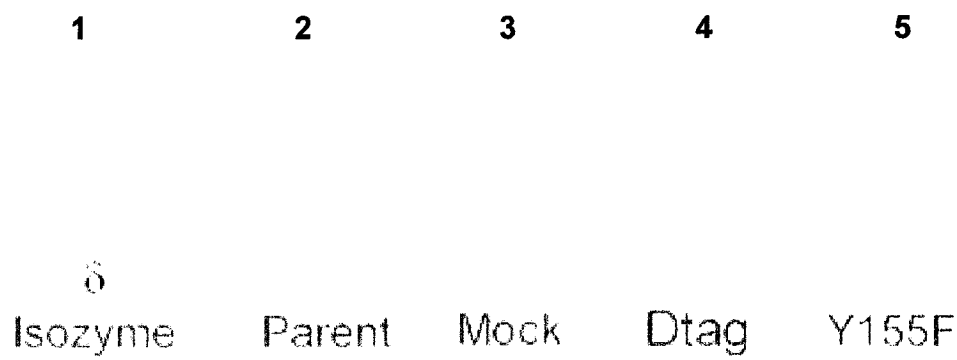

Figure 1B Legend: Western blot analysis using PKC delta antibody pre-incubated with blocking peptide at 1:5 concentration for 1hr prior to the western immuno-blotting. Lane 1 was loaded with purified PKC delta isozyme, 50ng/lane. Lanes 2-5 were loaded with 42ug of whole cell extracts. The primary antibody dilution was 1:300 and the dilution for the 2^{o} antibody, was 1:5000.

Detection of transfected PKC delta expression in cultured cells with anti-PKC epsilon antibody.

In the previous experiment, both endogenous and transfected delta were detected with an anti-delta antibody (Figure 1A). To detect over-expression transfected wild-type and Y155F delta, a PKC epsilon antibody capable of recognizing the 12 amino acid PKC epsilon Tag on the vector was used for detection. Results show that endogenous epsilon migrated at a MW of 90 KD whereas endogenous delta and over-expressed delta with an epsilon tag migrated at a lower MW of 85 KD(Figure 1C). Combined, the results obtained using either the anti-delta and anti-epsilon antibodies, demonstrate a marked over-expression of comparable amounts of transfected wild-type and Y155F delta (Figure 1A and 1C).

Figure 1C: Western blot analysis of whole cell extracts with anti-PKC epsilon Antibody

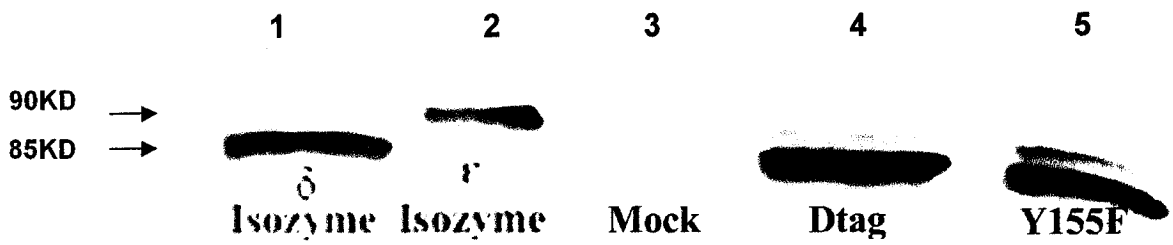

Figure 1C Legend: Western blot analysis of whole cell extracts with anti-PKC epsilon antibody. Lane 1 is delta isozyme 50ng/lane, probed with delta antibody at a dilution of 1:300. Lane 2 is epsilon isozyme 50ng/lane, lanes 3-5 are whole cell extracts; 32ug/lane. Lanes 2-5 were probed with epsilon antibody at a dilution of 1:600. Dilution of the 2^{o} antibody is 1:5000. Higher MW bands denote endogenous epsilon expression, while the lower bands in 4 and 5 show over-expressed delta protein.

PKC delta potentiates the metastasis of B16F1 melanoma cells

To determine the effects of transfected mutant delta on metastasis of the weakly metastatic B16F1 melanoma, transfected and control cell lines were intravenously inoculated into host mice. Analysis of pulmonary metastasis three weeks later revealed that animals injected with cells over-expressing wild-type delta had significantly greater numbers of metastatic lesions than those injected with cells transfected with Y155F or Mock delta (Figure 2). These results show that over-expression of wild-type PKC delta regulates metastasis causing an increase in the number of metastatic nodules, and suggests that an intact tyrosine 155 is essential for this regulation.

Figure 2: Regulation of metastasis of B16F1 melanoma cells by PKC delta

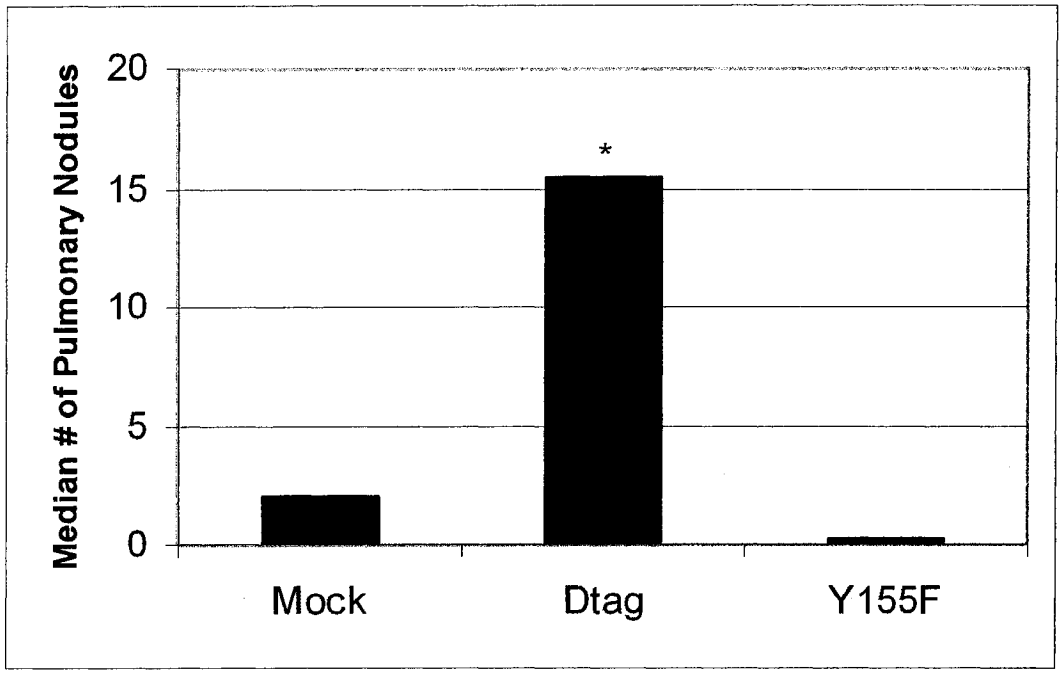

Figure 2 Legend : Analysis of pulmonary metastasis. Bars denote the median number of pulmonary metastases, N= 12 animals/group. The Wilcoxon test was used to determine significance. * denotes significant differences when compared to Mock and Y155F (P=0.005).

Over-expression of wild-type delta increases the size of pulmonary nodules

Qualitative examination of the lungs of mice inoculated with transfected TC strongly suggested that transfection with wild-type delta resulted in an increase in nodule size. Accordingly, we next sought to determine whether there were in fact differences in the incidence of large nodules (i.e. >1.0mm^2 in diameter). Results demonstrated that mice receiving cells transfected with wild-type delta had larger pulmonary nodules than animals inoculated with cells transfected with Mock or Y155F (P, 0.004), Table 1. This data suggested that wild-type delta may regulate key cellular processes such as cell proliferation or TC cell size in the microenvironment of the lung. It also suggested that intact tyrosine 155 is essential for this function.

Table I. The incidence of mice with large nodules ($1mm^2$)

Cell Lines	Mock	Dtag	Y155F
Incidence of large nodules>1mm	4/10	*10/10	2/10

Table 1 Legend: Incidence is expressed as the number of mice/group with nodules > than $1mm^2$). Significance was determined using the Fischer's Exact Test. *denotes significant differences compared with Mock and Y155F (P=0.004).

Transfection is stable in vivo

To determine if TC present in metastatic nodules over-express transfected wild-type PKC delta after being in the host for 3 weeks, cells from tumor nodules removed from the lungs of animals were subjected to cell lysis and western blot analysis as described earlier. The results show that the transfected cells are stable and that PKC delta is still over-expressed in cells that have metastasized in the host (Figure 3).

Figure 3: Detection of delta over-expression in metastatic nodules removed from lung

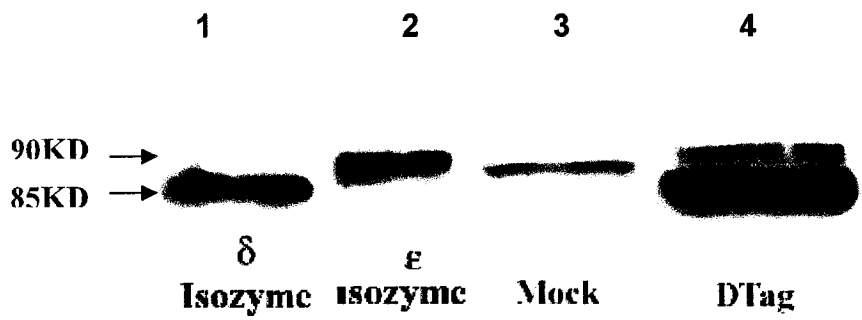

Figure 3 Legend: Western blot analysis of extracts of metastatic nodules. Lane 1 is 50ng delta isozyme probed with delta antibody 1:300. Lane 2 is epsilon isozyme, 50ng; lanes 3 and 4 are whole cell extracts of cells removed from pulmonary nodules; 41ug/lane. Lanes 2-4 were probed with epsilon antibody, 1:600. For all lanes, dilution of the secondary antibody was 1:5000. Higher MW bands denote endogenous epsilon expression, while the lower band in lane 4 show over-expression of wild type delta.

Effects of over-expression of PKC delta on the retention of viable TC in the lung during the early stages of metastasis

In the above experiments, metastasis was analyzed at a relatively late stage of metastasis and demonstrated that over-expression of wild-type delta increased both the frequency of pulmonary metastases as well as nodule size. Using cell tracker labeled cells, we next sought to determine whether delta over-expression might mediate these results by affecting the retention of TC in the lung at an early stage of pulmonary metastasis.

Preliminary experiments using Cell-Tracker labeled cells showed that TC fluorescence is lost upon cell death, and that fluorescently tagged cells showed no difference in metastatic potential from control untagged cells (data not shown). In the present study, we used cell-tracker labeled cells and quantitative confocal microscopy techniques, and analyzed the number as well as the area of fluorescent tumor cells/clumps present in the lungs 2hrs and 48hrs after IV TC inoculation. Results reveal that in all groups similar numbers of fluorescent cells/clumps were present at 2 hours (Figure 4A), and cell /clump sizes were similar. In all groups, significant numbers of TC were cleared from the lungs between 2hr and 48hrs (Figure 4A). Interestingly, however, over-expression of wild-type delta increased both the number and the size of metastatic loci in the lungs 48 hrs after inoculation when compared with the Y155F and mock groups (Figure 4A and 4C). Representative confocal micrographs are shown in Figure 4B.

Figure 4A: Quantitative analysis of numbers of loci containing live TC in the lungs 2hrs and 48 hrs after IV inoculation of fluorescently labeled transfected melanoma cells.

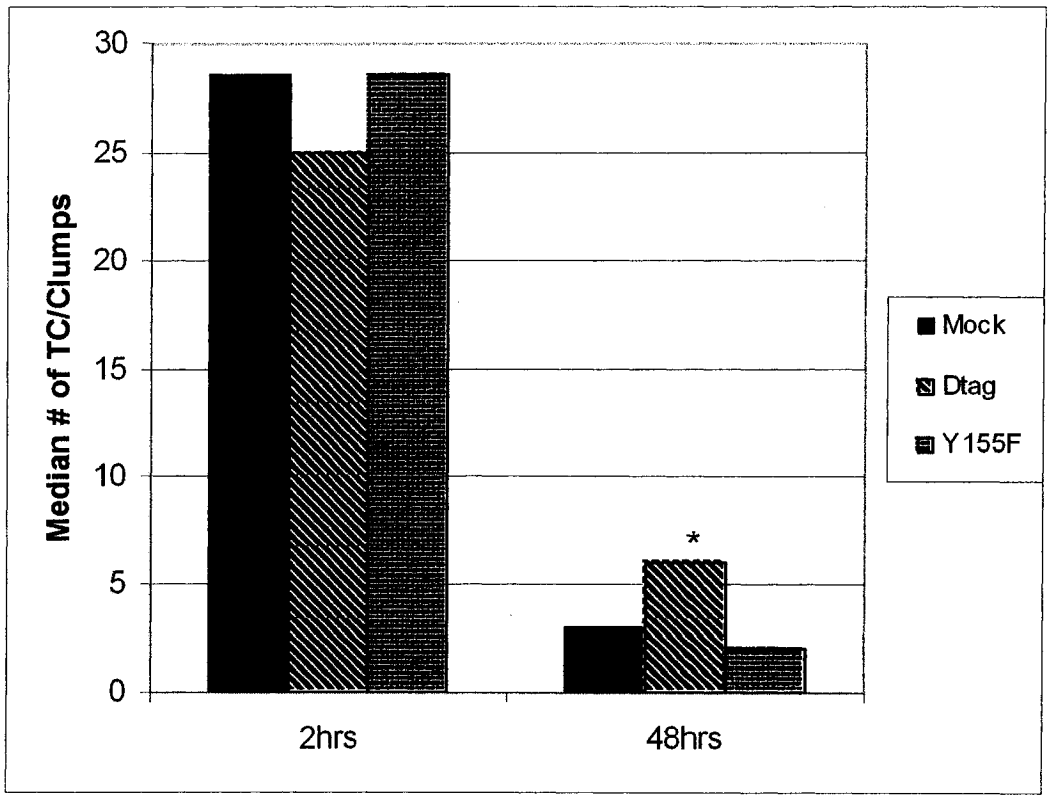

Figure 4A Legend:

2hrs: Mock was not significantly different than Dtag (P=0.094), Y155F (P=0.172). Y155F was not different than Dtag (P=0.915)

48hrs: *denotes that animals injected with Dtag cells had more TC clumps than mock (P=0.001) and Y155F (P=0.001).

Figure 4B: Fluorescent micrograph of cells transfected with wild-type delta present in the lung 2hrs and 48 hrs after IV tumor cell inoculation

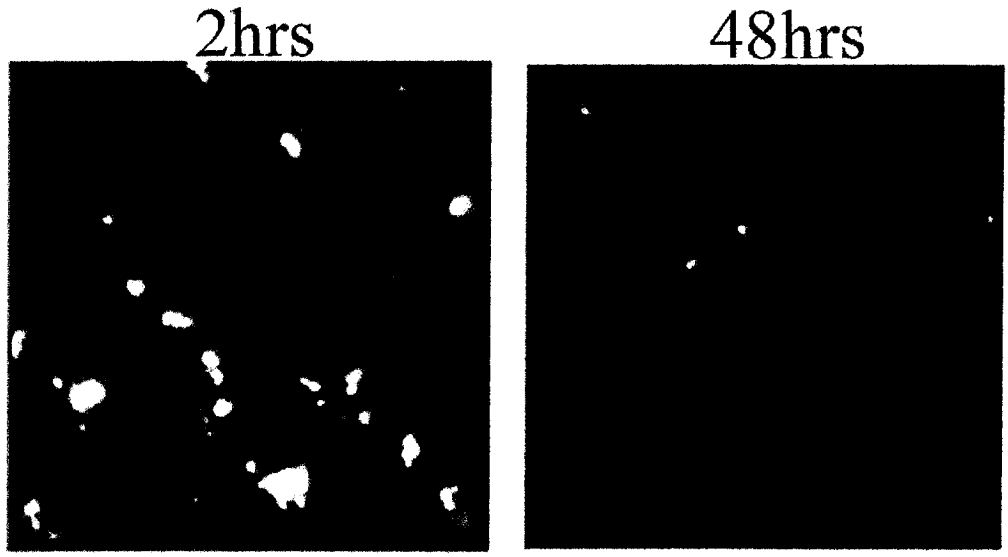

Figure 4B Legend: Analysis of the area of fluorescent TC/clumps in lungs 48 hrs after IV inoculation.

Figure 4C : Analysis of TC/ Clump area

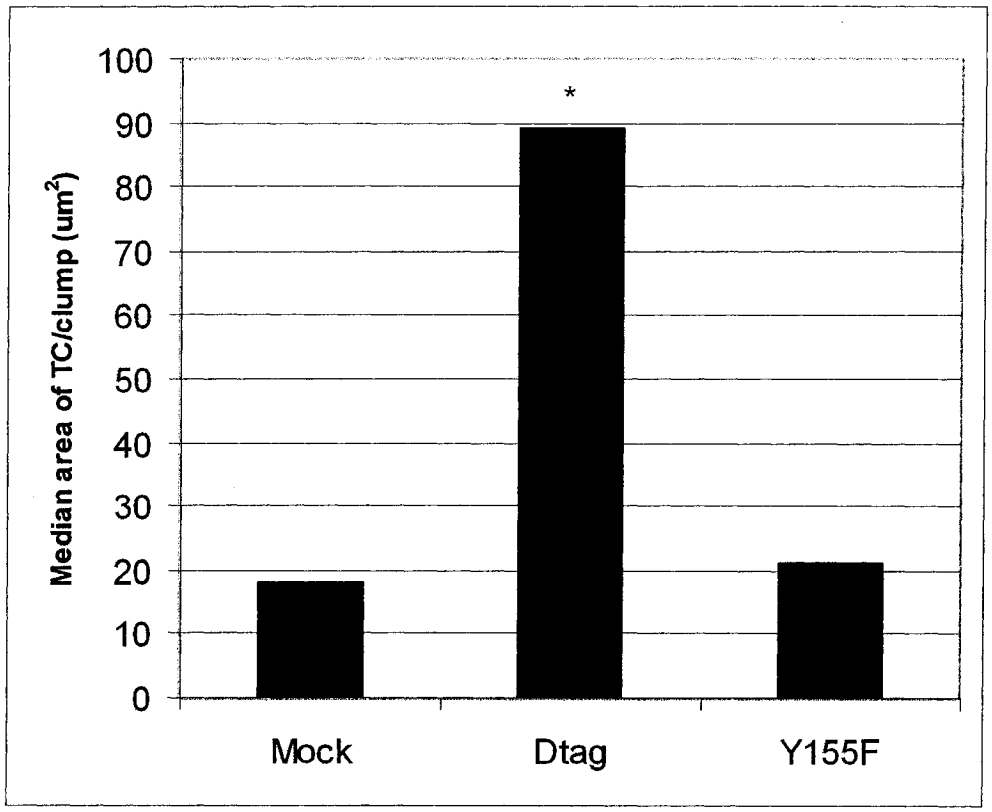

Figure 4C Legend: Bars denote the median area of TC/TC clumps present in the lung at 48hrs. The Wilcoxon test was used to determine significance. *denotes that more Dtag cells are retained than mock (P=0.001) and Y155F (P=0.025).

The effects of delta over-expression on tumor cell size

The retention and metastasis data (Figure 2 and 4A) demonstrated an increased number and size of TC deposits in the lung during an early and late stage of metastasis, respectively. These findings suggested that over-expression of PKC delta may affect either TC growth (cell size), proliferation, and/or survival in the lung. Therefore, we next sought to determine whether PKC delta could in fact regulate the size of B16F1 melanoma cells using matrigel or fibronectin as a substrate.

To determine whether the increased size of fluorescent TC loci in the lung at 48 hours in cells transfected with wild-type delta reflected an increase in TC size, quantitative confocal microscopy was used to determine the area of fluorescent cells grown on biological matrices. Results show that over-expression of Dtag had little or no effect on cell size on a matrigel matrix, and actually decreased cell size on fibronectin (Figure 5B). On both substrates, over-expression of Y155F results in increased cell size when compared to Dtag (Figure 5A and 5B). This data suggests that PKC delta can regulate cell size, and that this regulation depends on substrate and whether or not tyrosine 155 is present.

Figure 5A Quantitative analysis of B16F1 cell size on matrigel

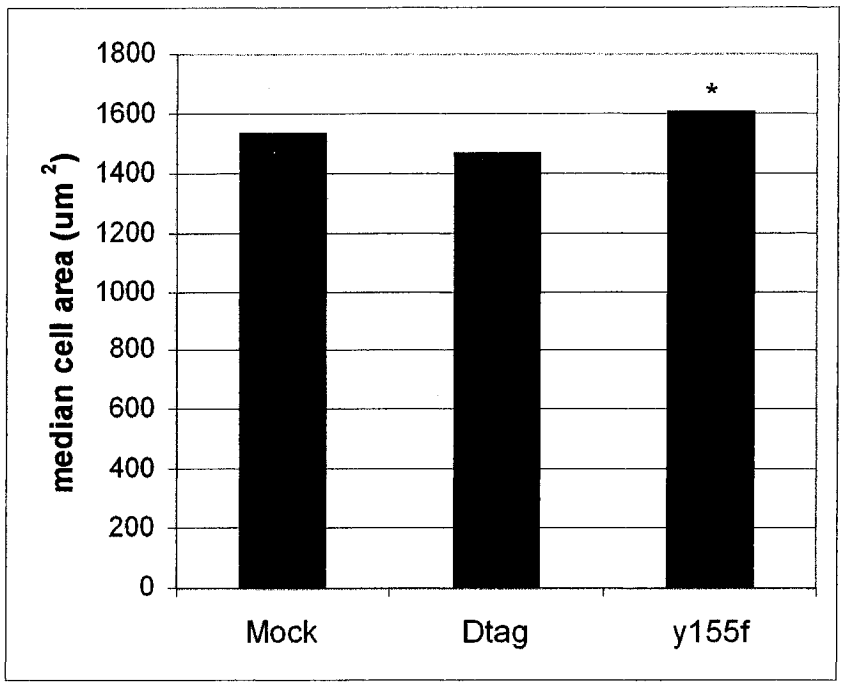

Figure 5A Legend: *denotes that Y155F has a larger cell size than Dtag (P=0.007).

Figure 5B Quantitative analysis of B16F1 cell size on Fibronectin

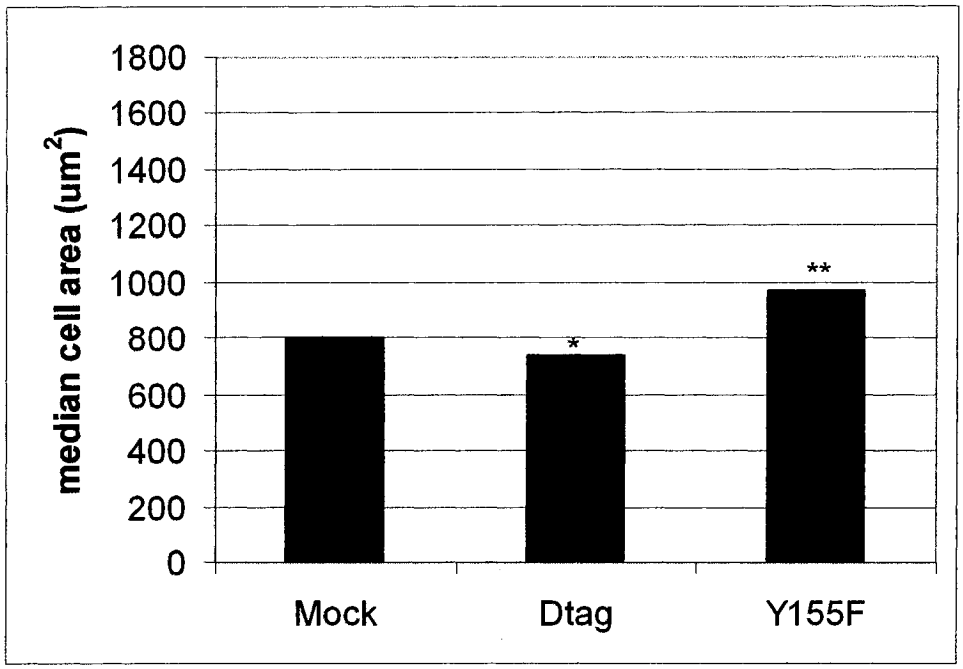

Figure 5B Legend: *denotes that Dtag cells are smaller than Mock (P=0.001).

**Y155F cells are larger than Mock (P=0.001) and Dtag (P=0.001).

Examination of adhesion properties of transfected cell lines

Adhesion of circulating TC to the endothelium is a critical early event during metastasis. Others have shown that blood borne TC can attach to the lung endothelium soon after entry into the circulation (Al-Medhi et al 2000, Wong et al 2002). To determine whether the increased numbers of fluorescent TC in the lungs of animals inoculated with cells transfected with wild-type delta might reflect an increased adhesion of these cells in the microcirculation, we next sought to determine whether over-expression of wild-type PKC delta can affect TC adhesion to matrigel or fibronectin. Analysis of cell adhesion revealed that cells that over-express wild-type delta are markedly more adherent to matrigel than control and Y155F cells (Figure 6a and 6 B), while cells that over-express wild-type delta or Y155F are markedly less adherent to fibronectin than control cells (Figure 6B). Thus, over-expression of PKC delta can produce differential effects on adhesion depending upon the presence or absence of tyrosine 155, and/or on substrate.

Figure 6a Quantitative analysis of B16F1 cell adhesion to matrigel

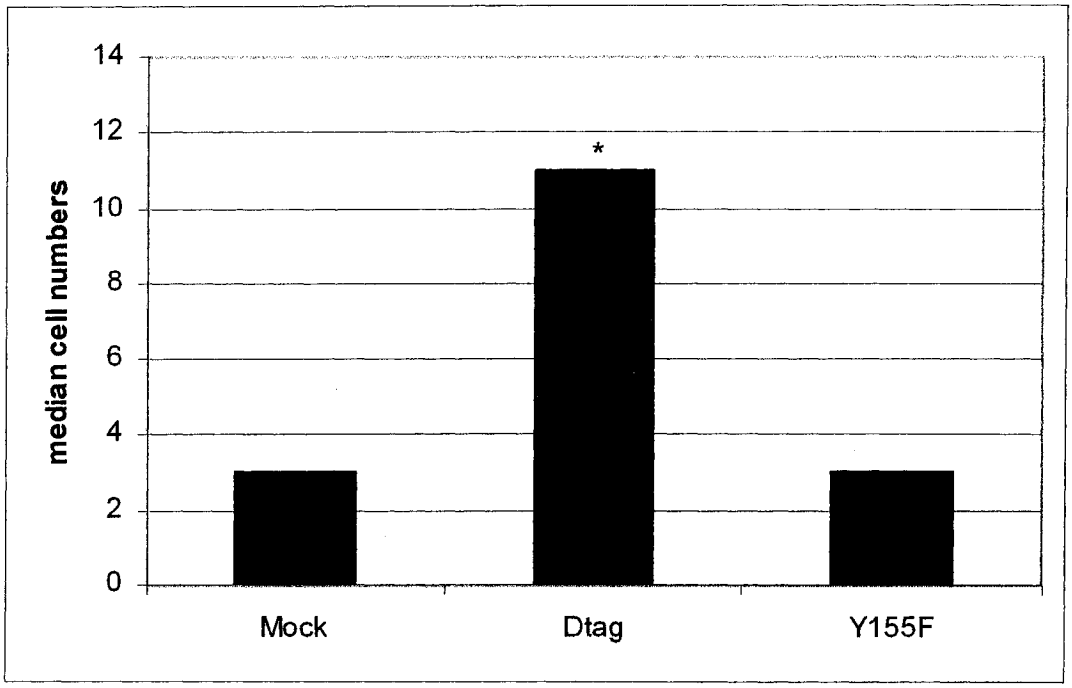

Figure 6A Legend: *denotes that Dtag is more adherent to matrigel than Mock (P=0.001) or Y155F (P=0.001).

Figure 6B Quantitative analysis of B16F1 cell adhesion to Fibronectin

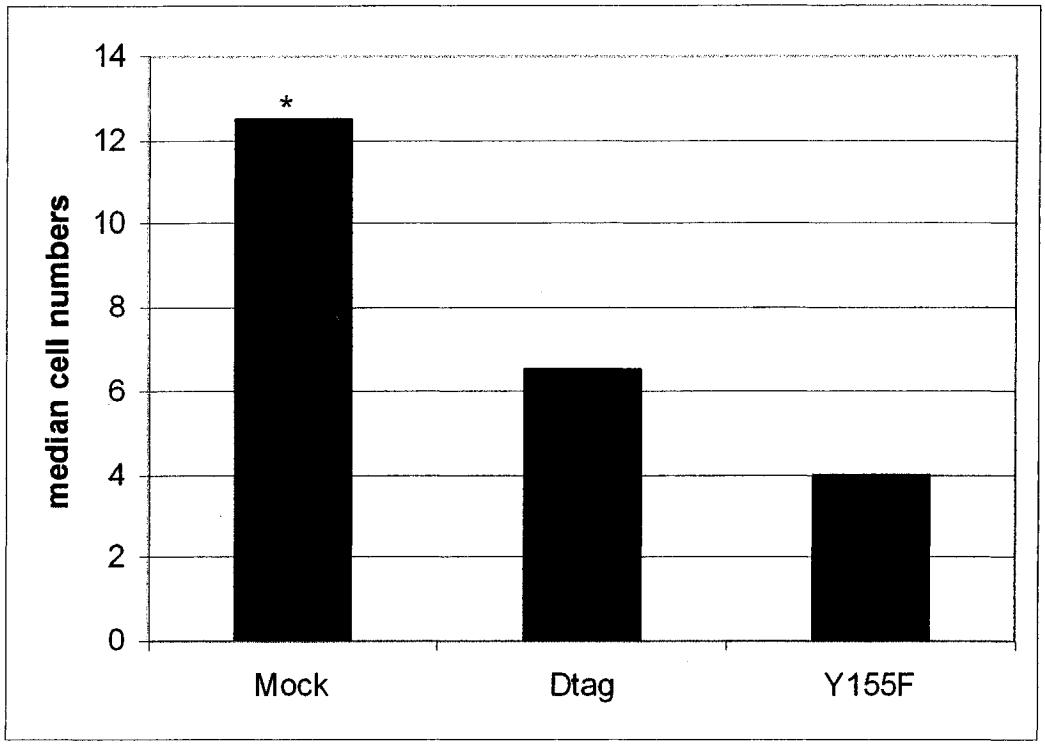

Figure 6B Legend: *denotes that Mock cells are more adherent to fibronectin than Dtag (P=0.002) and Y155F (P=0.001).

Effects of delta over-expression on tumor cell invasion

The process of invasion involves adherence of the tumor cells to the extra-cellular matrix (ECM), degradation of matrix components, and movement of the cell body. In the previous study, we showed that over-expression of wildtype delta can regulate the adhesion of tumor cells to biological matrices. To determine whether transfected delta is capable of regulating TC invasion, cells were plated in Boyden Chemotaxis chambers and the number of invasive cells were analyzed. Results show that over-expression of wild-type or Y155F delta had little or no effect on invasion (Figure 6).

Figure 7: Analysis of TC cell invasion through a Boyden chemotaxis chamber

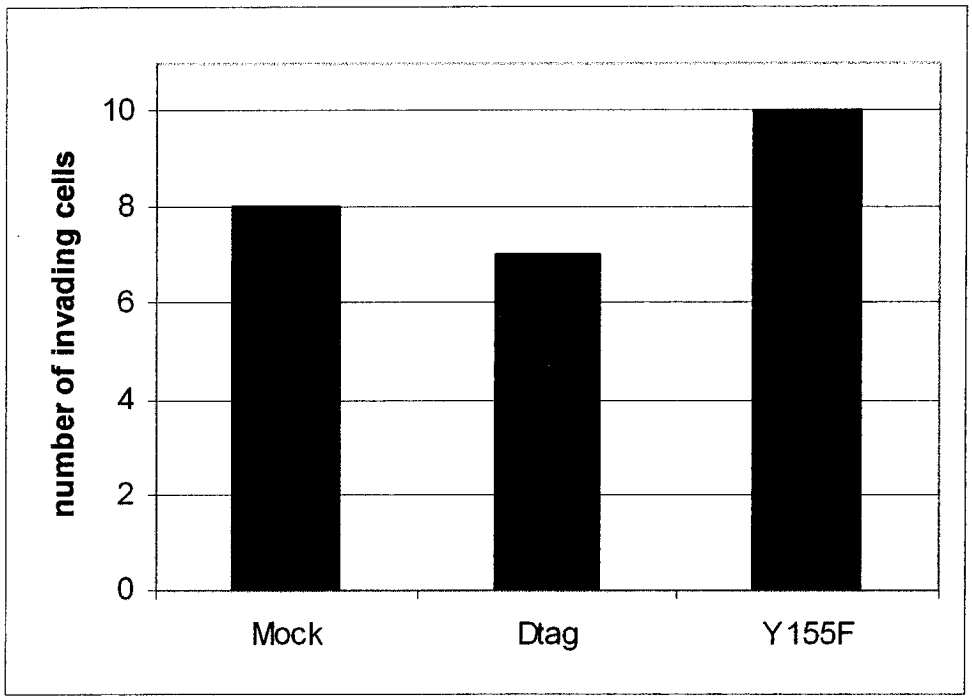

Figure 7 Legend: Bars denote median numbers of tumor cells that were invasive and attached to the surface of the underlying layer of fibronectin in the outer section of the Boyden Chamber.

Discussion:

In this chapter, we have shown that B16F1 cells are stably transfected and have observed the effects of over-expressed wild-type and Y155 F delta on metastasis, the early retention of TC in the lung, and on cell size, adhesion and invasion in vitro.

Western blot analysis of delta expression in extracts of cultured cells using the epsilon-specific primary antibodies demonstrated strong over-expression of both transfected Dtag and Y155F and use of the delta specific primary confirmed the presence of increased expression of Dtag and Y155F mutant delta. Thus, comparison of results between these two cell lines could provide information concerning the functional significance of the Y155F mutation.

Evaluation of metastasis 3 weeks after TC inoculation revealed that over-expression of wild-type PKC delta causes an increase in both the number and the size of metastatic nodules in the lungs of mice inoculated with cells over-expressing wild-type delta compared mock or Y155F. Quantitative evaluation of whole mounts prepared 48 hours after IV inoculation of TC demonstrates an increase in the number and size of fluorescent TC/and or small TC clumps in lungs from animals receiving cells over-expressing wild-type delta when compared with cells over-expressing Y155 Fdelta, or with mock. It is unlikely that the increased size of the clumps represent differences in TC size, as over-expression of wild-type delta was never observed to increase TC size, and in fact could even decrease cell size (Figure 5B). The findings of increased size and

number of metastatic TC deposits at both 2 days and 3 weeks suggests that the effects of delta on metastasis are likely due to regulation of key events in the metastatic cascade such as adhesion, cell proliferation, and/or invasion of blood borne TC during the very early stages of metastasis.

In vitro studies presented here demonstrate an increased adhesion of cells over-expressing wild-type delta to matrigel, a derivative of basement membrane material obtained from blood vessels, and would be consistent with a hypothesis that over-expression of wild-type delta may increase adhesion of blood borne TC to the endothelium of pulmonary vessels, and contribute to the increased frequency of pulmonary nodules. We hypothesize that these clumps represent early foci for development of many of the metastatic nodules observed 3 weeks later, while some of the very small nodules observed at 3 weeks may reflect a later event and be metastases of metastases. The observation of increased sizes of the TC/ clumps in the lung at 48 hrs and of nodules observed 3 weeks later would be consistent a hypothesis that wild-type delta stimulates proliferation of B16F1 TC. In fact, we have demonstrated that over-expression of wild-type delta does stimulate proliferation of B16F1 melanoma cells (Chapter 2).

Using different cell types, others have shown that mutations of specific tyrosine residues of the regulatory domain of PKC delta have varying effects on cellular functions. For example, tyrosine 155 regulates cell proliferation whereas tyrosine 187 regulates glutamine synthase expression in glial cells *(Kronfield et al 2000).* The contributions of the regulatory domain and catalytic domain of PKC

delta to it's function and activity have also been investigated in chimeric studies. Chimeras in which the regulatory portion of delta was combined to the catalytic portions PKC epsilon or alpha inhibited glial cell proliferation and glutamine synthase expression (Brodie *et al 1998 and Kronfield 2000).* Additionally, tyrosine phosphorylation of delta in response to PMA and PDGF stimuli only occurred in chimeras that contained the regulatory domain of PKC delta *(Brodie et al 1998).* Finally, the importance of the PKC delta regulatory domain in metastasis, has been illustrated in a mammary adenocarcinoma cell line, where over-expression of the regulatory fragment interferes with endogenous delta activity and phosphorylation of cytoskeletal substrates, alters subcellular localization and reduces metastasis *(Kiley et al 1999).* Our results are consistent with *Kiley et al's* observations on the importance of the regulatory region of PKC delta in regulation of metastasis.

It is important to note that only cells with increased levels of delta containing an intact tyrosine 155 are able to significantly increase the frequency and size of TC retained in the lung at 48hrs, the number and size of metastatic pulmonary nodules observed several weeks later, and adhesion. This data demonstrates the importance of tyrosine 155 for these functions, and suggests a possible role for tyrosine 155 phosphorylation in the PKC delta-mediated stimulation of B16F1 metastasis.

Chapter 2

Effects of over-expression of wild-type and mutant PKC delta on B16F1 melanoma cell proliferation and susceptibility to cytotoxic agents

Abstract

We previously demonstrated that over-expression of wild-type delta (Dtag) in B16F1 cells increases: 1) the number and size of viable tumor cells (TC)/ clumps in the lung of C57/Bl6 mice 48 hrs after intravenous inoculation, 2) the number and size of metastatic pulmonary nodules several weeks later, and 3) in vitro adhesion of TC to matrigel. These results were dependent upon the presence of delta tyrosine 155. In the present chapter we utilize B16F1 cell lines over-expressing wild-type delta (Dtag), or mutant delta containing a substitution of phenylalanine for tyrosine 155 (Y155F) to examine B16F1 proliferation and viability when subjected to adverse conditions including: 1) limiting serum concentrations, 2) cell crowding, and 3) exposure to cytotoxic agents. Over-expression of wild-type delta consistently increases proliferation when compared to Y155 mutant delta or control cells, and has only a marginal effect on cell viability. Y155F cell properties are highly dependent upon serum concentration and cell crowding, however, under most conditions, Y155F cells proliferate less than Dtag and show diminished viability. In contrast, cell death studies utilizing cycloheximide, camptothecin, or ethanol show that over-expression of Y155 mutant delta markedly increases TC survival in the presence of cytotoxic agents, whereas wild-type delta has little or no effect on induction of cell death when compared to control cells. The proliferation studies support the hypothesis that wild-type PKC delta potentiates metastasis by stimulating TC proliferation in the circulation and/or in the lung parenchyma, and suggest that phosphorylation of tyrosine 155 is required for this effect. The cell death studies would be

consistent with a hypothesis that phosphorylation of tyrosine 155 may increase TC susceptibility to cytotoxic agents.

Introduction:

Tumor cells (TC) must complete several critical steps in order to metastasize. These include migration from the primary site, intravasation into the bloodstream, proliferation, adhesion and arrest in the microvasculature of the target organ, extravasation into the surrounding tissue, induction of new blood vessels, and finally proliferation of microscopic metastases. During these steps, the TC is subject to stimuli capable of causing proliferation and/or cell death. Indeed, TC survival and successful metastasis depend upon the rate of proliferation exceeding that of cell death. Thus, favorable TC responses to stimuli capable of regulating proliferation and apoptosis are essential to successful metastasis.

Several studies have shown that the fate of blood-borne TC can be determined within 48 hrs of their entry into the vasculature. During the early stages of experimental metastasis there is a rapid adhesion of TC to the endothelium, and attachment of TC to the lung endothelium occurs as early as 4hrs after arrival in the microvasculature *(Al-Medhi et al 2000).* Classical studies suggested that increased adhesion of TC to the pulmonary vasculature would facilitate extravasation, and promote metastasis. In addition, more recent work suggests that during attachment or adhesion to the endothelium, TC may receive critical signals that influence survival *(Al-Medhi et al 2000, Wong et al 2002).*

More than 90% of blood-borne TC are lost and cleared from the circulation within 24hrs of injection *(Fidler et al 1970, 1976 1977, 1978, Luzzi et*

al 1998). In addition, Wong et al showed that apoptosis of TC occurs within 24hrs after IV injection. On the other hand, others have shown that surviving TC can begin to proliferate within 24hrs in the vasculature, before they have extravasated to the surrounding tissue *(Crissman et al 1988; Wong et al 2002).* Thus, it is clear that the metastasis of blood-borne TC is dependent on their response to stimuli that may induce proliferation or cell death.

Protein kinase C delta has been shown to exert varying effects on cell growth and apoptosis. It has a negative effect on proliferation of several cell types including CHO cells, NIH3T3 fibroblasts, smooth muscle cells, capillary endothelial cells, glial cells, and B16BL6 melanoma cells *(Watanbe et al 1992, Mischak et al 1993, Acs et al 2000, Kang et al ,Fukumoto et al 1997, Harrington et al 1997, Brodie et al 1998, Kronfield et al 2000, LaPorta et al 2001).* PKC delta has also been shown to arrest cell growth *(Perletti et al 1999, Watanbe et al 1992, Fukomoto et al 1997, Griffiths et al 1996, Li et al 1999)* and differentiation of keratinocytes (Szallasi et al 1994) and to induce apoptosis in response to various stimuli in keratinocytes and salivary gland acinar cells *(Reyland 1999).*

To date, little is known about how PKC delta affects the survival of B16F1 melanoma cells under adverse conditions. In chapter 1, we have shown that over-expression of wild-type delta increases the number and size of metastatic pulmonary nodules, the number and size of loci containing blood borne TC retained in the lung during the early stages (48hrs) of metastasis, and the adhesion of TC to matrigel. These effects were dependent upon the presence of

tyrosine 155. The above data is consistent with the hypothesis that over-expression of delta may increase nodule frequency by increasing adhesion of TC to the endothelium, and suggests that a delta-mediated regulation of cell proliferation and/or cell death might account for increased nodule size and/or nodule numbers. In the present in vitro study, we examine how over-expression of wild-type and mutant Y155F delta effects: 1) TC proliferation and viability under conditions of cell crowding and limiting serum concentrations and, 2) TC survival in response to cytotoxic agents.

Methods and Materials

Constructs:

Description of constructs are as described in Chapter 1.

B16F1 melanoma cells were transfected with either wild-type delta (Dtag), delta containing a substitution of phenylalanine for tyrosine 155 (Y155F), and control cells (mock) transfected with an empty vector. All transfected genes contain a 12 amino acid epsilon tag on the C-terminus of the PKC delta gene.

Cell culture

All cell lines were grown in MEMα media (Gibco BRL -Life Technologies), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100ug/ml streptomycin, 0.250ug/ml Amphotericin and 200ug/ml Geneticin (Gibco BRL-Life Technologies) at 37^0C in humidified incubator with 5% CO^2. Cells were detached by trypsinization. Cell numbers were determined with a hemocytometer and the Trypan blue exclusion test.

Proliferation Assay

Cells were cultured as described above using either a 'low' concentration (10^4 cells /ml) or a 'high' concentration ($5x10^4$ cells), plated in triplicate in the presence of 0%, 0.1%, 1% or 10% FBS, and incubated for 24, 48, or 72 hrs. Cells were then trypsinized and the total number of cells present in each dish determined using a hemocytometer and the Trypan blue exclusion test. Statistical significance was determined with a Wilcoxon T test.

Cell Death Assay

$5x10^4$ /ml cells were plated in 24 well micro-titer in complete media. Experimental cells were treated with either 100ug/ml cycloheximide, 20umM camptothecin or 2.5% ethanol in media containing 1% serum for 20hrs or 48hrs. Control cells were incubated in media lacking cytotoxic drugs. Cells were then trypsinzed and numbers of viable and dead cells were determined by subtracting the percent cell death of the controls from the percent cell death of drug treated cells, where the % cell death = % of drug induced cell death - %cell death of control.

Results:

TC survival in response to cytotoxic agents

The retention study from Chapter 1 (figure 4a & 4c), illustrated that over-expression of wild-type delta increases the number and size of viable tumor cells (TC)/ clumps in the lung of C57/Bl6 mice 48 hrs after intravenous inoculation, and suggested a possible effect of delta on TC survival in the circulation or in the lung parenchyma. To test whether delta is capable of affecting cell survival, TC were treated with the potent cell death inducers: cycloheximide, camptothecin or ethanol for 20 or 48hrs.

Over-expression of Y155 delta confers protection against cell death induced by cycloheximide, camptothecin, and ethanol

Cycloheximide:

Studies have shown that cycloheximide (CHX) can either promote or inhibit apoptosis in different cell types, in response to various death stimuli, by inhibiting protein synthesis *(Pap et al 2002)*. We therefore sought to determine the effect of cycloheximide on B16F1 melanoma cells. Transfected B16F1 cells were exposed to 100ug/ml of cyclocheximide for 20hrs and 48hrs. Results show that cycloheximide causes cell death in control cells, and that over-expression of wild-type delta offered little or no protective effect against cell death after 20 hrs of exposure to cycloheximide, and only a slight protective effect by 48hrs when compared to mock and Y155F (Figure 1). In contrast, cells that over-express Y155F had markedly less cell death than mock at 20 and 48 hrs, and were even more resistant to the cytotoxic affects of cycloheximide than cells transfected with wild-type delta.

Figure 1: Cell death induction by cycloheximide

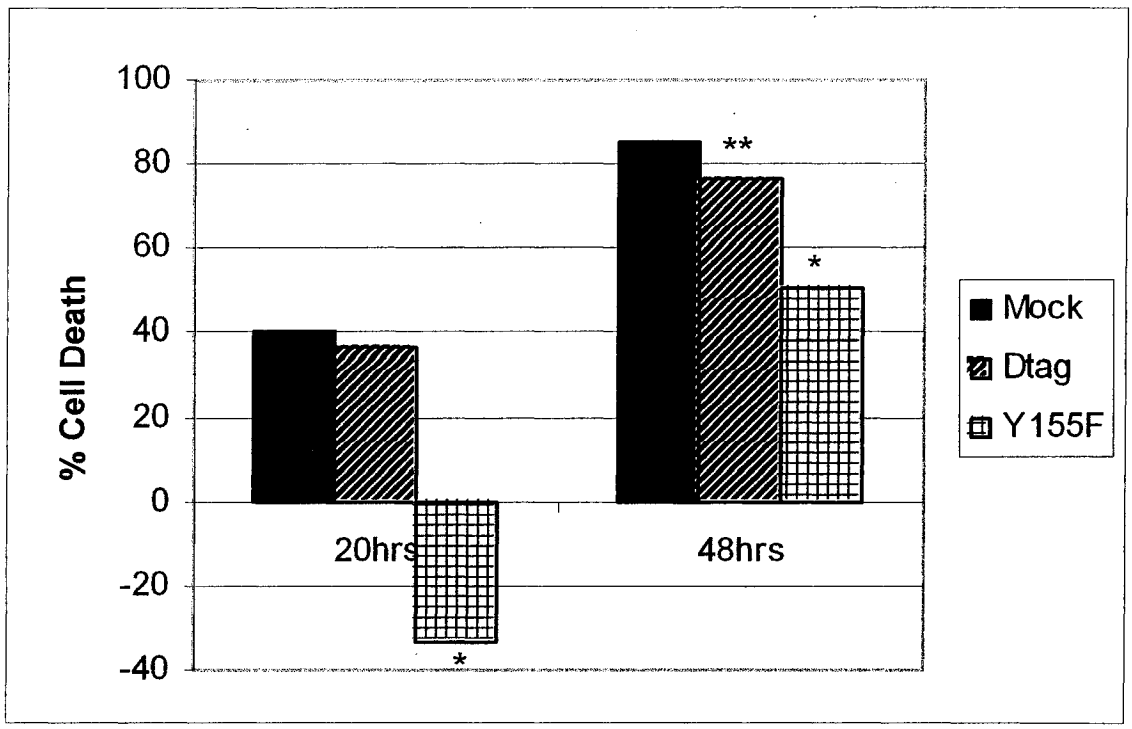

Figure 1 Legend:

20hrs: *denotes that Y155Fhas less cell death than mock (P=0.001) and Dtag (P=0.001)

48hrs: *denotes that Y155F has less cell death than mock (P=0.001) and Dtag (P=0.001); ** Dtag has less cell death than mock (P=0.001)

Camptothecin:

Camptothecin (CPT) is a potent anti-cancer agent which can induce apoptosis by inhibiting topoisomerase I *(Cazuwara-Ladykowska et al 2001, Pondarre et al 1997).* Camptothecin had a slight or modest effect on cell death of control cells after 20 and 48 hrs, respectively (Figure 2). Even after 48hrs of exposure to 20uM campothecin, we observed that over-expression of wild-type delta had little or no significant affect upon cell death. However, at both 20 and 48hrs, over-expression of Y155F markedly decreased cell death when compared to mock or Dtag, P=0.001, (Figure 2).

Figure 2: Cell death induction by camptothecin

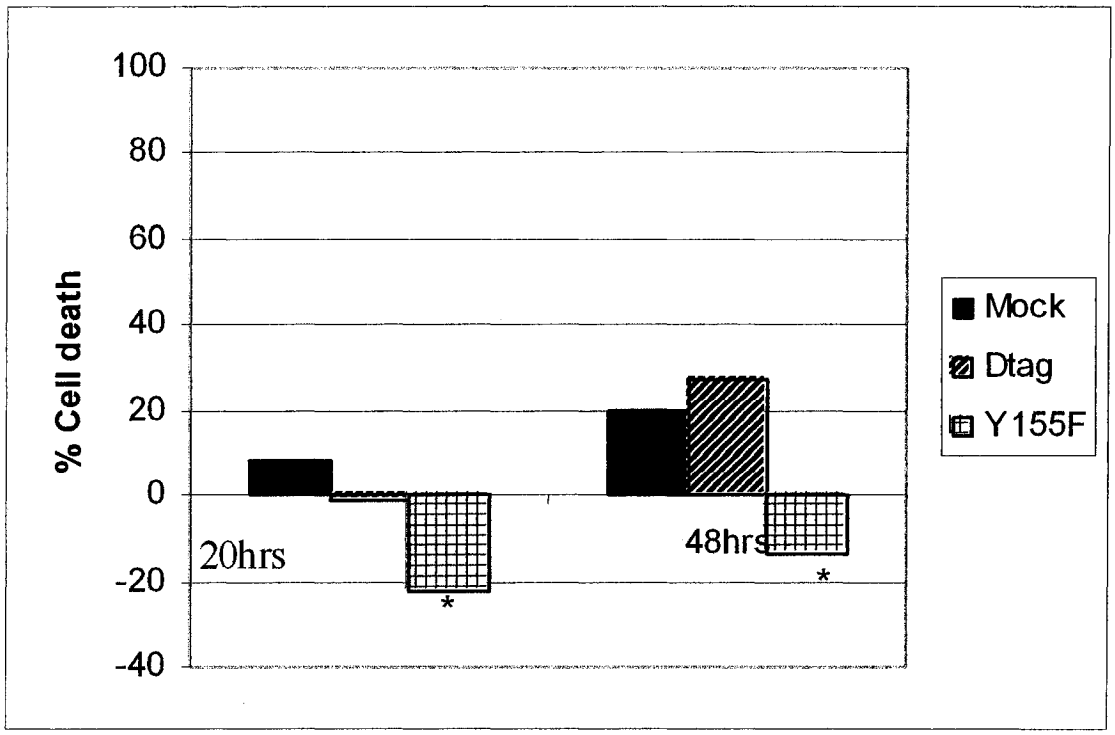

Figure 2 Legend:

20hrs: * denotes that Y155F has less cell death than mock (P=0.001) and Dtag (P=0.001).

48hrs: *Y155F has less cell death than mock (P=0.001) and Dtag (P=0.001).

Ethanol:

Studies have shown that ethanol can induce cell death in different cell lines, in various ways *(Chen et al 1996, Luo et al 1997, 1998, Castaneda et al 2000).* We sought to examine the effects of ethanol on the B16F1 cells that had been exposed to 2.5% ethanol for 20 and 48hrs. Figure 3 illustrates that ethanol has a modest effect on cell death of mock transfected cells, and over-expression of wild-type delta potentiates an early cell death in response to ethanol. In contrast, transfection with Y155F delta offered significant protection at both early and later time periods compared with mock or Dtag cells (Figure 3).

Figure 3: Cell death induction by ethanol

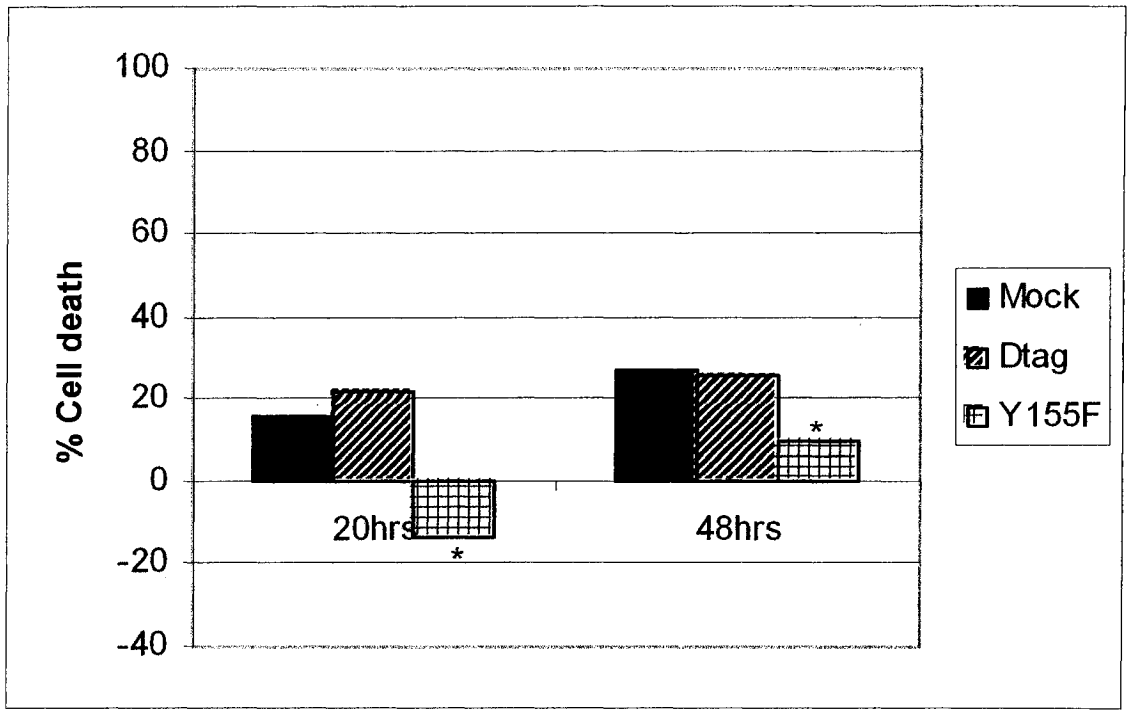

Figure 3 Legend:

20hrs: *denotes Y155F has less cell death than mock (P=0.002) and Dtag (P=0.001); **denotes that Dtag has more cell death than mock (P=0.004)

48hrs : *denotes that Y155F has less cell death than mock (P=0.008) and Dtag (P=0.006).

Proliferation and viability of "low" concentrations of cells (10^4 cells/ml) in the presence of varying serum concentrations

During metastasis tumor cells are exposed to varying degrees of cell crowding, as well as varying concentrations of growth-promoting factors including those present in serum. In order to determine whether over-expression of PKC delta may be capable of affecting cell proliferation or survival during metastasis, these processes were examined in vitro using serum concentrations varying from 0 to 10 % and either a low or a higher concentration of TC, (10^4 or 5X 10^4cells/ml, respectively).

Results:

TC viability decreases in the absence of serum (0%), and the decrease is greatest for Y155F cells

To determine if the presence of serum plays a critical role in the growth and survival of B16F1 cells, cells were grown without serum for to up to 72hrs. Examination of proliferation and viability data using low cell concentrations shows that in the absence of serum, little or no proliferation occurs in any of the cell lines, and in all cell lines viabilities decreased with time, (Figure 4A and 4B), demonstrating a requirement of serum for survival. Y155 cells showed the greatest dependence on serum for survival.

Figure 4A: Proliferation in the absence of serum (0% serum)

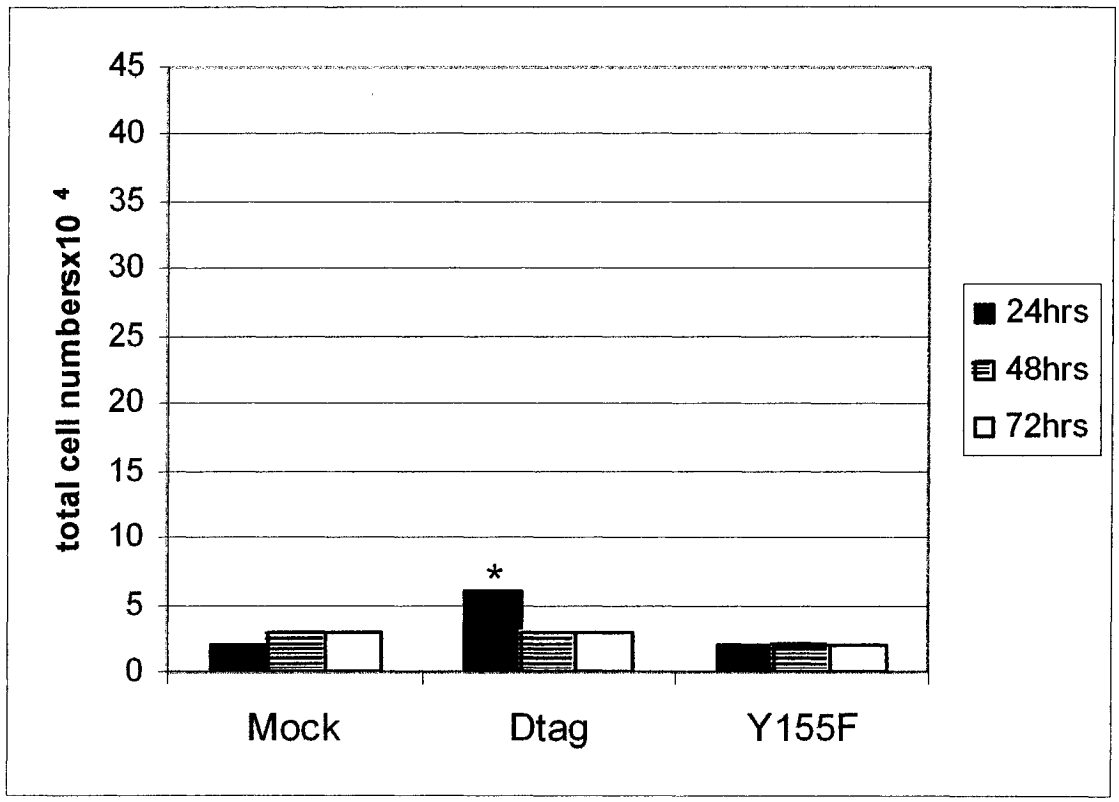

Figure 4A Legend:

*denotes that more Dtag cells are present at 24hrs than at 48hrs, or 72hrs

Figure 4B: Viability in the absence of serum (0% serum)

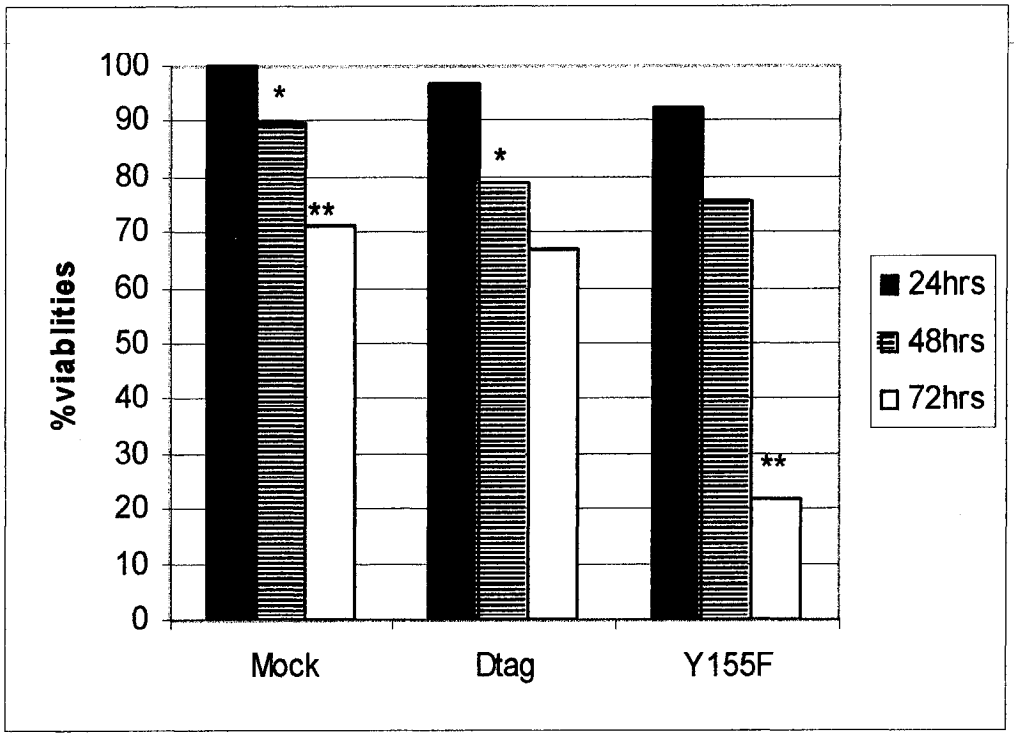

Figure 4B Legend:

*denotes that at 48hrs cells are less viable than at 24hrs Mock (P=0.002) and Dtag (P=0.001).

**denotes that at 72hrs cells are less viable than at 48hrs Mock (P=0.002) and Y155F (P=0.002)

Over-expression of wild-type delta potentiates proliferation of B16F1 cells in the presence of low serum (0.1%)

In Figure 4a and 4b, we showed that B16F1 cells fail to grow or survive in the absence of serum. We next sought to determine if the presence of very low serum levels (0.1 %) would be sufficient to induce growth and maintain cell viability. Results show that at all time intervals mock and Y155F cells showed little or no proliferation. In contrast, cells over-expressing wild-type delta showed an increased proliferation at 48 hrs at which time viabilities remained high and were comparable in all cell lines. (Figure 5A and 5B). Results demonstrate that for up to 48 hours, 0.1% serum can maintain high viability in all cell lines, and increased proliferation of Dtag cells, but not of Y155F cells. This suggests that an intact tyrosine 155 is necessary for the proliferation effect.

Figure 5A: Proliferation in 0.1% serum

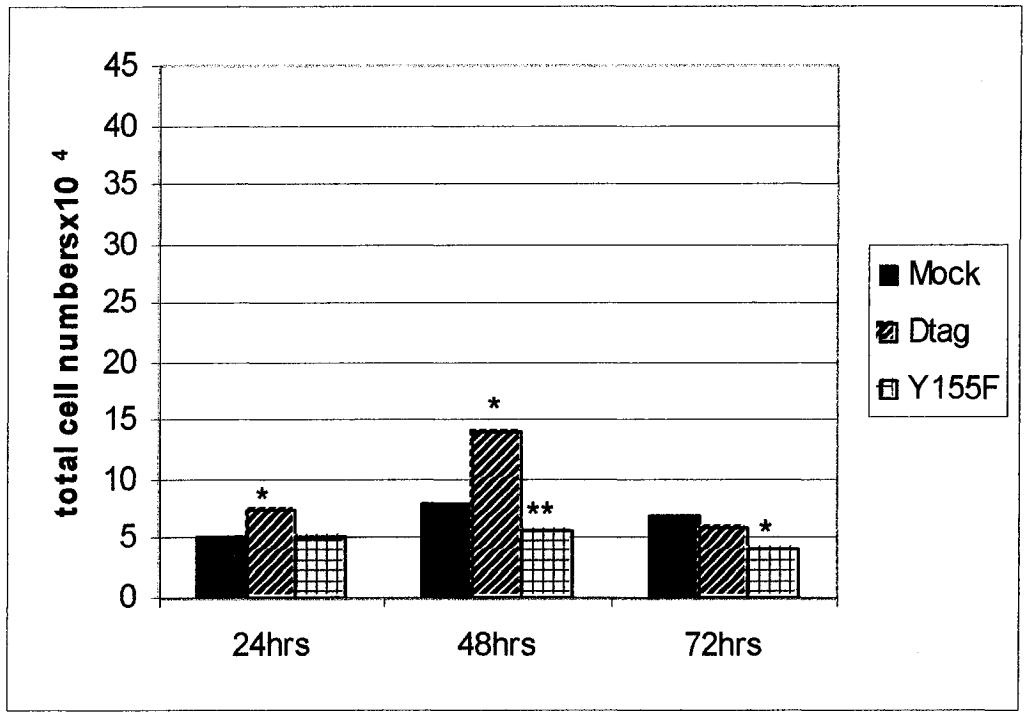

Figure 5A Legend:

24hrs:*denotes that Dtag proliferates more than Mock (P=0.001) and Y155F (P=0.001)

48hrs: *denotes that Dtag proliferates more than mock (P=0.008) and Y155F (P=0.001).**denotes that Y155F proliferates less than Mock (P=0.011).

72hrs:*denotes that Y155F proliferates less than Mock (P=0.003) and Dtag (P=0.001).

Figure 5B: Viability in 0.1% serum

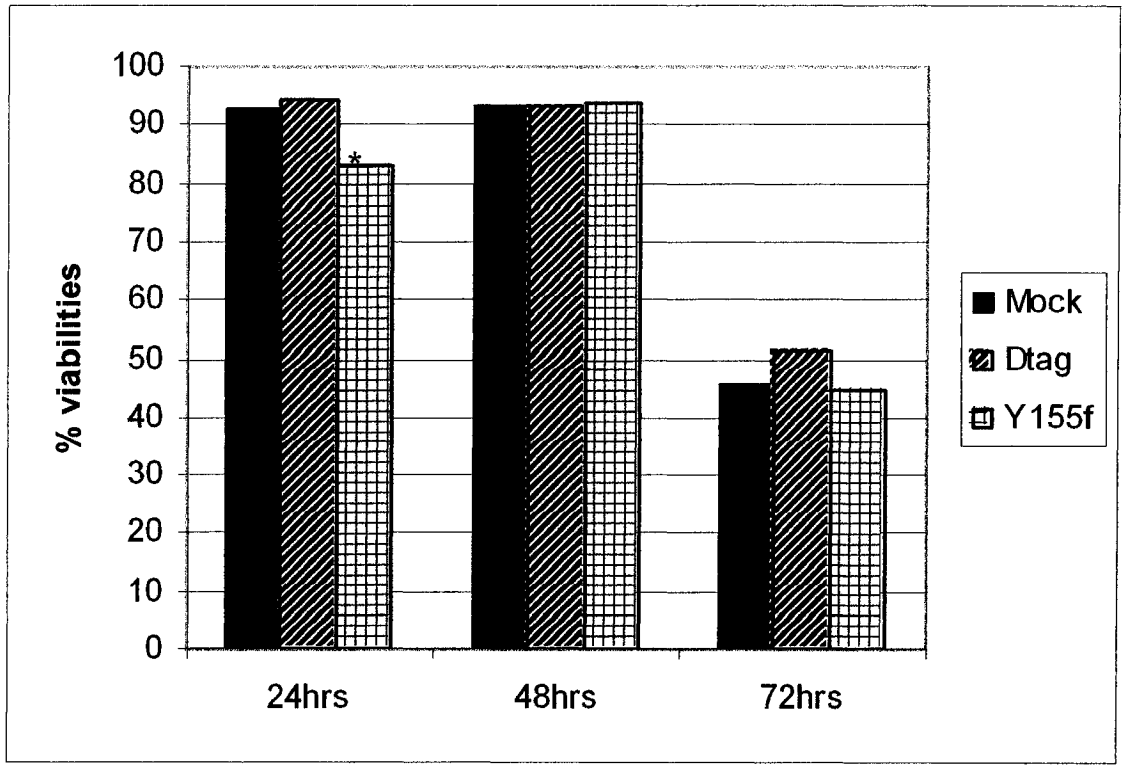

Figure 5B Legend:

24hrs: * denotes that Y155F is less viable than mock and dtag

Intact tyrosine 155 delta promotes cell proliferation and cell survivability in the presence of 1% serum

Examination of cell growth and viability in the presence of 1% serum showed that at all time intervals proliferation was greatest with cells over-expressing wild-type delta. By 72 hours, proliferation of Y155 cells was less than that observed in the control (mock) group (figure 6a). Survival of cells transfected with wild-type delta was the same as in the control group, while survival was lowest for cells transfected with Y155F delta (figure 6b). As with the experiments in 0.1% serum, these results demonstrate that the ability of cells over-expressing wild-type PKC delta to stimulate proliferation when compared with mock, is independent of an effect on survival. Results also demonstrate that tyrosine 155 is necessary for sustained cell survival.

Figure 6A: Cell proliferation in 1% serum

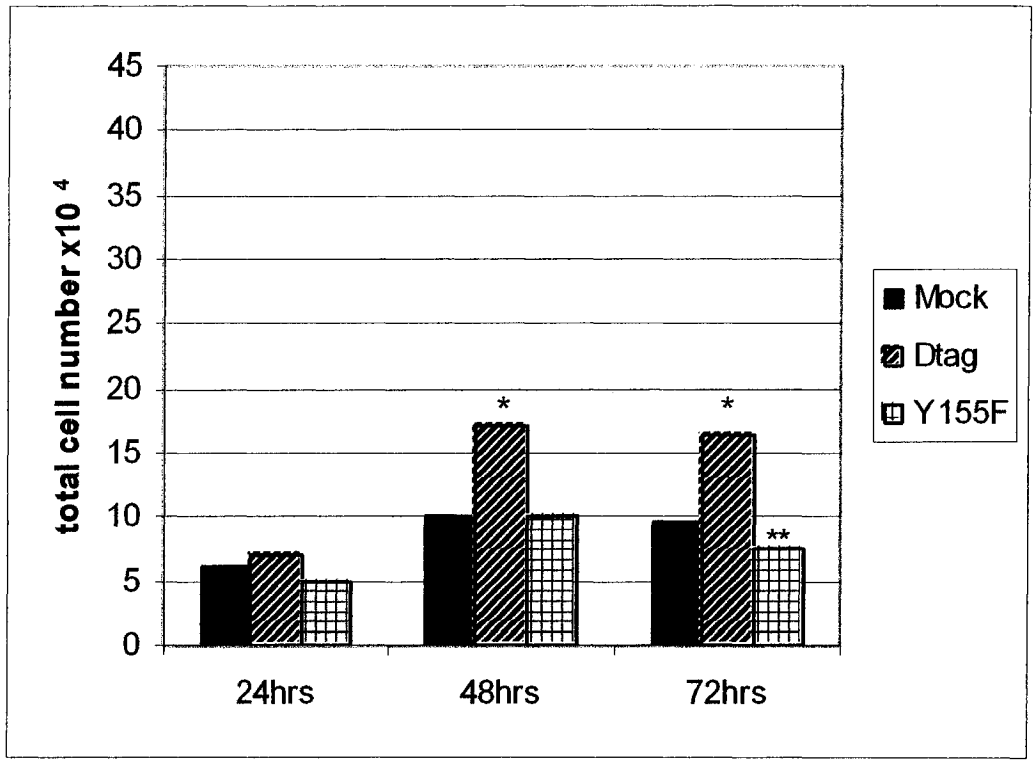

Figure 6A Legend:

48hrs:*denotes that Dtag proliferates more than Mock (P=0.001) and Y155F (P=0.001)

72hrs:*denotes that Dtag proliferates more than Mock (P=0.001) and Y155F (P=0.001). **denotes that Y155F proliferates less than Mock (P=0.005).

Figure 6B: Cell viability in 1% serum

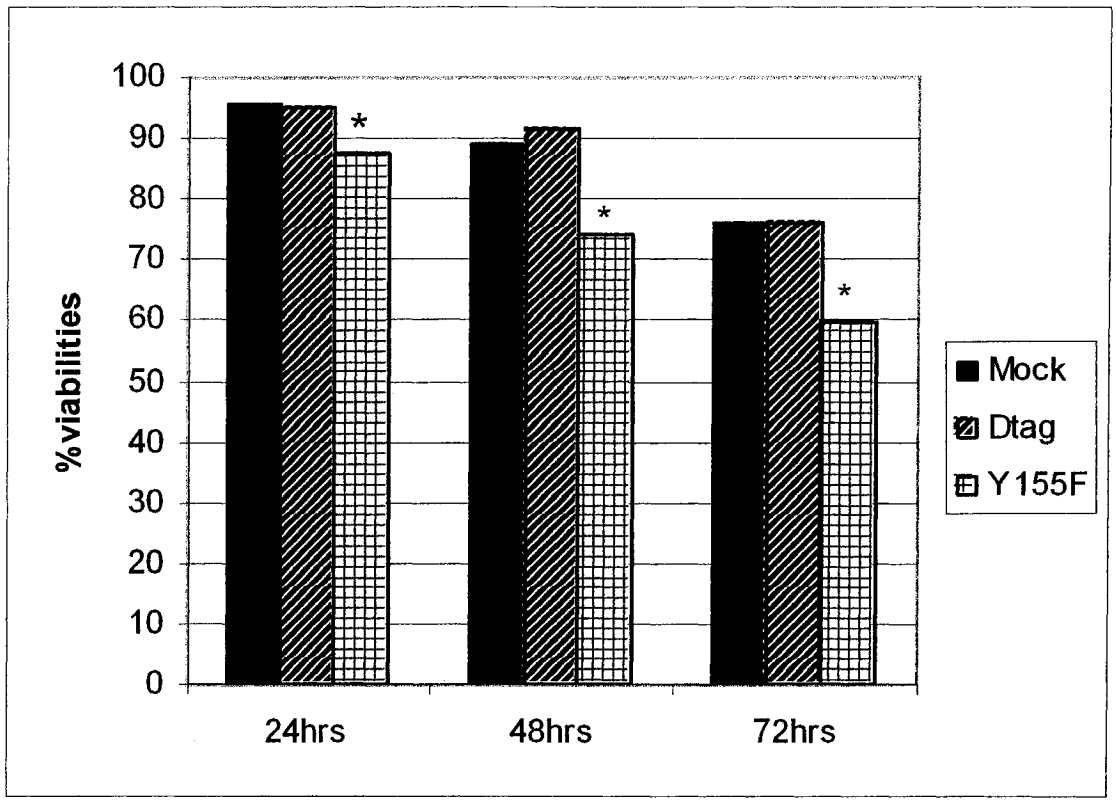

Figure 6B Legend:

24hrs: *denotes that Y155F proliferates less than Mock (P=0.001) and Dtag (P=0.001)

48hrs: *denotes that Y155F proliferates less than Mock (P=0.001) and Dtag (P=0.001)

72hrs: *denotes that Y155F proliferates less than Mock (P=0.003 and Dtag (P=0.001)

High serum (10%) markedly increases proliferation and viability of all B16F1 cell lines but transfected tyrosine 155 is required for maximal proliferation

To determine the effects of high serum levels on cell proliferation and viability, cells were grown in the presence of high serum (10%) for up to 72hrs. Results show that at all time intervals over-expression of wild-type delta markedly increased proliferation when compared with mock or Y155F cells, and that proliferation of Y155F cells was similar to that observed for mock. Similar and high viabilities were observed in all groups at all time intervals demonstrating a stimulatory effect of over-expressed wild-type delta on proliferation compared with mock and Y155F, and an inhibitory effect of Y155F on proliferation when compared with Dtag that is independent of cell viability (Figure 7a and 7b). This data also shows that the decreased viability often observed with Y155F over-expression can be overcome with high serum concentrations.

Figure 7A: Proliferation in 10% serum

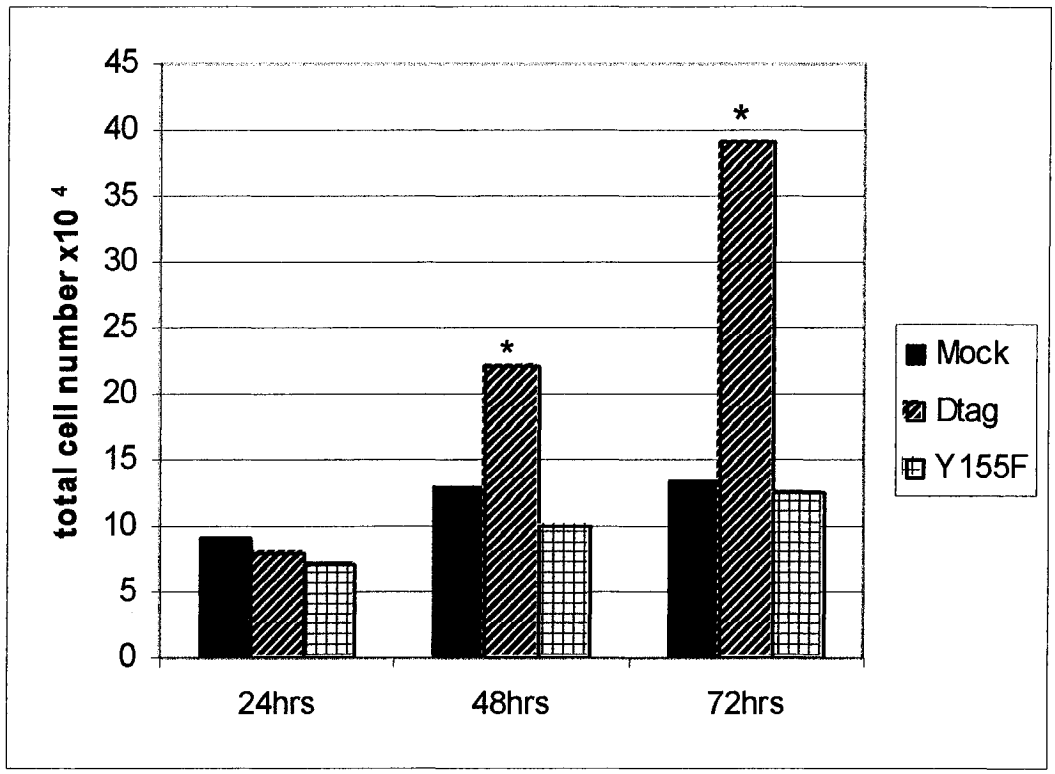

Figure 7A Legend:

48hrs:*denotes that Dtag proliferates more than Mock (P=0.001) and Y155F (P=0.001)

72hrs:*denotes that Dtag proliferates more than Mock (P=0.001) and Y155F (P=0.001).

Figure 7B: Viability in 10% serum

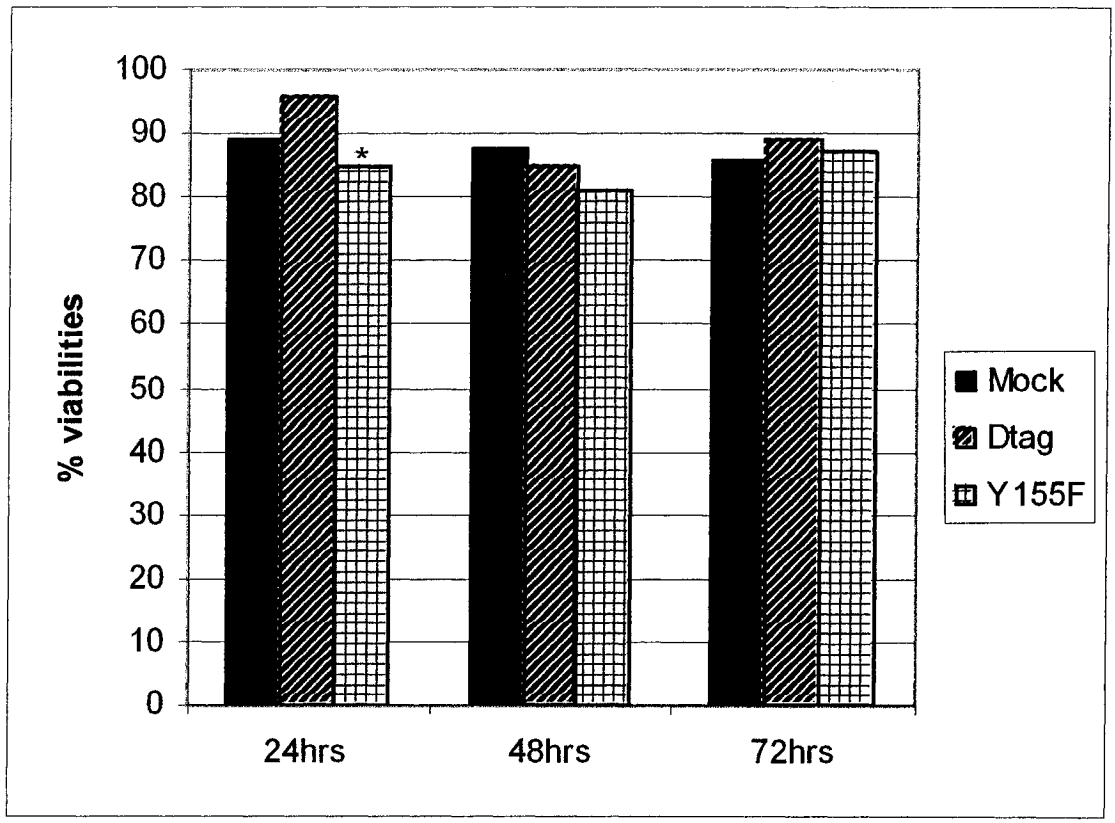

Figure 7B Legend:

24hrs:*denotes that Y155F is less viable than Dtag (P=0.002)

Proliferation and viability of cells grown at "high" cell concentrations ($5X10^4$ cells/ml) with varying serum concentrations

In this series of experiments we sought to examine the effects of delta over-expression on cell proliferation and survival under conditions of increased cell crowding (i.e., $5x10^4$ cells/ml)

Proliferation and survival of cells grown under conditions of increased crowding requires serum

Figure 4a and 4b had shown that at least a low concentration of serum is necessary for proliferation and survival of low concentrations of all cell lines. Figure 8 shows that similar results occur with greater cell crowding; little or no proliferation occurs in any cell line in the absence of serum, and a generally comparable decrease in cell survival occurs in all cell lines with time.

Figure 8A: Proliferation in the absence of serum (0% serum)

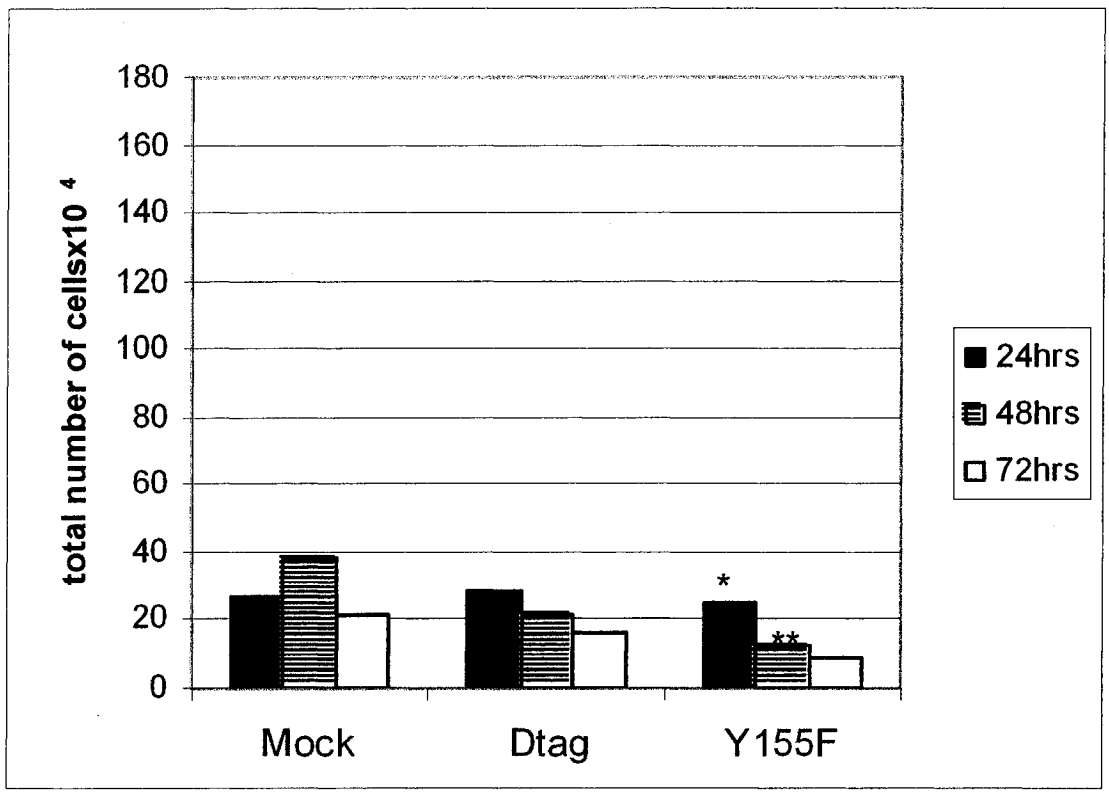

Figure 8A Legend:

Y155F: *denotes that more cells are present at 24hrs than at 48hrs (P=0.002).

**denotes more cells present at 48hrs than at 72hrs (P=0.004)

Figure 8B: Viability in the absence of serum (0% serum)

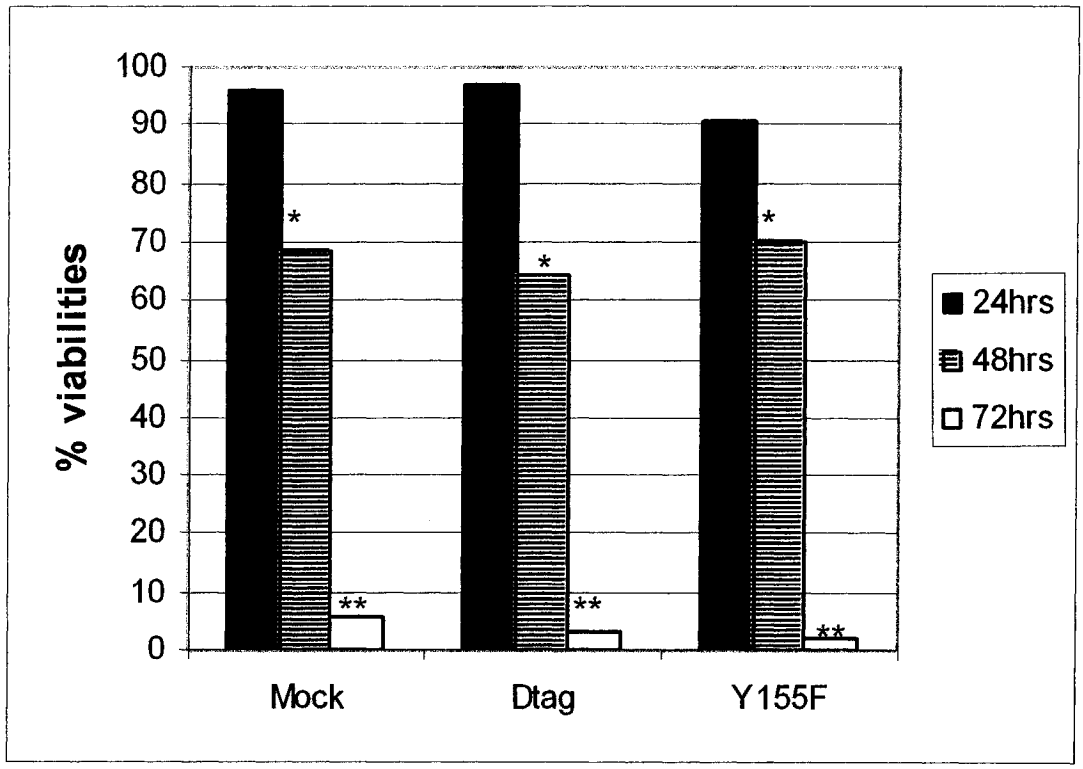

Figure 8B Legend:

*denotes that at 48hrs cells are less viable than at 24hrs Mock (P=0.001, Y155F (P=0.001) and Dtag (P=0.001)

** denotes that at 72hrs cells are less viable than at 48hrs Mock (P=0.001), Y155F (P=0.001) and Dtag (P=0.001)

Over-expression of wild-type delta increases proliferation in 0.1%serum

Using 10^4 cells, we observed that at 48 hrs, over-expression of wild-type delta potentiated cell proliferation in 0.1% serum, and viabilities of all cell lines remained high (Figure 5A). Examination of the effects of increased cell crowding on cell proliferation and viability in 0.1% serum are essentially the same as those seen using lower cell concentrations, except that viability only remained high for 24 hrs(Figure 9b).

Figure 9A: Proliferation in 0.1% serum

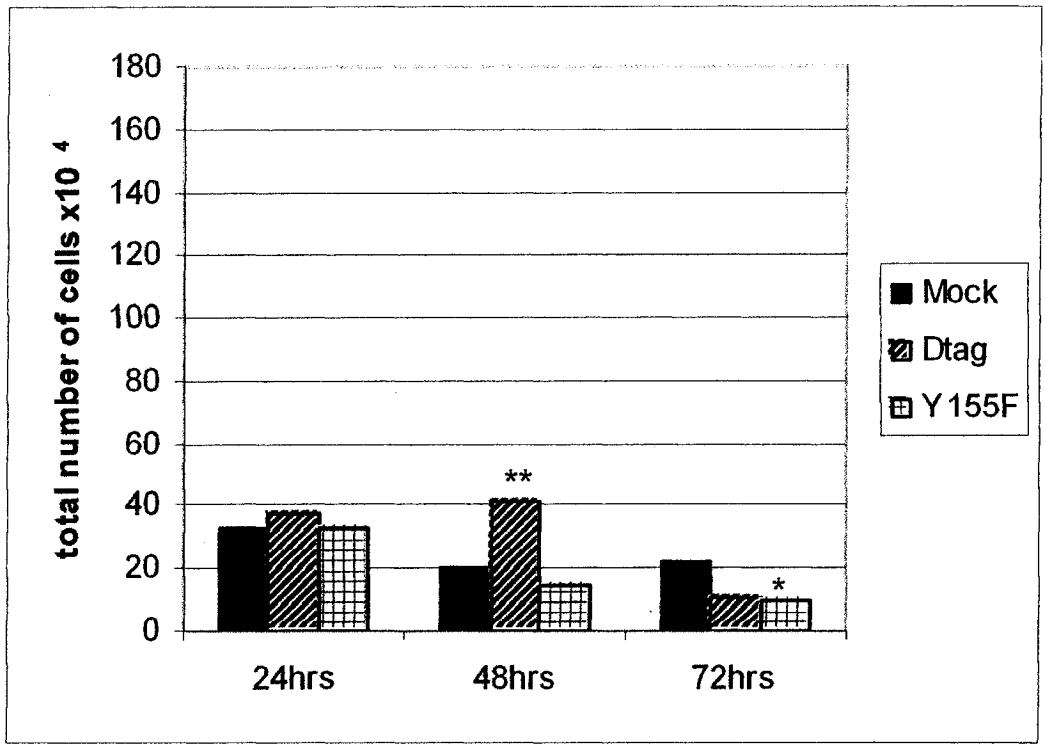

Figure 9A Legend:

48hrs: **denotes that Dtag proliferates more than mock (P=0.007) and Y155F (P=0.001)

72hrs: *denotes that Y155F proliferates less than mock (P=0.001)

Figure 9A: Viability in 0.1% serum

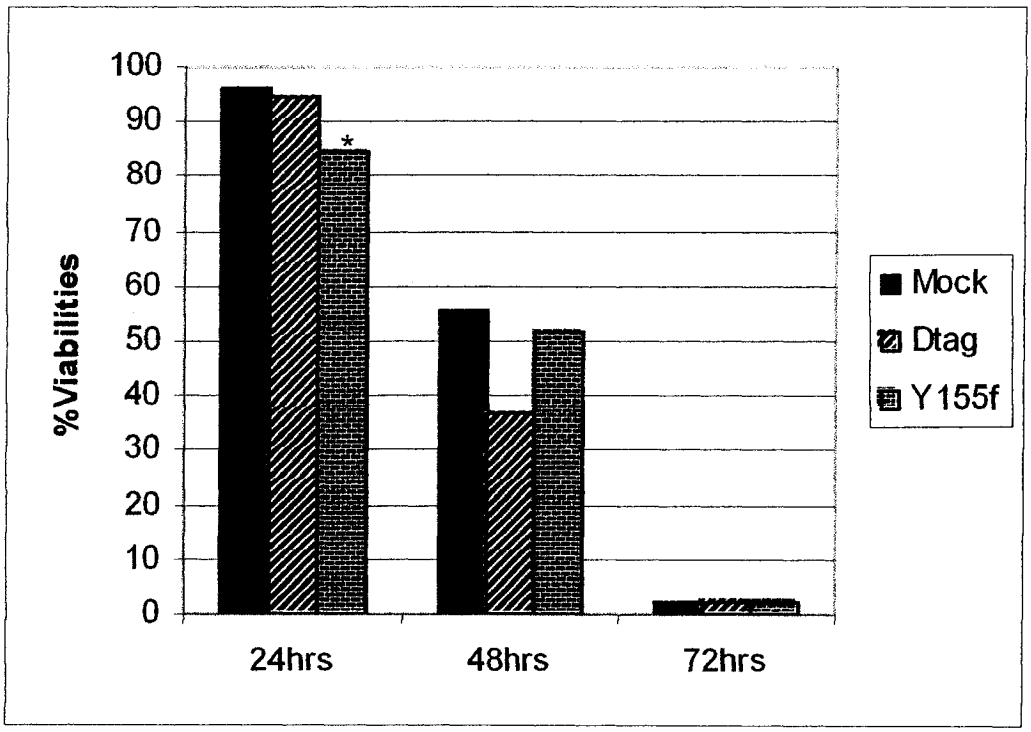

Figure 9B Legend:

24hrs: **denotes that Y155F is less viable than mock (P=0.001) and Dtag (P=0.003).

Cell crowding increases the proliferation and viability of wild-type and Y155F transfected cells in the presence of 1% serum

Using 10^4 cells/ml, we showed that in the presence of 1% serum, over-expression of wild-type delta potentiated cell growth whereas over-expression of Y155F delta resulted in decreased viability (Figure 6). In Figure 10, we examine the effect of increased cell crowding on proliferation and viability in 1% serum. Results show that proliferation was greatest for Dtag (Figure 10B) at all time intervals. At 24 and 48 hrs viability remained high in all groups although a slight but significant decrease was seen for Y155F cells. Although both wild-type and Y155F cells showed increased proliferation by 72 hrs compared with mock (Figure 10A) proliferation was still greatest for Dtag.

Figure 10A: Proliferation in 1% serum

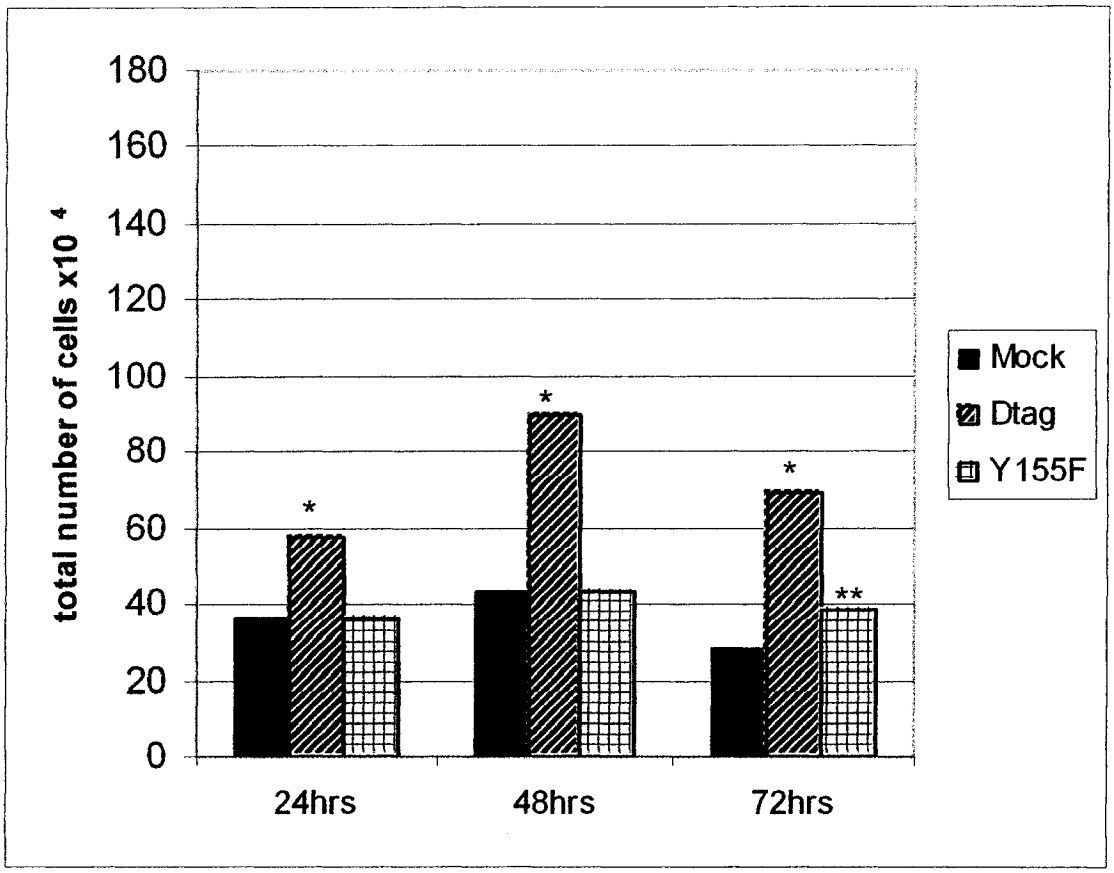

Figure 10A Legend:

24hrs:*denotes that Dtag proliferates more than mock (P=0.001) and Y155F (P= 0.001).

48hrs: *denotes that Dtag proliferates more than mock (P=0.001) and y155F (P=0.001)

72hrs:*denotes that Dtag proliferates more than mock (P=0.001) and Y155F (P=0.001). ** denotes that Y155F proliferates more than mock (P=0.001).

Figure 10B: Viability in 1% serum

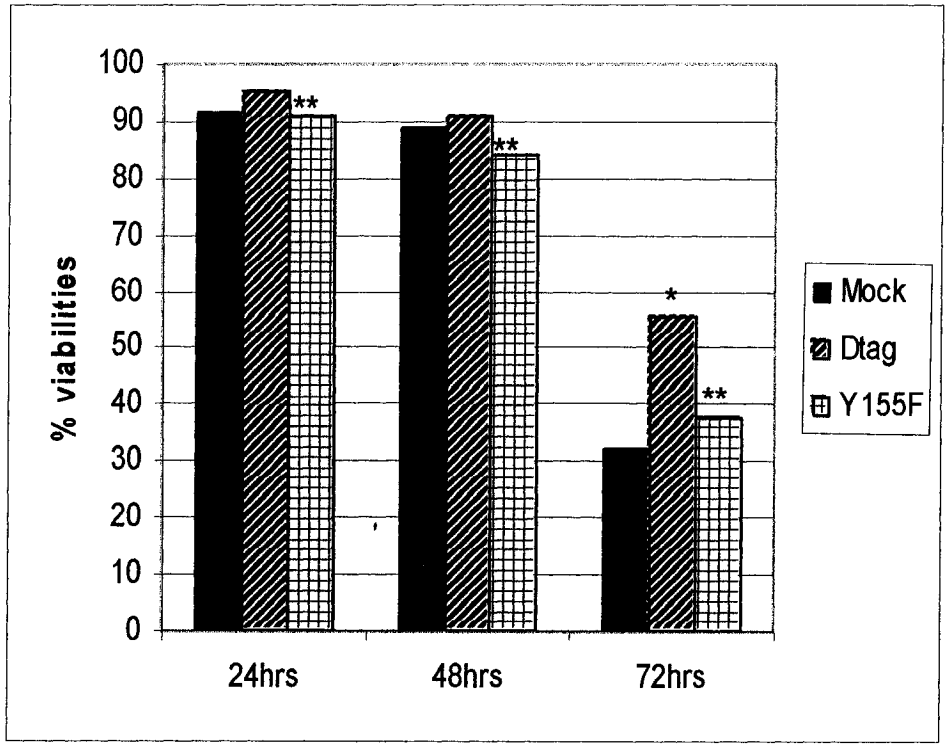

Figure 10B Legend:

24hrs: **denotes that Y155F is less viable than Dtag (P=0.001).

48hrs: **denotes that Y155F is less viable than mock (P=0.002) and Dtag (0.001).

72hrs: *denotes that Dtag is more viable than mock (P=0.001). **denotes that Y155F is more viable than mock (P=0.007) and less viable than Dtag (P=0.001)

Cell crowding markedly increases proliferation and viabilities of cells over-expressing wild-type and Y155F PKC delta in 10% serum

Using 10^4 cells we show that 10% serum increases the proliferation of Dtag cells (figures 7A and 7B), and that cell viabilities remain high and comparable at all time intervals. We now examine the effects of increased cell crowding on cell proliferation and viability at this high serum concentration. Figure 11 A shows that at 48 and 72 hrs, proliferation increased in all cell lines. Although proliferation was greatest for Dtag, the proliferation of Y155F cells was significantly greater than observed for mock. Although slight differences were observed for viability, it remained greater than 85% for all cell lines (Figure 11B).

Figure 11A: Proliferation in 10% serum

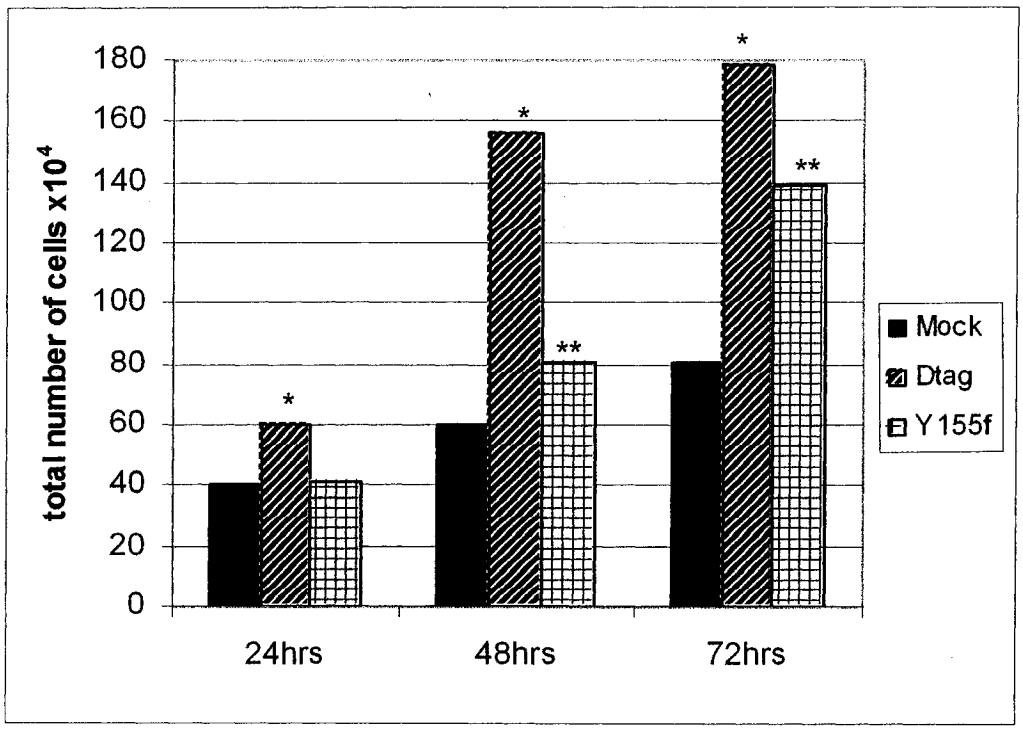

Figure 11A Legend:

24hrs: *denotes that Dtag proliferates more than mock (P=0.001) and Y155F (0.001).

48hrs:*denotes that Dtag proliferates more than mock (P=0.001) and Y155F (P=0.001). **Y155F proliferates more than mock (P=0.011).

72hrs:*denotes that Dtag proliferates more than mock (P=0.001) and Y155F (P=0.001). **denotes that Y155F proliferates more than mock (P=0.001).

Figure 11B: Viability in 10% serum

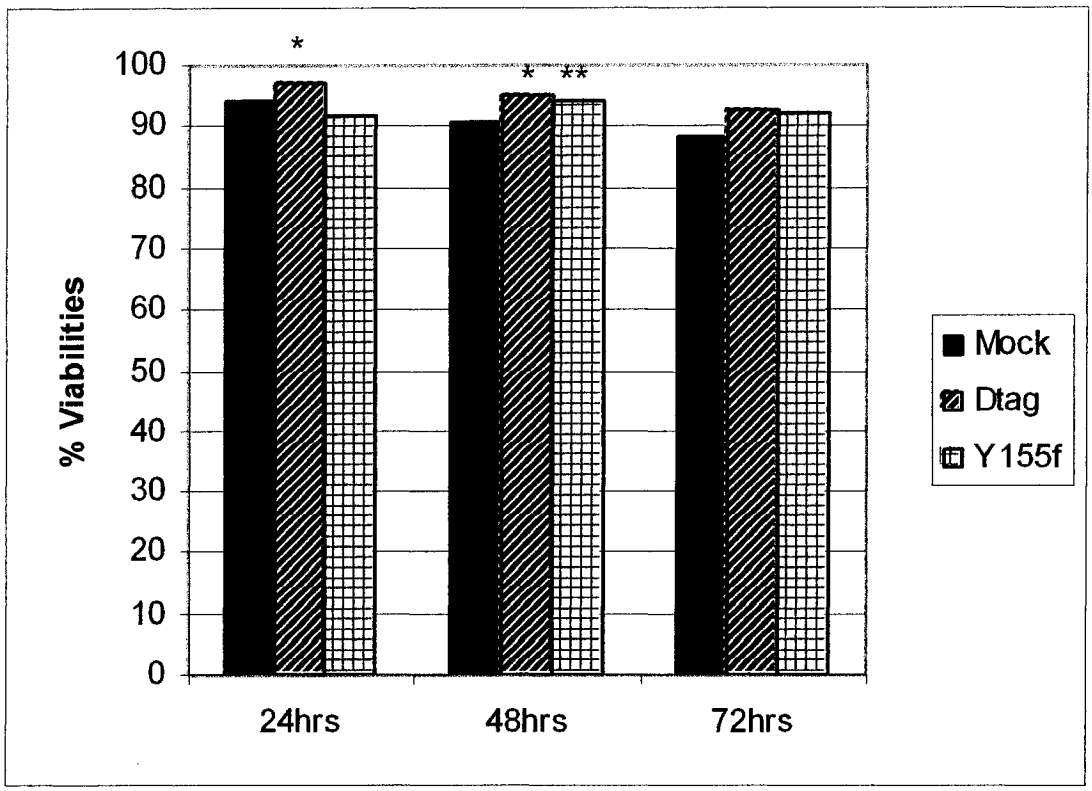

Figure 11B Legend:

24hrs: *denotes that Dtag is more viable than mock (P=0.003) and Y155F (P=0.001).

48hrs: *denotes that Dtag is more viable than mock (P=0.001). **denotes that Y155F is more viable than mock (P=0.002).

Discussion:

The pathogenesis of metastasis includes several complex steps that a TC must complete to successfully establish metastatic colonies. Tumor cells must overcome host immune cell killing while carrying on critical functions such as proliferation, invasion, migration, and the induction of angiogenesis. During metastasis, the rate of proliferation verses cell death is a critical factor in TC survival. Several studies have shown that PKC delta can regulate proliferation and/or apoptosis of various cell lines *(Watanbe et al 1992, Mischak et al 1993, Acs et al 2000, Kang et al ,Fukumoto et al 1997, Harrington et al 1997, Brodie et al 1998, Kronfield et al 2000, LaPorta et al 2001,Reyland 1999, Emoto et al 1995, Denning et al 1998, Fujii et al 2000, Konishi et al 1999 Shao et al 1997*). In this chapter, we examined the effects of delta over-expression on TC proliferation and viability under conditions of cell crowding and limiting serum concentrations, as well as TC survival in response to cytotoxic agents.

In the cytotoxic studies, cells were treated with cycloheximide, camptothecin or ethanol. All three cytotoxic agents are potent cell death inducers that have been shown to affect different pathways or steps involved in apoptosis. Cycloheximide (CHX) can either promote or inhibit apoptosis in different cell types in response to various death stimuli by inhibiting protein synthesis *(Pap et al 2002)*. It can contribute to apoptotic processes either in conjunction with another agent (e.g. tumor necrosis factor-alpha) or on its own, and primarily involves a FADD-dependent mechanism *(Tang et al 1999)*. Camptothecin (CPT) is a potent anti-cancer agent which induces apoptosis by

inhibiting topoisomerase I *(Cazuwara-Ladykowska et al 2001, Pondarre et al 1997),* and ethanol can induce cell death in several ways including disordering the cell membrane *(Chen et al 1996)* or interfering with the action of growth factors *(Luo et al 1997, 1998, Castaneda et al 2000).*

The cytotoxicity studies in the present study demonstrate that all three cytotoxic agents are capable of inducing variable degrees of cell death of all B16F1 cell lines and that over-expression of delta can have varying effects on the B16F1 cell response to these cytotoxic agents. While it is not clear which cell death pathways are induced by these cytotoxic agents in B16F1 cells, the ability of all 3 agents to induce cell death in these TC suggests that PKC delta may regulate more than one cell death pathway in this cell.

Over-expression of wild-type delta offers only slight protection against cell death induced by cycloheximide, and little or no protection from camptothecin or ethanol. Importantly, over-expression of Y155F delta consistently protected these TC from induction of cell death induced by all of the cytotoxic agents examined.

Several tyrosine phosphorylation sites have been identified for PKC delta, in both the regulatory and catalytic domains *(Brodie et al 1998).* Phosphorylation of these sites has been shown to have varying affects on delta activity and function *(Kronfield et al 2000).* Tyrosine phosphorylation of delta has been shown to be essential for induction of apoptosis in response to etoposide. Tyrosine 155 has been shown to be an in-vivo phosphorylation site for the Src kinase Lyn, and

has been implicated in the regulation of tumorgenicity, and anchorage independent growth in NIH3T3 fibroblasts *(Acs et al 2000).*

Interestingly, our studies show that PKC delta with a loss of an intact tyrosine 155 consistently potentiates cell survival in response to all cytotoxic agents, suggesting the importance of in vivo phosphorylation of delta tyrosine 155 in potentiation of B16F1 cell death. To our knowledge, this is the first study to demonstrate that a mutation of delta tyrosine 155 can increase cell survival in response to cytotoxic agents.

In the present study, we also examined the effects of serum concentration and cell crowding on TC proliferation and survival. In the presence of serum (0.1 – 10.0%), and regardless of cell crowding, over-expression of wild-type delta consistently increases cell proliferation compared with mock and Y155F. These results are consistent with a hypothesis that wild-type PKC delta contributes to increased metastasis by increasing TC proliferation, and suggest that phosphorylation of delta tyrosine 155 stimulates proliferation.

In general, at low serum concentrations, and using 10^4 cells/ml, over-expression of Y155 tends to decrease proliferation and/or viability compared with controls (Figures 5,6) . However, at higher serum concentrations, or with more crowded cells (Figures 7, 10, 11), the detrimental effect of the Y155 mutation is less apparent, and proliferation and viabilities are comparable to the mock controls. Furthermore, in 10% serum, the higher cell concentration of Y155F cells was actually capable of potentiating proliferation when compared with mock (Figure 11). We postulate that one or more factors present in serum, and/or

autocrine or paracrine factors may override the detrimental effect of the Y155 mutation on cell viability, and consequently on cell proliferation.

It is interesting that mutation of tyrosine 155 affects survival such that resistance to cytotoxicity is increased while viability during cell proliferation is decreased. It is noteworthy that the conditions of cell and serum concentration used during the cytotoxicity assays is the same as that shown in Fig. 10B, and that at 24 hours and 48 hours, cell viabilities are high for all cell lines, with only a small drop in viability for Y155F cells at 48 hrs, conditions that promote viability of Y155F during proliferation.

Taken together, the above results demonstrate the importance of an intact delta tyrosine 155 to the regulation of cell proliferation or viability, and suggest the importance of Y155 phosphorylation for this regulation. It is interesting though, that even in the absence of tyrosine phosphorylation (i.e., in the Y155F mutant) proliferation and viability may be increased, and may be dependent upon the presence of one or more signals found in serum and/or produced by other TC under conditions of crowding. Thus, depending upon the presence or absence of these factors, PKC delta and tyrosine 155 phosphorylation may exert differing effects on cell proliferation or viability.

Chapter 3

Localization of PKC delta in B16F1 melanoma cells

Abstract:

In chapters 1 and 2, we used B16F1 tumor cells (TC) and showed that PKC delta is capable of regulating metastasis, early retention of TC in the lung, cell adhesion, proliferation, and cell death induced by cytotoxic agents. Tyrosine 155 was shown to play an important role in delta's regulation of these activities. In various cell lines, others have shown that PKC delta can localize to many sub-cellular sites, where the isozyme is presumed to interact with specific substrates. These substrates differ with cell type, and localization of isozyme to particular sites is believed to determine isozyme function in cell. In the present study, we use immuno-cytochemistry with confocal microscopy to examine the patterns of localization of PKC delta in control cells (mock) and cells that over-express wild-type or mutant Y155F delta, in order to begin to identify specific substrates which may play a role in the delta-mediated regulation of B16F1 cell activity. Results show that in the wild-type and Y155F transfected cell lines, PKC delta localizes to several cellular regions including: 1) a thin detergent soluble rim around the cell periphery, 2) detergent resistant cytoplasmic granular structures of varying sizes and 3) detergent resistant particles in the perinuclear region and within the nucleus. Most of the granular cytoplasmic delta co-localized to mitochondria, with no obvious differences in localization of wild-type or Y155 delta. However, we found that Y155F cells showed marked localization of delta to the nucleus, and often exhibited striking alterations of nuclear morphology including large complex multi-lobed nuclei associated with ring-like arrangements of delta-rich particles. These observations suggest that in the absence of

phosphorylation of tyrosine 155, PKC delta localizes to nuclear structures and results in an alteration of nuclear structure and possibly nuclear function.

Introduction:

PKC is a major intracellular receptor for DAG and tumor promoting phorbol esters such as TPA. PKC activation causes phosphorylation of cytoplasmic and nuclear substrates on serine and threonine residues, and typically causes translocation of the enzyme from the cytosol to the membrane and/or *to* various detergent resistant regions of the cell. The localization of specific isozymes to particular substrates differs in different cell types, and is believed to determine isozyme function in a particular cell (*Rotenberg & Weinstein 1991, Goodnight et al 1995; Li et al 1999, Goodnight et al 1995; Li et al 1994, Mochly-Rosen et al 1998;, Kiley et al 1999, Kronfeld et al 2000)*. Thus, identification of specific cellular sites containing isozyme could provide important information concerning isoform function in that cell.

PKC has been shown to regulate the morphology and function of the B16 cytoskeleton (*Timar et al 1992, 1993),* and delta has been shown to localize to, and/or phosphorylate, several cytoskeletal proteins including adducin, in a mammary adenocarcinoma cell line *(Kiley et al 1999)*, vimentin in a murine melanoma cell line *(Szalay et al 2001)*, HL60 cells *(Owen et al 1996),* and focal adhesion molecules in Swiss 3T3 cells *(Barry et al 1994).* Other studies have shown that in un-stimulated cells PKC delta localizes to the cytoplasm in U937 leukemia cells and mammary adenocarcinoma cells (*Goodnight et al 1995, Majumder et al 2000 Kiley et al 1999)* and to the cytoplasm and Golgi in fibroblasts *(Wang et al 1999, Perego et al 2002)*. However, treatment of these cells with TPA results in translocation of delta to other cellular compartments

such as the cell processes, cell periphery, and/or to the mitochondria *(Goodnight et al 1995, Majumder et al 2000).*

Localization of PKC delta may be dependent on the cell cycle (*Perego et al, 2002).* In unstimulated or starved B16L cells, delta localized to the cytoplasm and to the perinuclear region during G_0. During S phase, delta shifted to the nuclear matrix and nucleoli, and in the G2M phase, delta was associated with the golgi (*Perego et al 2002).* Over expression of delta causes arrest in the G2M phase of the cell cycle in CHO cells *(Watanbe et al 1992)*

In previous chapters we showed that over-expression of wild-type delta increases metastasis and the retention of blood borne tumor cells in the lung, increases cell proliferation, and regulates cell size, adhesion, and the cellular response to cytotoxic agents. We have also shown that an alteration of tyrosine 155 of PKC delta can play a critical role in determining the effect of PKC delta on these properties. In this chapter, we examine the localization patterns of PKC delta in control B16F1 cells and in cells that over-express mutant and wild-type delta.

Methods and Materials:

Constructs:

Description of constructs are discussed Chapter 1. Briefly, B16F1 melanoma cells were transfected with either wild-type delta (Dtag), delta containing a substitution of phenylalanine for tyrosine 155 (Y155F), and control cells (mock) transfected with an empty vector. All transfected genes contain a 12 amino acid epsilon tag on the C-terminus of the PKC delta gene.

Cell culture

All cell lines were grown in MEMα media (Gibco BRL -Life Technologies) , supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100ug/ml streptomycin, 0.250ug/ml Amphotericin and 200ug/ml Geneticin (Gibco BRL-Life Technologies) at 37 C in a humidified incubator with 5% CO^2 and grown to 70% confluence. Cells were detached by trypsinization. Cell numbers were determined with a hemocytometer and the Trypan blue exclusion test.

Immunocytochemistry:

PKC delta specific rabbit IgG primary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was a FITC conjugated goat anti-rabbit IgG (Sigma Chemical Co., Mo). A non specific control which consisted of purified rabbit IgG (Sigma) was used instead of the PKC delta primary, then treated with the secondary antibody.

Whole cell preparations: cells were fixed in 3.7% formaldehyde for 5 min at room temperature and then permeablized with 0.5% Triton X-100 for 5min. After fixation, cells were rinsed 3x in PBS-Tween (PBST; PBS with 0.5% tween-20),

blocked with 10% goat serum for 30 min at 37°C, and then incubated with PKC delta Ab (4ug/ml) or a nonspecific rabbit primary antibody IgG (4ug/ml) for 1hr at 37^0C. After incubation, cells were rinsed 3x in PBST and incubated with the secondary antibody (11ug/ml) for 1hr. at 37c, rinsed 5x in PBST, and mounted with gelmount (Biomedia). Cells labeled with Mitotracker (Molecular Probes,OR) were incubated with 0.8nM of Mitotracker for 30min at 37 C. Cells were then rinsed with warm media with 10% FBS and fixed in 3.7% formaldehyde at room temperature for 5 min. Cells were then permeablized and incubated with primary and secondary antibodies as described above.

Cytoskeletal preparations: The cytoskeletal preparation is a fixation protocol involving simultaneous fixation, and the extraction of detergent soluble cellular components. Cells were treated with buffer A containing 135 mM NaCl, 5mM KCl, 20mM Hepes, 5mM MgCl2, 1mM AEBSF, 0.5% Triton X-100, and 2% formaldehyde for 5 min at room temperature. Cells were further fixed in Buffer A without triton x-100 for 5 min, rinsed 3x in PBST, then blocked for 30 min with 10% goat serum in pbsT. Incubation in primary and secondary antibodies was as described above.

Confocal Microscopy:

The Meridian Ultima laser scanning confocal microscope equipped with an argon laser. A 530/30 bandpass filter for FITC fluorescence and 580/30 bandpass filter was used for Mitotracker fluorescence. Digitized images of thick optical slices were obtained by scanning cells at a 20x magnification and a 225uM pinhole. These images give an overview of the distribution of total cell fluorescence and

analysis of cell morphology. For serial sections or thin optical slices, cells were scanned at 60x, pinhole :100uM and a z step size of 0.5uM. Images of the thin optical slices are of optical planes that exhibit the maximum fluorescence intensity. Co-localization images are of serial sections where PKCδ and Mitotracker are seen on the same focal plane. Images of PKCδ and Mitotracker are first shown separately, then the Meridian overlay option is used to illustrate areas of co-localization. A cross-over compensation analysis is used to correct for spill over of FITC fluorescence into Mitotracker fluorescence and vice versa.

Results:

All cells contain detergent resistant delta-rich structures, and cells transfected with Y155F delta display large, complex, multi-lobed delta-rich nuclei.

Analysis of cell morphology and delta fluorescence in cells transfected with wild-type or mutant delta was first examined at low magnifications (i.e. 20X objective) in order to obtain an over-view of the distribution of cells present in each cell line. This analysis revealed that over-expression of Y155F results in striking alterations of cellular and nuclear morphology. Y155F cells are frequently several times larger than control cells and nuclei are often large, complex, and multi-lobed (Figures 1 and 2). The results of Figure 2 also revealed that for all cell lines, a considerable amount of delta is present in detergent insoluble structures and to a specific definite pattern of fluorescence associated with the nucleus (Figure 2).

Figure 1: Delta fluorescence in whole cell preparations (low magnification)

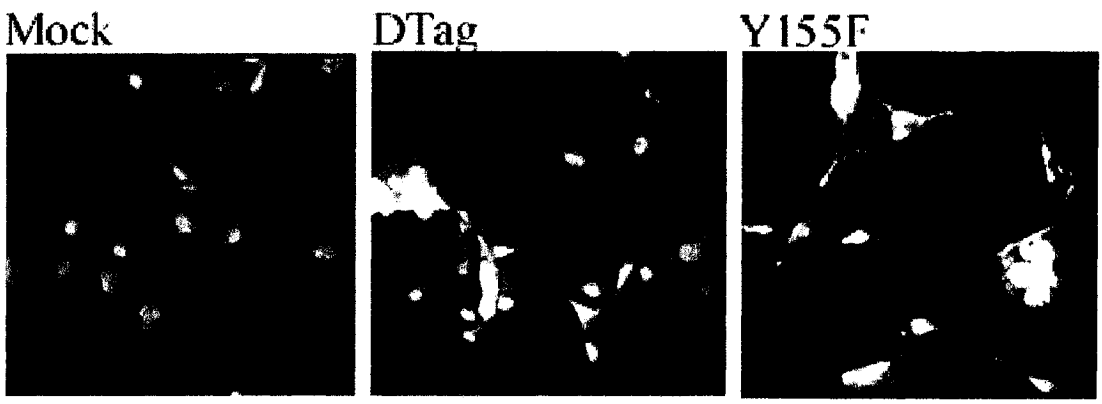

Figure 1 Legend: Fluorescence micrographs of mock, wild-type and Y155F PKCδ in whole cell preparations. Micrographs are of thick optical slices of cells fixed in formaldehyde and viewed at 20x mag. Note the large Y155F cells with large, complex, multi-lobed nuclei.

Figure 2: Detergent resistant delta fluorescence in cytoskeletal preparations (low magnification)

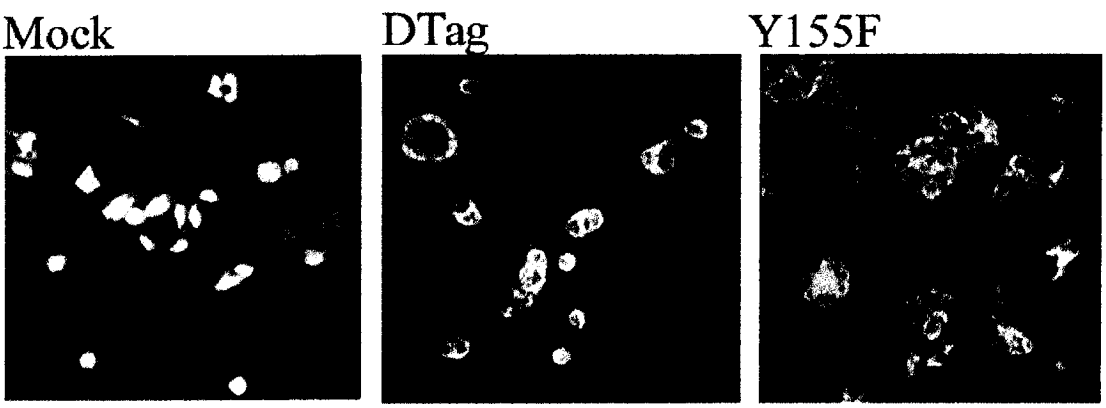

Figure 2 Legend: Fluorescence micrographs of detergent resistant mock, wild-type and Y155F PKCδ. Micrographs are of thick optical slices of cells treated with a cytoskeletal medium and viewed at 20x mag.

Localization of PKC delta to detergent resistant cytoplasmic and perinuclear granules, and to complex ring-like nuclear structures in Y155F cells

Examination of cells at high magnification revealed that in whole cell preparations, delta is present as discrete small granules, varying in size throughout the cytoplasm, in and/or around the nucleus and in the cellular processes. Fluorescent ring-like structures are observed in and/or around the nuclei of Y155F cells (Figure 3). Comparison of cytoskeletal preparations of these cells with formaldehyde fixed whole cell preparations revealed that some delta-rich structures observed in whole cell preparations are detergent soluble while others are detergent resistant. Detergent resistant delta has the appearance of discrete granular structures in the cytoplasm of all cell lines, especially in the perinuclear region (Figure 4), while fluorescence at the edges of cell processes in whole cell preparations is detergent soluble, and presumably membrane associated, while the fluorescent ring-like structures observed in the nuclei of Y155F cells appears to be detergent resistant (Figure 4).

Figure 3: Delta localization in whole cell preparations (high magnification)

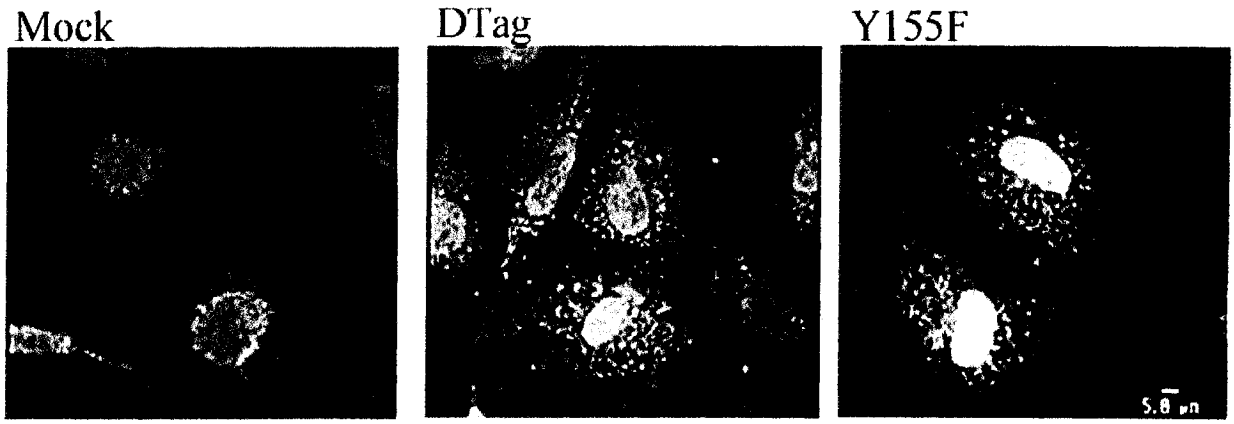

Figure 3 Legend: Fluorescence micrographs of mock, wild-type and Y155F PKCδ in whole cell preparations. Micrographs are of thin optical slices of cells fixed in formaldehyde and viewed at 60x .with oil immersion.

Figure 4: Delta fluorescence in cytoskeletal preparations (high magnification)

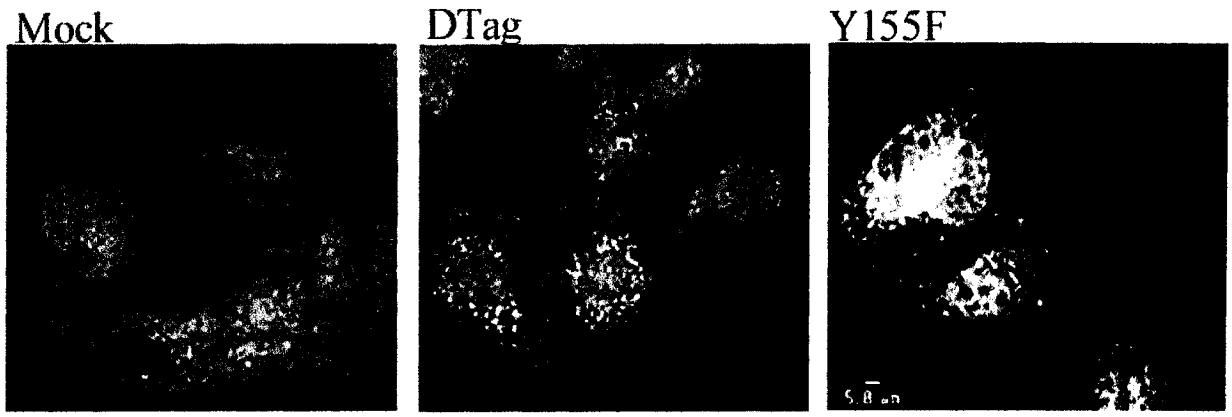

Figure 4 Legend: Fluorescence micrographs of detergent resistant mock, wild-type and Y155F PKCδ. Micrographs are of thin optical slices of cells treated with a cytoskeletal medium and viewed at 60x mag.

PKC delta localizes to the periphery of B16F1 cells

Detergent soluble PKC delta is present at the cell periphery. In focusing on the cell periphery, we examined a different focal plane the only than those were selected when looking at cytoplasmic granules. We found that in cells fixed in formaldehyde, delta is localized to a thin rim at the cell periphery in all cell lines (Figure 5). In contrast, serial sections through CSK preps revealed little or no fluorescence at the cell periphery (data not shown).

Figure 5: Localization of PKC delta to the cell periphery

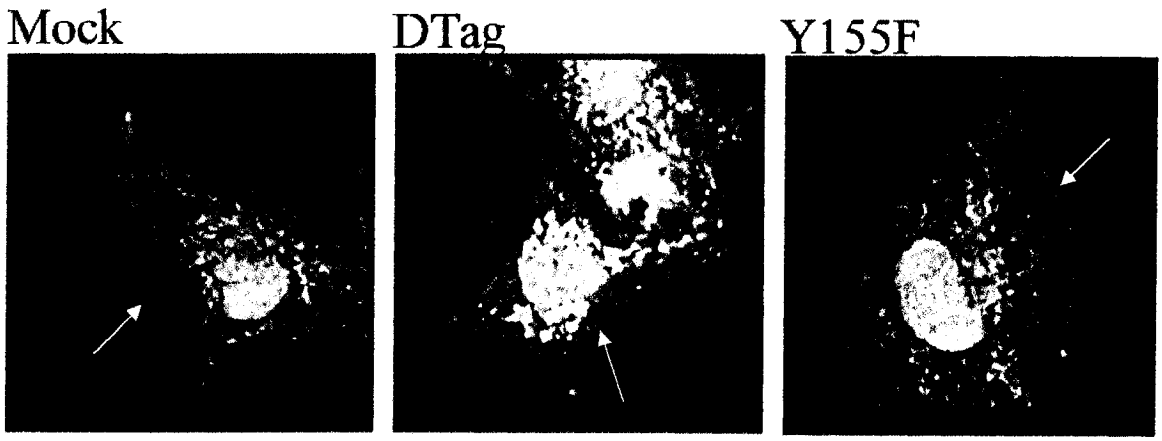

Figure 5 Legend: Fluorescence micrographs of PKC delta localized to the cell periphery. Micrographs are of thin optical slices of cells fixed in formaldehyde and viewed at 60x mag.

Altered nuclear and cytoplasmic delta localization in Y155F cells

The large nuclei seen in whole cell preparations of Y155F cells at low magnifications are shown in greater detail at higher magnifications. Most of the cells observed were very large and often had nuclei that appear to be multi-

lobed. In addition to the localization of delta to granular structures of varying size in the cytoplasm (Figure 6A), marked fluorescence was seen in the form of ring-like structures in the nucleus (figure 6B), around lobes of the nucleus (Figure 6C), and to a sub-population of melanin-rich cytoplasmic structures of varying sizes (Figure 6D). However, delta did not localize to all melanin rich structures observed. Thus, substitution of Y155 with a phenylalanine residue can have marked effects, suggesting that in vivo phosphorylation of tyrosine 155 regulates key cytoplasmic and nuclear activities.

Figure 6: Transfection with Y155F delta results in altered nuclear morphology and altered PKC delta localization

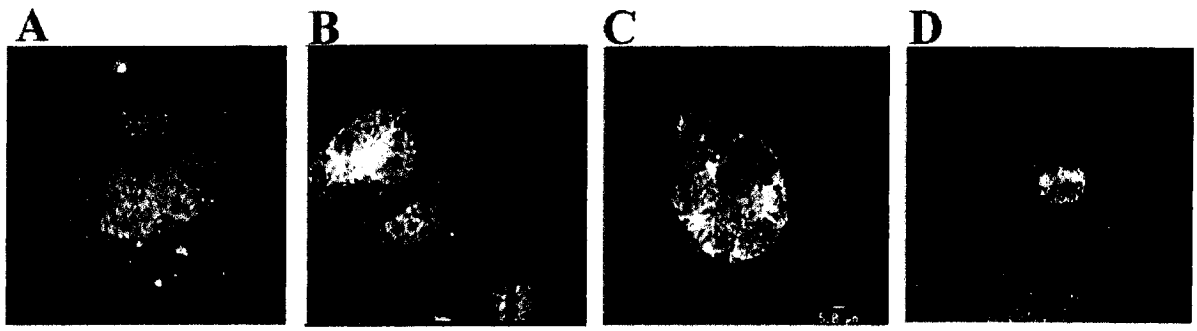

Figure 6 Legend: Micrographs of Y155F cells with altered nuclear and cytoplasmic delta localization

A) Delta localizes to ring-like structures in the nuclear region, the perinuclear region and to granular structures in the cytoplasm

B) Localization in a rounded cell that has very little cytoplasm visible and a lobed nucleus. Intense delta staining is observed around nuclear lobes.

C) A large cell with either a lobed nucleus or multiple nuclei. Delta is localized to the nuclear region as well as to numerous cytoplasmic granules of varying sizes.

D) Delta localization to a melanin containing structure

Immunocytochemical staining suggests that PKC delta and mitochondrial stains identify mitochondria in Dtag cells

Several studies have shown that PKC delta can localize to various organelles, including the mitochondria. We have shown that in the B16F1 cell lines, PKC delta localizes to cytoplasmic granules found throughout the cell (Figure 7a), and concentrated around the nucleus. We next used the fluorescent stain, Mito-tracker, to examine the localization of mitochondria and found that mitochondria showed the same pattern of staining and localization as PKC delta throughout the cytoplasm and concentrated around the nucleus (Figures 7a and 7B).

Figure 7: Identification of PKC delta and mitochondria in cells transfected with wild-type delta

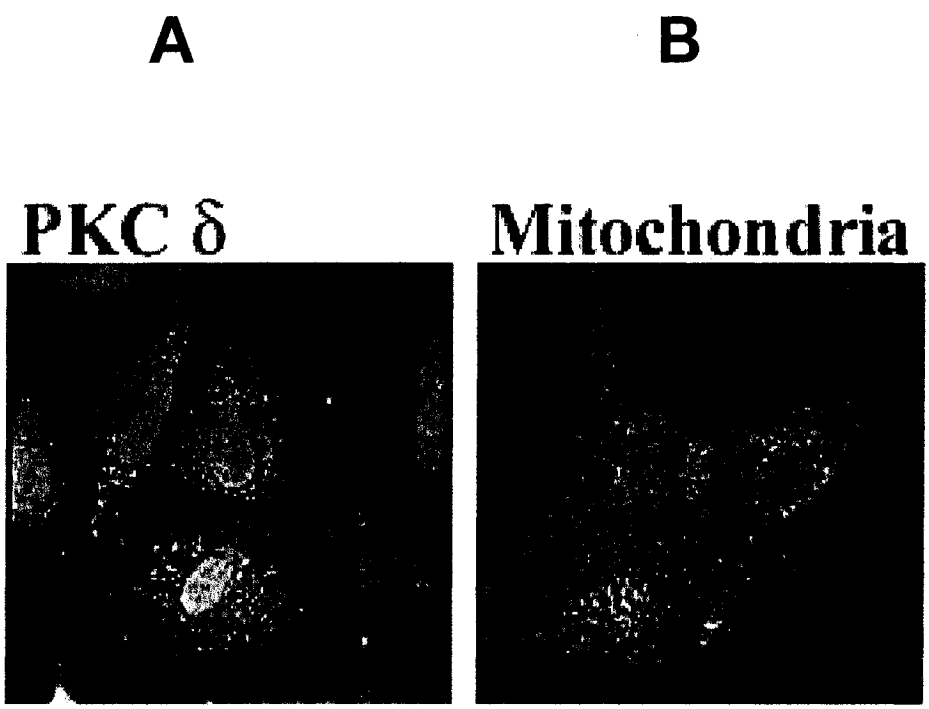

Figure 7 Legend: Micrographs of PKC delta or mitochondrial staining in Dtag cells. Micrographs are of thin optical slices of cells fixed in formaldehyde and viewed at 60x magnification. (A) Dtag cells probed with anti-PKC delta antibody. (B) Dtag cells probed with MitoTracker

Co-localization of PKC delta to the mitochondria

In Figure 7a and 7b we showed and the mitochondrial localization patterns were similar to the localization of PKC delta. We next used dual imaging to determine whether PKC delta and mitochondria co-localize to the same structures. The results show that PKC delta co-localizes to mitochondria throughout the cytoplasm and in the perinuclear region as well as to additional cytoplasmic structures in all the B16F1 cell lines (Figure 8).

Figure 8: PKC delta co-localizes to mitochondria

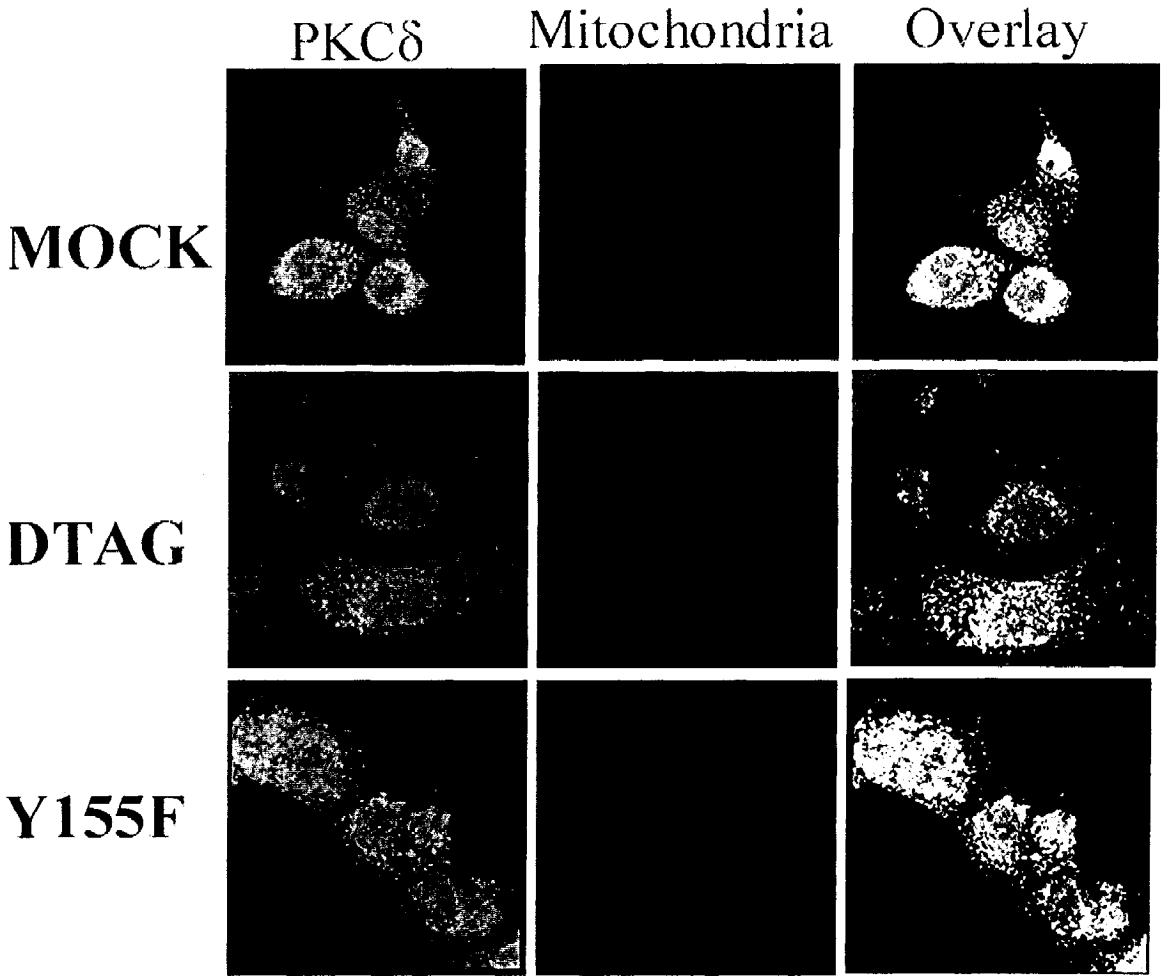

Figure 8 Legend: Micrographs of delta localization to the mitochondria. Micrographs are of thin optical slices of cells fixed in formaldehyde and viewed at 60x. Cells were probed with anti-PKC antibody, then stained with Mitotracker. (A) denotes delta localization. (B) denotes staining pattern of mitochondria after being stained with Mitotracker. (C) denotes overlay of delta localization and mitochondrial staining.

Discussion:

In this chapter, we examine the localization patterns of wild-type and mutated Y155F delta using confocal microscopy and immuno-cytochemical techniques. Our study shows that in all cell lines PKC delta localizes to a detergent soluble ring around the periphery of the cell, presumed to be cell membrane. In addition, all cell lines showed localization of delta to numerous detergent-resistant granular structures of varying size throughout the cytoplasm and to detergent-resistant concentrations of particles in the perinuclear region. In addition, Y155F delta localized to nuclear structures and the nuclei in these cells were atypical

In all cell lines fluorescent mitochondria display the same cytoplasmic distribution and appearance as delta, and the co-localization studies show that PKC delta co-localize. The co-localization of delta and mitochondria suggests a possible effect of delta on aerobic respiration and/or on cell survival in the presence of cytotoxic stimuli, and is consistent with our findings of altered responses of cells transfected with Y155F to cytotoxic stimuli. In human myeloid leukemia cells, localization of PKC delta to the mitochondria results in the release of cytochrome c and the induction of apoptosis *(Majumder et al 2000).* Others have shown that localization of PKC delta to the perinuclear region can be the result of co-localization to the golgi network *(Wang et al 1999, Perego et al 2002),* however, present study does exclude this as an additional localization of delta in B16F1 cells. It is noteworthy that in the present study, co-localization of cytoplasmic delta appeared to be similar in all cell lines, even though different

responses to cytotoxic agents were noted, (chapter 2). Thus, the findings presented in the present chapter suggest that if altered mitochondrial function contributes to the protective affect of Y155F delta, this mutation may not to affect localization, but may affect a mitochondrial action occurring subsequent to localization. The fact that no obvious qualitative differences were observed in the localization of wild-type or mutant deltas to the mitochondria, may suggest that tyrosine 155 is not required for localization to these structures. However, it is also possible that differential localization within the mitochondria may occur but be beyond the limit of resolution afforded by these light microscopic studies.

In chapter 2, we found that the Y155 mutation can have a profound protective affect on cell survival after exposure to agents capable of inducing cell death. It is possible that the Y155 mutation may confer a protective effect against cytotoxic agents as a result of this mutants ability to alter the regulation of key nuclear events. In this regard, it is interesting to note that we find that over-expression of Y155F delta results in a marked localization of delta to the nucleus and striking alterations of nuclear morphology including the formation of large, complex multi-lobed nuclei associated with ring-like arrangements of delta-rich particles. These observations may suggest an important role for tyrosine 155 phosphorylation in the regulation of normal nuclear structure and function. It should be noted however, that it is not known whether the nuclear changes observed in this mutant cell line reflect changes associated with a particular stage of the B16F1 cell cycle. However, multi-lobed nuclei have been reported in cells which have undergone growth arrest *(Bahar et al 2002)*.

Appendix

Effects of wild-type and Y155F delta on *In-vivo* Proliferation

The metastasis, lung retention, and proliferation data suggest that over-expression of wildtype delta may increase metastasis of blood borne TC by increasing the proliferation of TC either intravenously and/or in the lung. Due to experimental and technical limitations, we sought to determine how over-expression of delta would affect growth of tumor cells in the host. Female C57BL6 mice were injected subcutaneously in to thigh flanks with $5x10^4$ DTAG, Mock, or Y155F cells. When tumors became palpable, the area tumors were measured with a vernier caliper 3 times a week, until the time of sacrifice.

The In vivo studies show that while over-expression of wild-type causes latency of sub-cutaneous tumor growth, final tumor sizes are the same for all groups (Figure 1). These results are consistent with the in vitro observations that a delta-mediated regulation of B16F1 proliferation is influenced by environmental factors, and illustrates the importance of the micro-environment on TC growth.

Figure 1: Graph of subcutaneous tumor growth

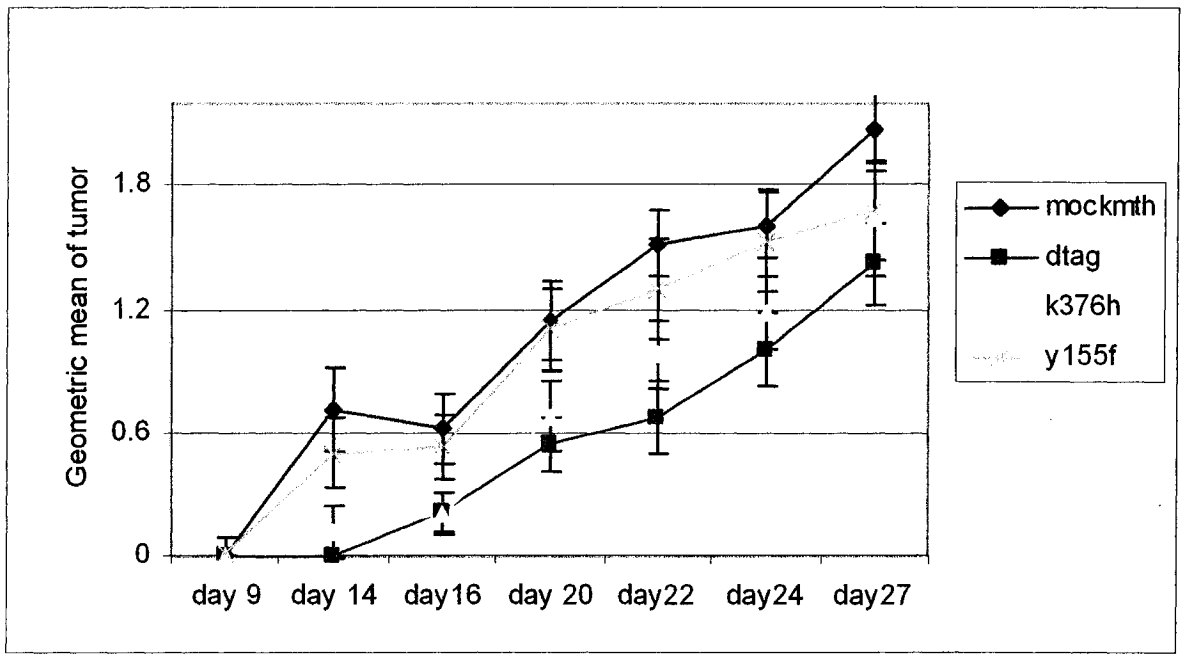

Figure 1 Legend: Points represent median tumor size for each time period.

Discussion:

The effects of mutant deltas on proliferation and viability are variable and may depend upon signaling factors present in serum and/or on autocrine/paracrine signals released by the TC. An investigation of TC growth in-vivo revealed that over-expression of wild-type delta inhibits early tumor growth but can have a stimulatory effect on TC growth in the in-vitro studies. This data strongly is consistent with other data showing that the host micro-environment can influence TC properties such as invasion, and metastatic potential. Our in vivo sub-cutaneous growth data therefore would be consistent with the hypothesis that a tumor's microenvironment can play an important role in TC survival and proliferation. In fact, In view of the larger growth of pulmonary nodules observed in animals inoculated IV with TC over-expressing wild-type delta, it is reasonable to assume that the micro-environment of the lung differs from that of subcutaneous tissue, and is supportive of increased growth in the case of wild-type transfected cells.

Summary and Conclusions:

The goal of this study was to examine the role of transfected wild-type and Y155F PKC delta in the regulation of B16F1 metastasis, and properties that govern key regulatory events during metastasis. We examined the in vivo effects of transfection with wild-type delta and mutated Y155 delta on metastasis and TC retention in the lung, and in vivo effects of transfection on adhesion to components of the extra-cellular matrix, cell size, invasion, proliferation under adverse conditions, and survival in response to cytotoxic agents. In addition, the cellular localization of wild-type and mutant delta was examined using confocal microscopy, and immuno-cytochemical analysis of formaldehyde fixed preparations, and cytoskeletal preparations.

In chapter 1, we showed that that over-expression of wild-type delta significantly increased metastasis causing an increase in both the incidence and size of pulmonary nodules, and increased the number and size of TC retained in the lung during the early stages of metastasis (48hrs). Results obtained with quantitative confocal microscopy showed that over-expression of wild-type delta does not increase cell size. Thus, we hypothesize that the lung retention results at 48 hrs resulted from increased TC proliferation and/or adhesion, or with increased survival in the presence of cytotoxic stimuli. In fact, our in vitro results suggest that transfection with wild-type delta does increase adhesion to matrigel and TC proliferation, and can offer slight protection against cell death induced by some cell death inducers. Furthermore, the cytotoxicity results would be consistent with a hypothesis that increased expression of PKC delta could

increase resistance to cytotoxic stimuli if phosphorylation of tyrosine 155 was blocked or decreased in vivo.

To determine if PKC delta regulates cell proliferation or survival, B16F1 cells were grown in adverse conditions and at varying cell concentrations, or exposed to cytotoxic agents. In Chapter 2, we report that transfected PKC delta can regulate B16F1 cell viability, proliferation, and susceptibility to cytotoxic agents. Results suggest that increased cell viability and proliferation may result from increased phosphorylation of tyrosine 155, while the cytotoxicity studies suggest that increased survival in response to cytotoxic agents may result from an inhibition of tyrosine 155 phosphorylation. Results in Chapter 2 also demonstrate that environmental factors and cell crowding can markedly influence regulation of the above properties, and also support the hypothesis that in the presence of serum, over-expression of wild-type delta is capable of increasing B16F1 proliferation, and adhesion to a matrigel substrate likely accounting for the increased frequency and size of pulmonary nodules during metastasis . It is noteworthy, that all the above regulatory functions appear to depend upon an intact tyrosine 155, and presumably upon in vivo phosphorylation of this residue. It is interesting to note that the cytotoxic studies suggest that under conditions that might prevent phosphorylation of tyrosine 155, increased PKC delta expression could also increase cell survival in response to cytotoxic stimuli.

In chapter 3 our localization studies show that in B16F1 cells, delta localizes to periphery of the cell and that localization to this region is detergent soluble, and presumably the cell membrane. Delta also localizes to detergent

resistant cytoplasmic strucutures varying in size, including perinuclear particles. Co-localization studies reveal that many of these 'particles' are mitochondria. Similar localization patterns to these regions were observed for all cell lines, except that cells that over-expressed mutant Y155F had striking alterations of nuclear morphology including the formation of large, complex multi-lobed nuclei associated with ring-like arrangements of detergent resistant delta-rich particles.

In summary, the combined results of this thesis support studies by others showing that PKC delta can regulate metastasis (*Kiley et al 1999, LaPorta et al 2000).* Our studies suggest PKC delta regulates the metastasis of B16F1 by increasing the adhesion of blood borne tumor cells to the endothelium and/or by facilitating the proliferation, all presumably potentiated by phosphorylation of tyrosine 155. The proliferation and viability appear to be strongly influenced by factors in the host microenvironment, such as cell-cell signaling, as well as growth factors present in serum and/or autocrine or paracrine factors produced by the TC. In addition increased PKC expression could possibly increase the survival of TC challenged with cytotoxic agents, under conditions inhibiting phosphorylation of tyrosine 155. Thus, collectively results in this thesis suggest that phosphorylation of tyrosine 155 can markedly and differentially effect many of the key regulatory events essential to the metastatic cascade.

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