REGULATION OF PHORBOL ESTER-INDUCED RAS/RAF/ERK

SIGNALING PATHWAY IN EL4 CELLS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of SHUJIE HAN find it satisfactory and recommend that it be accepted.

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Chair

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REGULATION OF PHORBOL ESTER-INDUCED RAS/RAF/ERK

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Abstract

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The mechanisms of cellular responses to the tumor promoter phorbol 12-myristate 13-acetate (PMA) were examined in a panel of EL4 lymphoma cells. We hypothesized that 1) RasGRP1 confers the PMA-sensitive phenotype in EL4 cells; 2) integration of the PI3K/Akt signaling pathway at Raf-1 and feedback regulation of Raf-1 by Erk contribute to the differences between PMA-sensitive, -resistant and intermediate phenotypes; and 3) Raf kinase inhibitor protein (RKIP) is involved in regulating Erk activation, migration and invasion in EL4 cells.

First, we characterized PMA-induced Ras/Raf-1/Erk activation in representative EL4 cell lines. We manipulated RasGRP1 levels using RasGRP siRNA in WT2 cells, and then tested PMA-induced Ras/Raf-1/Erk activation, proliferation, and IL-2 production. Based on these findings, we conclude that RasGRP1 confers the major features of the PMA-sensitive phenotype in EL4 cells.

The second study examined the regulation of Ras/Raf/Erk signaling pathway by the PI3K/Akt and Erk pathways. Based on experiments using pharmacologic inhibitors, we conclude

that negative regulation of Raf-1 at Ser259 by PI3K/Akt and auto-inhibitory feedback of Raf-1 at Ser289/296/301 by Erk are responsible in part for the differences in PMA-induced Raf/Erk activation between EL4 phenotypes.

The third study was conducted to determine the potential contribution of RKIP to the enhanced metastasis of V7 cells. We used RKIP siRNA to knock down RKIP expression in V7 cells, and then assessed PMA-induced Raf-1/Erk phosphorylation, migration and invasion assay. Based on the results, we conclude that RKIP likely plays a role in the enhanced metastasis of V7 cells.

Together these results suggest that multiple factors contribute to the distinct PMA responses in EL4 cells. RasGRP1 confers PMA-sensitive responses in EL4 cells; negative regulation of the Ras/Raf/Erk signaling pathway by PI3K/Akt at Raf-1 Ser259 and auto-inhibition of Raf-1 by Erk at Ser289/296/301 contribute to the distinct characters of PMA-sensitive, -resistant and intermediate phenotypes; and RKIP is, at least partially, responsible for enhanced migration and invasion of PMA-resistant cells. These studies illustrated how differences in the expression of key signaling proteins can either enhance or suppress events involved in tumor progression.

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KEY TO ABBREVIATIONS

Akt/PKB: protein kinase B

DAG: diacylglycerol

EGF: epidermal growth factor

Erk: extracellular signal-regulated kinase

FAK: focal adhesion kinase

GEF: guanine nucleotide exchange factor

GTP: guanosine triphosphate

KSR: kinase suppressor of Ras

MAPK: mitogen activated protein kinase

MEK: MAPK/Erk kinase

PAGE: polyacrylamide gel electrophoresis

PAK: p21 activated kinase

PDK: 13'-phosphoinositide-dependent kinase

PIP2: phosphatidylinositol biphosphate

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PMA: phorbol 12-myristate 13-acetate

PP2A: protein phosphatase 2A

PTEN: Phosphatase and Tensin homologue deleted on chromosome 10

Raf: protein serine/threonine kinase that activates MEK

Ras: small GTP-binding protein that activates Raf

RasGRP: Ras guanyl releasing protein

RKIP: Raf kinase inhibitor protein

RT-PCR: reverse transcriptase-polymerase chain reaction

SDS: sodium dodecyl sulphate

siRNA: small interfering RNA

WT: wild type

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 GENERAL OVERVIEW

EL4 is a murine thymoma/lymphoma cell line that may be derived from a natural killer (NK) cell tumor (Gays *et al.,* 2000). It was originally derived from a C57BL/6N mouse that had been treated with 9,10-dimethyl-1,2-benzanthracene (Herberman, 1972). The EL4 cell line, in its variant forms, serves as a useful model system in which to examine molecular mechanisms of malignant transformation. The EL4 cell line is unique in its utility as a model system in which to identify signaling pathways involved in responses to phorbol ester tumor promoters. The cell lines mentioned here were developed in our lab by clonal selection from naturally occurring variants of the wild-type (WT) EL4 cell line (Ku and Meier, 2000). EL4 cells exist in phorbol ester-sensitive (WT, WT2, WT3, WT5 phenotypes), –resistant (V5, V7, V9, V11 phenotypes), and intermediate variants (V3 and V10). Sensitive cells respond to phorbol 12-myristate 13-acetate (PMA), a diacylglycerol analog, with activation of Erks (Meier *et al.*, 1991; Gause *et al.*, 1993; Sansbury *et al.*, 1997), synthesis of interleukin-2 (IL-2) (Farrar *et al.*, 1980; Sando *et al.*, 1982; Pearlstein *et al.*, 1983; Harrison *et al.*, 1987; Rayter *et al.*, 1992), growth arrest (Sando *et al.,* 1982; Harrison *et al.,* 1987; Desrivieres *et al.,* 1997; Sansbury *et al.,* 1997), and eventual death. Resistant cells proliferate normally in the presence of PMA (Resnick *et al.,* 1997; Sansbury *et al.,* 1997; Ku and Meier, 2000), and are resistant to PMA-mediated Erk activation (Meier *et al.,* 1991). Intermediate phenotype cell lines can proliferate upon PMA treatment, while still having a moderate level of Erk activation. The molecular mechanisms involved in Erk activation are recognized to be important with respect to cell proliferation and transformation. This project addresses the effects of naturally-occurring and experimentally induced variations in

gene expression on the phenotype of a lymphoma cell line.

EL4 variants provide a spectrum of phenotypes that are particularly useful in extending the results to other cell types, including adherent cells. PMA-resistant EL4 cells can adhere to tissue culture plastic, while PMA-sensitive cells grow in suspension. EL4 cells present a well-suited model in which to define the signaling pathways most critical for the pro-proliferative and anti-apoptotic effects of tumor promoters. Our long-term goal is to define the pathways most critical for the distinct characteristics of EL4 cell clones. The potential to examine integration of major signaling pathways in EL4 cells greatly broadens the scope of the system. Such knowledge will contribute to our understanding of tumorigenesis, directing therapeutic approaches to the signaling components that are most critical in tumor cells.

1.2 MECHANISM OF PMA-INDUCED TOXICITY

The concept of tumor promoter is derived from studies in which the application of low doses of a DNA reactive (genotoxic) carcinogen did not result in the development of tumors until the animals were treated repetitively with another non-DNA reactive (nongenotoxic) chemical that was itself known not to induce large numbers of tumors. The first chemical has been called an initiator and the second chemical refered to as the tumor promoter (Tennant, 1999). One of the most potent tumor promoters *in vivo* is PMA (tested in mouse skin model) (Furstenberger *et al.,* 1981). PMA is an *in vitro* artificial diacylglycerol (DAG) analogue, which binds and activates protein kinase C (PKC) and other receptors possessing C1 domains.

Our lab previously investigated the mechanism of PMA-induced cell death in EL4 cells.

PMA inhibited proliferation of WT2 cells, as noted previously. Another group subsequently reported that PMA specifically induces cell cycle arrest in WT EL4 cells, resulting in eventual death. PD 098059, a MEK inhibitor, blocks PMA-induced Erk activation and growth arrest in WT cells (Sansbury *et al.,* 1997). This study established that Erk activation is required for PMA-induced toxicity.

1.3 EXPRESSIONS AND REGULATION OF PKC ISOFORMS IN EL4 CELLS

Conventional forms of PKC (α, $\beta1$, $\beta2$, γ) are activated by DAG and calcium, whereas novel forms (δ, ϵ, η, θ) are activated by DAG but not calcium (Liu *et al.,* 1998). Both of these families of PKC isozymes have C1 domains, protein modules containing specifically arranged cystein, and histidine residues that coordinate zinc. C1 domains serve as membrane recruitment modules, and they bind to DAG with low nanomolar affinity. The C1 domains of conventional and novel PKC forms also bind the phorbol ester class of DAG analogs, members of this class have been extensively studied *in vivo* as tumor promoters and used *in vitro* as artificial DAG stimuli in various experimental protocols (Martiny-Baron *et al.,* 2007). Atypical forms of PKC, such as ι and ζ, are not regulated by DAG or calcium, and are not targets for phorbol esters (Teixeira *et al.,* 2003).

In view of the fact that PKC isoforms are considered the "major" receptors for PMA, PKC isoform expression was examined in sensitive and resistant EL4 cells (Sansbury *et al.,* 1997). PKCs α, ϵ, η, θ, and ξ were expressed in both PMA-sensitive and –resistant EL4 cells. In NV cells, PKCη levels were significantly lower, while PKCα levels were slightly lower. PMA

caused rapid membrane translocation of all PKC isoforms, followed by down-regulation of most, indicating that PKCs are PMA-responsive in both cell types.

To determine whether under-expression of PKCη was responsible for PMA resistance, PKCη was transiently over-expressed in NV cells (Sansbury *et al.,* 1997). The enzyme translocated to the membrane in response to PMA. Immunofluorescent localization of PKCη confirmed expression increased. PKCη appeared to be localized to the ER//Golgi region in WT and tranfected NV cells, as reported for other cell types. Transient transfections were similarly performed for PKCα. PMA-induced Erk activation was not enhanced in PKCη- or PKCα-transfected NV cells. Thus, differences in the expression levels of PKCη and/or PKCα are not responsible for differences in PMA- induced Erk activation between sensitive and resistant EL4 cells.

1.4 ROLE OF PKCs IN ERK ACTIVATION

The extracellular signal-regulated MAPKs, Erk1 and Erk2, are activated by a Ras/Raf/MEK/Erk cascade (Weinstein-Oppenheimer *et al.,* 2000). Erk activation is a nearly universal response to phorbol esters that activate PKC. Erk activation has been shown to be involved in the mechanism of action of diverse mitogens, and is an attractive target for therapeutic intervention in tumors. However, the mechanisms by which PKC activation is coupled to the Erk cascade remain unclear. Possible mechanisms include phosphorylation of Raf by PKC, phosphorylation of Ras effector proteins by PKC, PKC-mediated generation of lipid co-factors for Raf, and PKC-mediated activation of tyrosine kinases. Furthermore, while PKCs

have been the focus of most work on PMA responsiveness, there are additional receptors for PMA that can be coupled to Erk activation in a PKC-independent manner, such as RasGRP (Kazanietz *et al.,* 2000), Rac GTPase-activating proteins α– and β– chimaerins (Caloca *et al.,* 1997), and scaffolding proteins Unc-13/Munc-13 (Lackner *et al.,* 1999; Nurrish *et al.,* 1999).

1.5 ROLE OF RASGRP IN ERK ACTIVATION

Tumor-promoting phorbol esters bind to receptors possessing C1 domains, which were first characterized in protein kinase C (PKC) isoforms. In addition to binding PKCs, phorbol esters bind to additional receptors with equivalent affinity. These receptors possess C1 domains homologous to those present in PKCs. Of particular interest here, RasGRP, a guanine nucleotide exchange factor, can confer PMA-induced Erk activation by directly activating Ras in response to PMA.

RasGRP (Ras guanyl releasing protein) is a guanine nucleotide exchange factor (GEF) for Ras (Hogquist, 2001). RasGRP contains a C1 domain, as found in PKCs (Tognon *et al.,* 1998; Kazanietz, 2002), and binds PMA and diglyceride with affinities similar to that of classical PKC isoforms. RasGRP also has a pair of EF hands that bind calcium. RasGRP is highly expressed in hematopoietic cells, where it can initiate Erk activation, independent of PKCs (Lorenzo *et al.,* 2001; Hogquiest, 2001). RasGRP1 is essential for thymocyte differentiation and TCR signaling (Dower *et al.,* 2000; Ebinu *et al.,* 2000). RasGRP3 is required in coupling phospholipase C-γ2 to Ras in B cell receptor signaling (Oh-hora *et al.,* 2005; Teixeira *et al.,* 2003). RasGRP4 can potentially contribute to myeloid leukemia (Yang *et al.,* 2002; Reuther *et al.,* 2002, Li *et al.,*

2003).

Previous work in our lab showed, using immunoblotting, that RasGRP is expressed at much higher levels in PMA-sensitive, WT-derived cell lines than in PMA-resistant cell lines (Knoepp, reference to his doctoral thesis). Transient transfection of RasGRP into PMA-resistant clone V5 cells resulted in increased levels of RasGRP protein (95kDa), as detected by immunoblotting. Additional data indicated that PMA-induced Erk activation is increased in RasGRP transfected V5 EL4 cells.

1.6 RAS/RAF/ERK ACTIVATION IN EL4 CELLS

The Ras/Raf/Erk signaling cascade plays an important role in the development of human malignancies as well as in the normal growth process. The Ras/Raf/Erk signaling pathway evolved to rapidly activate nuclear transcription factors in response to extracellular stimuli, such as EGF, PMA. The terminal kinases Erk-1 and Erk-2, are evolutionarily conserved signaling molecules, that can phosphorylate and activate AP1 proteins (Jun/Fos). Erk can influence diverse cellular functions and biological processes including cell growth, proliferation, differentiation, apoptosis, and tumor progression (Trakul, *et. al,* 2006; Klysik et al., 2008).

PMA-sensitive and –resistant EL4 cells differ with respect to the extent of PMA-induced Ras/Erk activation. PMA-induced Erk activation is much more prominent in PMA-sensitive EL4 cells than in resistant cells. In fact, PMA-induced Erk activation was not detectable in our earliest studies done by our lab (Gause *et al.,* 1993), due to the relatively low sensitivity of the assays used at that time. Subsequent work showed that, in resistant and intermediate phenotype

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cells, Erk activation is relatively weak and delayed (Ku and Meier, 2000).

1.7 ERK ACTIVATION AND CELL CYCLE ARREST

Continuously elevated Erk signaling during the G1, S or G2 phases of the cell cycle leads to upregulation of cyclin-dependent kinase inhibitors and induces arrest or senescence, while uncontrolled Erk activation during the M phase leads to bypass of the spindle assembly checkpoint and generation of chromosomal abnormalities (Eves *et al.,* 2006; Rosner, 2007). The need for precise titration of the Erk signal ensures the integrity of the spindle assembly process.

1.8 REGULATION OF RAF ACTIVATION

The Raf-1 kinase is an important signaling molecule, functioning in the Ras pathway to transmit mitogenic, differentiative, and oncogenic signals to the downstream MEK and Erk. Because of its integral role in cell signaling, Raf-1 activity is precisely controlled.

The regulation of Raf-1 activity is complex, involving inter- and intramolecular protein interactions as well as phosphorylation/dephosphorylation process (Dougherty *et al.,* 2005). Studies focusing on Raf-1 phosphorylation document both positive and negative effects of Raf-1 phosphorylation. For example, p21 activated kinase (PAK) and Src mediate Raf-1 phosphorylation on positive regulatory sites that augment Raf-1 activity (Balan *et al.,* 2006). Negative regulation of Raf-1 has also been reported. Phosphorylation of Ser259 can inactivate Raf-1, probably by promoting the formation of a Raf autoinhibitory complex via association with 14-3-3 (Heckman *et al.,* 2005). The kinase responsible for Raf-1 Ser259 phosphorylation is a

subject of considerable controversy, although several candidate kinases, including PKA, Akt, AMP-activated kinase, have been proposed.
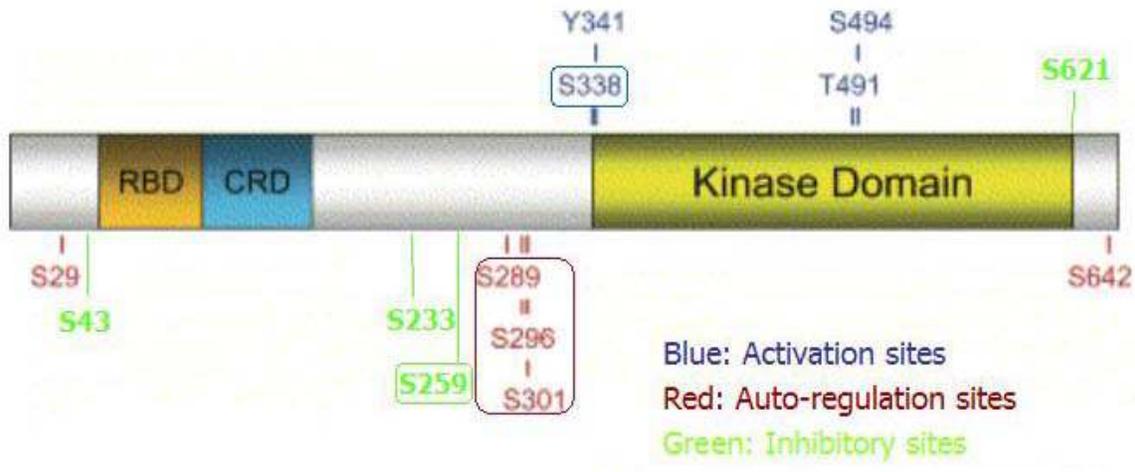
Figure 1-1. Schematic representation of Raf-1 phosphorylation sites.

The elaborate network of Raf-interacting proteins such as PKC, inhibitory molecule RKIP, accessory molecules 14-3-3, Hsp90, and kinase suppressor of Ras (KSR) constitute key regulatory elements of the Raf/MAPK cascade (Klysik *et al.,* 2008). Raf activation is also regulated by multiple phosphatases, such as phosphatase 1, protein phosphatase 2A, and protein phosphatase 5, which dephosphorylate specific residues.

1.9 INTEGRATION OF PI3K/AKT AND RAF/ERK SIGNALING PATHWAYS

Phosphoinositide 3-kinase (PI3Ks) are central to the control of cell growth, proliferation and survival, and drive the progression of tumors by activating Akt (protein kinase B) (Aeder *et al.,*

2004). PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$, which are generated by PI3K, are the lipids that are crucial for activation of Akt (Meuillet *et al.,* 2004). Akt is activated by ligation of PtdIns(3,4,5)P$_3$ and subsequent phophorylation on Thr308 by 3'-phosphoinositide-dependent kinase 1 (PDK1) at the membrane. Maximal activation requires phophorylation of Ser473 by an enzyme called PDK2 (Wymann *et al.,* 2003). Akt is a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation. Akt can also be activated in PI3K-independent manner, such as through cAMP/PKA or Ca2+/calmodulin-dependent kinase (Song *et al.,* 2005). It is reported that a negative feedback loop involving Raf and Akt exists, in which Akt phosphorylates Raf at serine 259 and inhibits Raf/Erk signaling. The inhibitory effect of Akt on Raf activity appears to be dependent on cell type and stage of differentiation (Chang *et al.,* 2003).

1.10 RKIP IN TUMOR METASTASIS

RKIP, also known as phosphatidylethanolamine-binding protein (Bernier *et al.,* 1986), influences intracellular signaling cascades, cell cycle regulation, differentiation, programmed cell death, and metastasis (Keller *et al.,* 2004; Trakul *et al.,* 2005). RKIP has been shown to modulate the Raf/MEK/MAPK cascade (Sasaki *et al.,* 2003; Matheny *et al.,* 2004; Wakioka *et al.,* 2001). Previous studies have suggested that RKIP inhibits activation of Raf-1 by blocking phosphorylation of Raf-1 by p21-activated kinase (PAK) and Src kinases (Trakul *et al.,* 2005). RKIP binds to Raf-1 and competitively interferes with MEK binding to Raf (Yeung *et al.,* 2000; Zhu *et al.,* 2005). It was reported that protein kinase C, activated by either phorbol ester or

epidermal growth factor (EGF), phosphorylates RKIP at Ser 153, and that this phosphorylation causes the dissociation of RKIP from Raf-1 and the subsequent activation of MAPK pathway (Klysik *et al.,* 2008; Corbit *et al.,* 2003).

RKIP may regulate cell cycle and mitosis through Raf-1 inhibition. RKIP depletion leads to a decrease in mitotic index; MEK inhibition can rescue the effects of RKIP depletion (Eves *et al.,* 2006), indicating that the response is due to uncontrolled activation of the Raf/MEK/Erk pathway. RKIP can indirectly influence the Aurora B kinase and spindle checkpoints, and thus profoundly impact the fidelity of the cell cycle (Eves *et al.,* 2006).

Recently, RKIP has been identified as a metastasis suppressor gene, and this function has been shown to correlate with Erk activity (Fu *et al.,* 2003). The report by Fu indicates that cell lines derived from metastatic prostate cancers have decreased levels of RKIP mRNA and protein as compared with primary tumor cell lines. A decrease in RKIP was also detected in melanoma and breast cancer, where the increased metastasis potential elicited by RKIP depletion was due, at least in part, to elevated MAP kinase activity (Schuierer *et al.,* 2004; Hagan *et al.,* 2005). RKIP may serve as an immune surveillance cancer gene, in that, its low expression or absence can allow tumors to escape host immune cytotoxic effector cells (Baritaki *et al,* 2007).

On the other hand, there is substantial evidence that RKIP contributes to enhanced metastasis. It is reported that RKIP knockdown suppresses cell motility in Madin-Darby canine kidney (MDCK) epithelial cells, while RKIP overexpression promotes cell migration (Bement *et al.,* 2005). RKIP expression changes the basic features of epithelial cell morphology, causing MDCK cells to lose cell-cell adhesions and generally resemble more motile cell types. RKIP

expression was found to be higher in a tumorigenic and metastatic murine fibrosarcoma cell line than in the weakly tumorigenic and nonmetastatic parental cell line from which it was derived (Hayashi *et al.,* 2005). Further studies have confirmed that RKIP is involved in the positive regulation of epithelial cell migration. RKIP-overexpressing MDCK cells grow like fibroblasts without cell-cell contact, becoming highly migratory cells with long protrusions (Zhu *et al.,* 2005). There is no previous report regarding the function of RKIP in cells of hematopoietic origin, to our knowledge.

1.11 METASTASIS OF EL4 CELL LINES

Previous work by others established a mouse model for testing metastasis of EL4 cells (Zhang *et al.,* 1998). When EL4 cells are injected via the tail vein into syngeneic immune-competent mice, death occurs within 30 days. Tumor nodules (micrometastasis) are visible on the surface of the enlarged liver. In previous work in our lab (Knoepp *et al.,* 2008), various EL4 cell lines were used in the experimental metastasis model. Survival time and liver mass were quantified. This study showed that only PMA-resistant cell lines establish tumors. While liver tumors were most prominent, renal tumors were also noted.

1.12 GENERAL HYPOTHESIS AND PROJECT OVERVIEW

The goal of the work described here was to further define the signaling pathways responsible for the distinct signaling and tumorigenesis phenotypes in EL4 cells. The pathways involved are known to be critically important in tumor cell biology. The Erk MAPK cascade

plays a prominent roll in cell proliferation. Activation of Akt can occur during tumor progression, and has been shown to affect cell survival. The results of our study are anticipated to contribute to our knowledge of oncogenesis in two ways: 1) by establishing molecular mechanisms coupling diacylglycerol generation to activation of the Erk pathway, and 2) by further defining pathways that contribute to enhanced tumor formation by PMA-resistant cells.

Previous work in this lab has shown that sensitive and resistant cells differ in expression of several proteins involved in PMA response. Specifically, sensitive cells express RasGRP, a protein that activates Ras in response to PMA (independent of PKCs), at much higher levels than in resistant cells. Work described in this dissertation defined the role of RasGRP in EL4 cells, and also shows that the basal activity of Akt is higher in resistant cells, and that resistant cells express higher basal level of Raf kinase inhibitor protein (RKIP) than sensitive and intermediate cells. All of these proteins are likely to contribute to the variant phenotypes.

The major hypotheses addressed in this dissertation are as follows: 1) that RasGRP1 confers PMA-sensitive responses in EL4 cells, such as robust Erk activation and growth arrest; 2) that integration of PI3K/Akt signaling pathway at Raf-1 and auto-regulation of Raf-1 by Erk contribute to the distinct character of PMA-sensitive, -resistant and intermediate cell phenotypes; and 3) that RKIP contributes to the weak PMA-induced Raf/Erk activation in resistant cells, and is at least partially, responsible for enhanced migration and invasion of PMA-resistant cells. Thus, this project examines a variety of signals involved in PMA-induced responses in EL4 cells.

PROJECT OVERVIEW

Aim I. To elucidate the role of RasGRP in PMA-sensitive responses in EL4 cells.

Previous data had shown that sensitive cells express RasGRP, a protein that activates Ras in response to PMA, at much higher levels than in resistant cells. PMA induces robust Erk activation, growth arrest and cell death in PMA-sensitive EL4 cells, while Erk activation is rather weak in PMA-resistant EL4 cells. PMA induces a moderate level of Erk activation in intermediate cells. PMA does not cause growth arrest and cell death in PMA-resistant and intermediate cells. We hypothesized that RasGRP1 is the upstream component responsible for enhanced PMA-induced Erk activation in EL4 cells. The effects of overexpression and down-regulation of RasGRP1 was studied in representative EL4 cells.

Aim II. To study signals regulating Ras/Raf-1/Erk cascade at the level of Raf-1.

We discovered that Akt is constitutively active in resistant cells, while basal Akt phosphorylation is weak in sensitive cells and at a moderate level in intermediate cells. We studied the regulation of PMA-induced Raf-1/Erk phosphorylation by PI3K/Akt, by Erk-mediated auto-regulation, and by Raf kinase inhibitor protein (RKIP). We used LY294002 to block PI3K/Akt pathway, U0126 to inhibit MEK/Erk activation, and RKIP siRNA to knockdown RKIP expression. The effects of these manipulations on PMA-induced Raf-1/Erk phosphorylation, as well as *in vitro* migration and invasion, were assessed.

CHAPTER TWO

RASGRP1 CONFERS THE PHORBOL ESTER-SENSITIVE PHENOTYPE

TO EL4 LYMPHOMA CELLS

2.1 SUMMARY

The murine EL4 lymphoma cell line exists in variants that are either sensitive or resistant to the tumor promoter phorbol 12-myristate 13-acetate (PMA). In sensitive EL4 cells, PMA causes robust Erk mitogen-activated protein kinase activation that results in growth arrest. In resistant cells, PMA induces minimal Erk activation, without growth arrest. PMA stimulates IL-2 production in sensitive, but not resistant, cells. The role of RasGRP1, a PMA-activated guanine nucleotide exchange factor for Ras, in EL4 phenotype was examined. Endogenous RasGRP1 protein is expressed at much higher levels in sensitive than in resistant cells. PMA-induced Ras activation is observed in sensitive cells but not in resistant cells lacking Ras-GRP1. PMA induces down-regulation of RasGRP1 protein in sensitive cells but increases RasGRP1 in resistant cells. Introduction of small interfering RNA (siRNA) for RasGRP1 suppresses PMA-induced Ras and Erk activations in sensitive cells. Sensitive cells incubated with siRNA for RasGRP1 exhibit the PMA-resistant phenotype, in that they are able to proliferate in the presence of PMA and do not secrete IL-2 when stimulated with PMA. These studies indicate that the PMA-sensitive phenotype, as previously defined for the EL4 cell line, is conferred by endogenous expression of RasGRP1 protein.

2.2 INTRODUCTION

EL4, a cell line originated from a carcinogen-induced murine thymoma, provides a unique model system for the study of phorbol ester response and resistance. Responses of sensitive "wild-type" (WT) EL4 cells to phorbol 12-myristate 13-acetate (PMA) include protein kinase C

(PKC) activation (Kramer and Sando, 1986; Meier *et al.,* 1991; Baier-Bitterlich *et al.,* 1996; Sansbury *et al.,* 1997), tyrosine phosphorylation (Richardson and Sando, 1995; Luo and Sando, 1997), Erk mitogen-activated protein kinase activation (Meier *et al.,* 1991; Gause *et al.,* 1993; Sansbury *et al.,* 1997), adhesion (Resnick *et al.,* 1997), IL-2 production (Farrar *et al.,* 1980; Sando *et al.,* 1982; Pearlstein *et al.,* 1983; Harrison *et al.,* 1987; Rayter *et al.,* 1992), and growth arrest (Sando *et al.,* 1982; Harrison *et al.,* 1987; Desrivieres *et al.,* 1997; Sansbury *et al.,* 1997). To study the mechanisms by which PMA elicits these responses, we and other investigators have characterized PMA-resistant EL4 cells, which by definition proliferate in the presence of PMA (Resnick *et al.,* 1997; Sansbury *et al.,* 1997; Ku and Meier, 2000). Resistant EL4 cells activate PKCs in response to PMA but show minimal activation of Ras (Rayter *et al.,* 1992), Erks (Meier *et al.,* 1991), MEK (Gause *et al.,* 1993), pp90^{RSK} (Meier *et al.,* 1991), or JNK (Bradshaw *et al.,* 1996). Erk activation is required for PMA-induced growth arrest in sensitive cells (Sansbury *et al.,* 1997). This situation mimics that seen in thymocytes, in which rapid and robust Erk activation leads to negative selection (McNeil *et al.,* 2005). Some PMA-resistant lines have been developed via selection for growth in the presence of PMA. However, in the absence of selective pressure, this phenotype exists as a natural variant in the EL4 cell population (Sansbury *et al.,* 1997). Despite extensive characterization of PMA-sensitive and -resistant EL4 cell lines, the molecular basis for the phenotypes has not been fully established.

It is now clear that PKC isozymes are not the only receptors for tumor-promoting phorbol esters. Studies by our lab and others have indicated that differences in expression of PKC isoforms between sensitive and resistant EL4 cells are not responsible for the major differences in

PMA sensitivity (Resnick *et al.,* 1997; Sansbury *et al.,* 1997). Other proteins, such as chimaerins and RasGRP1 (Kazanietz, 2002), contain the diglyceride/phorbol ester binding sites (C1 domains) present in PKC isoforms and thus bind PMA with affinity similar to that of PKCs (Tognon *et al.,* 1998; Lorenzo *et al.,* 2001). RasGRP1 is a guanine nucleotide exchange factor for Ras that binds phorbol esters in a calcium-independent manner (Lorenzo *et al.,* 2000). This protein, which is highly expressed in T lymphocytes (Ebinu *et al.,* 2000), is essential for thymocyte differentiation (Dower *et al.,* 2000). RasGRP1 mediates PMA- and diglyceride-induced activation of Ras in T-cells (Ebinu *et al.,* 2000; Jones *et al.,* 2002) and in some other cell types (Lorenzo *et al.,* 2001). Recent studies have established a physiologic role for RasGRP1 in mediating diglyceride signals in lymphoid cells (Sanjuan *et al.,* 2003; Zheng *et al.,* 2005). RasGRP1 has been shown to play a critical role in T cell differentiation (Priatel *et al.,* 2006). There are three other members of the RasGRP family, each with distinct patterns of tissue expression (Ebinu *et al.,* 1998; Reuther *et al.,* 2002; Yang *et al.,* 2002; Li *et al.,* 2003). All, except for a mouse form of RasGRP4 (Li *et al.,* 2003), are regulated by phorbol ester and diglyceride.

In this report, we further delineate pathways responsible for PMA sensitivity in EL4 thymoma cells. In particular, we show that RasGRP1 plays a critical role in the phorbol ester responsive phenotype.

2.3 METHODS

Cell Culture

EL4 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine

serum (FBS; Atlanta Biologicals, Norcross, GA, or Summit Biotechnology, Fort Collins, CO). The original WT (PMA-sensitive) and variant (PMA-resistant) EL4 cell lines used in our lab were provided by Dr. David Morris (University of Washington, Seattle, WA). The derivation of clonal EL4 cell lines by our group was described previously (Ku and Meier, 2000). WT-derived (PMA-sensitive) clonal cell lines were maintained in suspension culture dishes (Sarstedt) to discourage selection for adherent cells; variant clones were maintained in standard tissue culture dishes.

Cell Proliferation Assays

For cell proliferation assays, growing cells ($\geq$95% viability) were seeded in 24-well tissue culture plates at 2 x 10^5 cells/well with 2 ml of complete medium (including serum). Cells were incubated with 100 nM PMA or 0.1% ethanol (vehicle) at 37°C for varying times, in complete medium. Cell number was determined by mixing cells with 0.02% trypan blue in phosphate buffered saline (PBS) and counting dye-excluding cells using a hemocytometer.

Immunoblotting and Immunoprecipitation

Antibodies were obtained from the following sources: phospho-Erk, Promega (Madison, WI); Erk-1, Santa Cruz Biotechnology (Santa Cruz, CA); phospho-Raf (Ser338), Cell Signaling Technology Inc. (Beverly, MA); Ras, Santa Cruz Biotechnology or Chemicon (Temecula, CA); and RasGRP, Santa Cruz Biotechnology.

EL4 cells were treated with and without PMA as described previously (Ku and Meier, 2000).

After treatment, cells were collected by centrifugation at 1200*g*. Adherent cells were harvested using a cell scraper before the centrifugation. Cells were lysed in a buffer containing 20 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Extracts were sedimented at 10,000*g* for 10 min at 4°C to remove insoluble material. Samples equalized for protein (100 μg), as determined by Coomassie Protein Assay (Pierce), were separated by SDS-PAGE on 12.5% Laemmli gels, transferred to polyvinylidene difluoride paper, incubated with antibodies, and developed using enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were imaged by densitometry and quantified using NIH Image software.

Ras Activation Assay

Raf-1 RBD agarose (Upstate Biotechnology, Lake Placid, NY), which specifically binds to GTP-bound Ras, was used to pull down active Ras. Whole-cell extracts (1000 μg in 1ml) were added to 10 μg of Raf-1 RBD agarose for 1 h at 4°C. Precipitates were washed three times with 1 ml of ice-cold lysis buffer, and then resuspended in 4x (Laemmli sample buffer for protein separation by SDS-PAGE. Immunoblotting was performed using an anti-Ras monoclonal antibody (Chemicon).

siRNA Experiments

RasGRP1 siRNA, control siRNA, RasGRP1 primer, siRNA transfection reagent and

transfection medium, and RasGRP antibody were obtained from Santa Cruz Biotechnology. Cells, grown to 60 to 80% confluence, were incubated with RasGRP1 siRNA or control siRNA for 5 to7 hours in the absence of serum. FBS was then added to a final concentration of 10%; cells were incubated for an additional 18 to 24 h under cell culture conditions. The medium was then changed to RPMI with 10% FBS, and the cells were incubated for an additional 48 h before incubation with and without 100 nM PMA. Protein levels for RasGRP, phospho-Erk, and actin (immunoblotting), and activated Ras (pulldown assay) were assessed in cell extracts using the methods described above. Levels of RasGRP1 mRNA were assessed by RT-PCR, using the following protocol. Total RNA was extracted from harvested cells using TRIzol solution (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using Thermo Script RT-PCR System (Invitrogen) in a reaction volume of 20 µl under the conditions recommended by the manufacturer. Total RNA (3 µg) was used as a template for cDNA synthesis. The resulting cDNA was used as a template for PCR. PCR was performed in a 50µl reaction volume with a buffer consisting of 10x PCR buffer without Mg, 50 mM MgCl$_2$, 10 mM dNTP mix, Platinum *Taq* DNA polymerase, and 1 µl of each primer. PCR was performed by initial denaturation at 94°C for 2 min followed by 40 cycles consisting of denaturation at 94°C for 30 s, annealing for 30 s at 55°C, and extension at 72°C for 30 s. RT-PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV illumination.

IL-2 Production Assays

WT2 and V7 cells were incubated in duplicate with or without 100 nM PMA for varying

times. Supernatants (medium) were obtained by centrifugation for 3 min using a microcentrifuge. Enzyme-linked immunosorbent assay plates were coated with IL-2 antibody (BD Pharmingen, San Diego, CA) in binding solution (0.1 M Na_2HPO_4/NaH_2PO_4, pH 9.0) overnight at 4°. Plates were washed three times with 0.05% Tween 20 in PBS, then blocked for 1 h with 200 μl of 1% bovine serum albumin in PBS at room temperature. Duplicate samples of culture supernatant (100 μl/well) were added and incubated overnight at 4°. Biotinylated IL-2 (BD Pharmingen, San Diego, CA) was then added. After 1 h, streptavidin-horseradish peroxidase was added. After 30 min, the reaction was developed using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) for 20 min. A microplate reader (Bio-Rad Laboratories, Hercules, CA) was used to quantify the results at 450 nm. Statistical significance was analyzed using InStat (GraphPad Software, San Diego CA).

Statistical Analysis

For quantitative data, values are expressed as mean $\pm$ S.D. of value obtained. Statistical significance will be assessed by Student's t-test, using Prism software.

2.4 RESULTS

Characterization of Clonal EL4 Cell Lines

To further explore the differences in PMA responsiveness between sensitive and resistant EL4 cells, we used a panel of clonal EL4 cell lines developed previously in our laboratory (Ku and Meier, 2000). The characteristics of these cells can be briefly summarized as follows.

WT-derived cells (WT2, WT3, and WT5) do not proliferate in the presence of PMA. Studies by another group have established that the mechanism involves PMA-induced growth arrest in sensitive cells (Desrivieres *et al.,* 1997). In contrast, most clones derived from a "variant" (V) cell line (V5, V7, V9, and V11) proliferate at a normal rate in the presence of PMA. Erk2 is robustly activated by PMA in all WT-derived clones but is activated to only a minor extent in the variant clones listed above. Two variant clones of "intermediate" phenotype, V3 and V10, are exceptions in that they show moderate Erk activation in response to PMA and are partially sensitive to PMA-induced growth inhibition. Clones WT2 and V7 have been routinely used in our lab as representative PMA-sensitive and -resistant cell lines, respectively. Erk activation in V7 cells, which is never as extensive as that seen in WT2 cells, requires higher doses of PMA and longer incubation times than for WT-derived cells (Ku and Meier, 2000). Incubation with 100 nM PMA for 15 min elicits the respective maximal responses in either cell type.

Ras Activation in Clonal EL4 Cell Lines

We first examined the ability of PMA to induce Ras activation in clonal EL4 cell lines. A Ras pulldown assay, in which GTP-bound Ras is detected, was used to examine the activation state of Ras. Initial experiments examined Ras and Erk activation at a single time point after PMA addition (15 min). As shown in Fig. 2-1A and quantified in Fig. 2-1B, the extent of Ras activation is much higher in WT2 (PMA-sensitive) cells than in V7 (PMA-resistant) cells. As previously reported (Ku and Meier, 2000), the extent of Erk activation is also higher in WT2 than in V7 (Fig. 2-1A).

A

PMA: -   +   -   +

V7   WT2

B
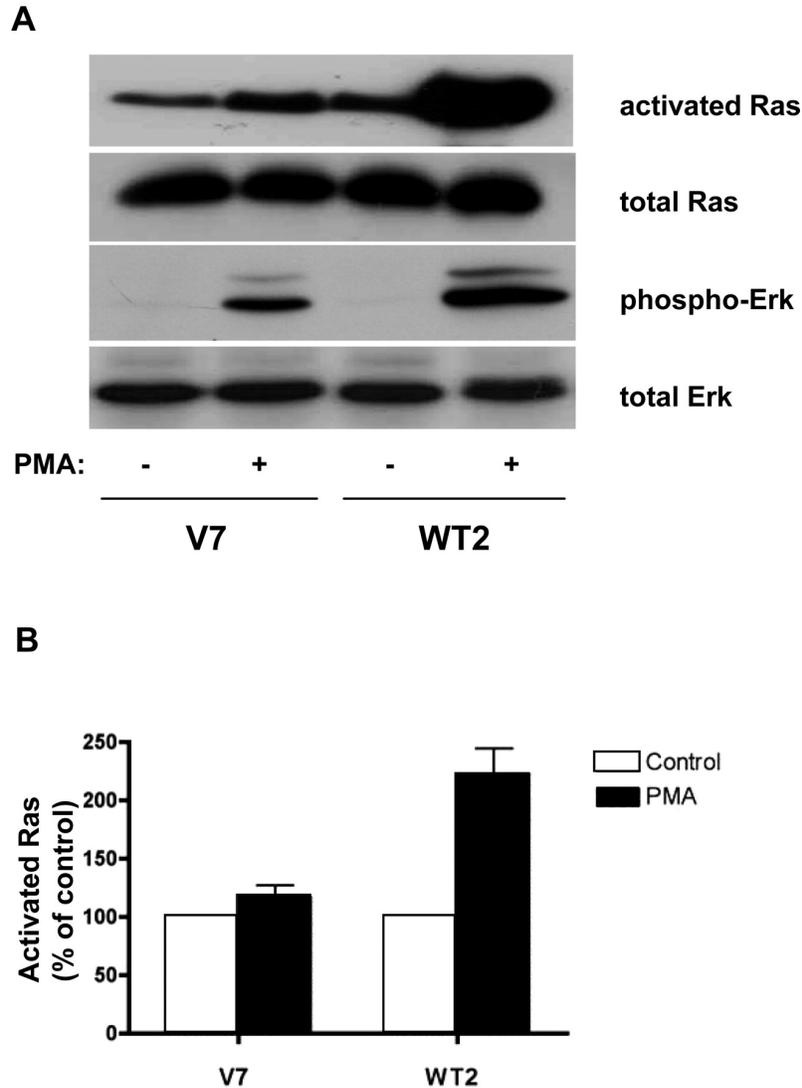
Figure 2-1. Effects of PMA on Ras and Erk activation in clonal El4 cell lines.
WT2 and V7 cells were treated with or without 100 nM PMA for 15 min. GTP-bound (activated) Ras was precipitated in a pulldown assay, as described under *Materials and Methods*, and detected by immunoblotting for Ras. Immunoblots were performed in the same experiment on whole-cell lysates for total Ras, phospho-Erk, and total Erk. A representative immunoblot is shown in A. Quantified results for Ras activation, normalized to total Ras, are shown in B. Data are expressed as percentage of the level of active Ras observed in each cell line without PMA treatment. Each data point represents mean ± S.E.M. from three separate experiments.

Time course experiments were performed with several EL4 cell lines to further examine the extent of PMA-induced Ras and Erk activation (Fig. 2-2). As shown in Fig. 2-2A, PMA induces Ras activation in WT2 cells within 5 min. The activation persists for at least 60 min. The time course of Erk activation, as detected using phospho-Erk antibody, was similar to that of Ras activation. In contrast, in V7 cells, PMA-induced Ras activation can only be detected (to a minor extent) after 15 min. Erk activation is detected in V7 cells by 5 min (Fig. 2-2B) and subsequently declines. Thus, in resistant cells, the time course of Erk activation seen in V7 does not correlate well with the time course of Ras activation. The magnitude of both Ras and Erk activation is severely blunted in V7 compared with WT2. V3 and V10 cell lines have the intermediate phenotype. In these cell lines (Fig. 2-2, C and D), PMA-induced Ras activation is minimal or nonexistent, even though there is a moderate level of Erk activation. In both V3 and V10 cell lines, Erk activation is less than that seen in WT2 cells and declines after 30 min. Taken together, these data suggest that PMA-induced Ras activation is correlated with the fulminate Erk activation observed in PMA-sensitive EL4 cells. In resistant and intermediate phenotype cells, there is little or no Ras activation in response to PMA, and Erk activation is comparatively weak.

Expression of RasGRP in Clonal EL4 Cell Lines

We next examined the expression of RasGRP in clonal EL4 cell lines. As shown in Fig. 2-3A, RasGRP protein (migrating as a doublet at ~85-90 kDa) is expressed at much higher levels in WT-derived than in variant-derived clones. The antibody used recognizes several isoforms of RasGRP, but the protein detected is presumed to be RasGRP1 because of its molecular size
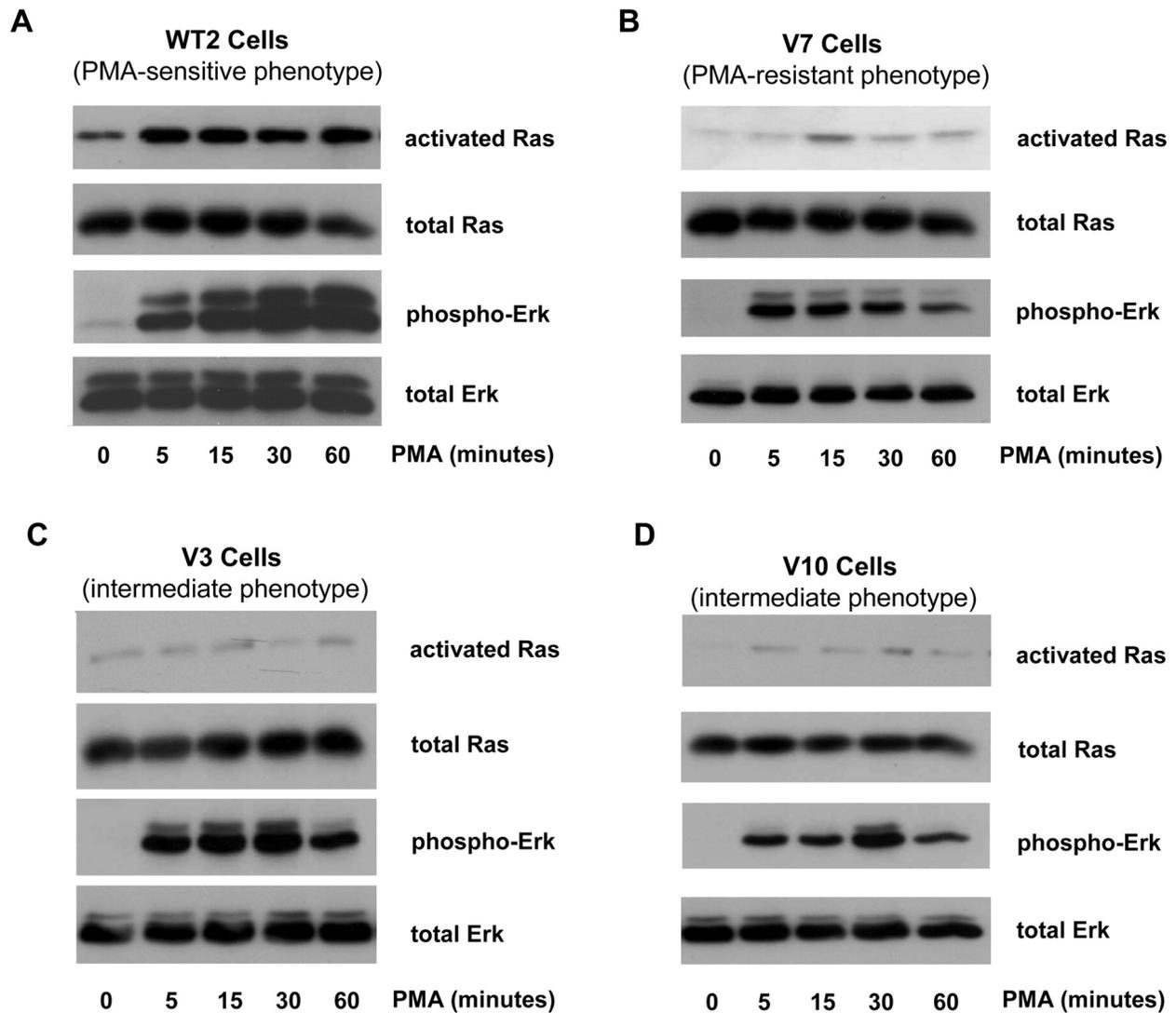
Figure 2-2. Time course of the effects of PMA on Ras and Erk activation in clonal EL4 cell lines.
WT2 (A), V7 (B), V3 (C), and V10 (D) cells were treated with or without 100 nM PMA for the indicated times. GTP-bound (activated) Ras was precipitated in a pulldown assay, as described under *Materials and Methods*, and detected by immunoblotting for Ras. Immunoblots were performed in the same experiment on whole-cell lysates for total Ras, phospho-Erk, and total Erk.

(RasGRP is 90 kDa; other members of the family are 69-75 kDa) and because this isoform is highly expressed in T lymphocytes (Ebinu *et al.,* 2000). Further validation is provided in Fig. 2-5. The effects of PMA on activation of Ras and Erk were tested in the same experiment. An incubation time of 15 min was used, based on the data obtained in Fig. 2-2. The results presented in Fig. 2-3A demonstrate that Ras was activated by PMA treatment in all of the cell lines expressing RasGRP1 (i.e., WT-derived cells). The basal level of active Ras was higher in RasGRP1-expressing (WT-derived) cells than in cells lacking RasGRP1. We were surprised to find that PMA-induced Ras activation was absent in intermediate clones V3 and V10, despite the fact that a modest level of Erk activation was evident in these cell lines. Thus, these data show that expression of RasGRP is positively correlated with PMA sensitivity in all clones except V3 and V10. These intermediate phenotype cell lines do not express RasGRP (Fig. 2-3A), but nonetheless activate Erks to a greater extent than resistant cells in response to PMA. An antibody recognizing Ser338 of Raf-1 was used to screen for Raf activation. Phosphorylation of this residue is important in the activation of Raf-1 by growth factors but is not sufficient for activation (Mason *et al.,* 1999). The results showed that Raf is phosphorylated to some extent in untreated WT-derived cells (Fig. 2-3A). After addition of PMA to sensitive cells, the phospho-Raf band shifted upward on the gel in all WT cell lines. The latter effect is typically indicative of phosphorylation of a protein on additional sites. These complex results, which are addressed in more detail in Chapter 3, are consistent with the fact that Raf-1 is regulated by phosphorylation on multiple sites by multiple kinases, including positive regulation by Erks (Balan *et al.,* 2006). In contrast, in PMA-resistant and intermediate cells, Raf was not phosphorylated under basal

conditions. Addition of PMA induced Raf phosphorylation on Ser338 in these cells, but the extent of phosphorylation and the mobility shifts were not as obvious as in WT-derived cells. The intensity of the phosphorylated band was greatest in the intermediate cells (V3 and V10), consistent with the greater extent of Erk activation observed in these cells. Finally, the results confirm that PMA-induced Erk activation is minimal in all of the resistant cell lines that lack RasGRP1 (V5, V7, V9, V11). In summary, the data presented in Fig. 2-3A establish that maximal PMA-induced activations of Ras, Raf, and Erk were only observed in EL4 cells expressing RasGRP1.

We next examined, using semiquantitative RT-PCR, whether the differences in RasGRP1 protein expression between sensitive and resistant cells reflected differences in mRNA expression levels. As shown in Fig. 2-3B, similar levels of mRNA for RasGRP1 were expressed in WT2 and V7 cells. Thus, the differences in protein expression may result from differences in post-transcriptional events between EL4 cell lines.

Regulation of RasGRP1 Expression

RasGRP isoforms, like PKC isoforms, can be down-regulated in response to PMA (Rambaratsingh *et al.,* 2003; Tuthill *et al.,* 2006). We therefore tested for long-term effects of PMA on RasGRP1 expression and signaling in EL4 cells (Fig. 2-4). As shown previously, PMA induced a high level of Erk activation in WT2 cells (Fig. 2-4A). This activation persisted for 24 h and then declined. RasGRP1 protein levels began to decline after 4 h, indicating down-regulation. It is noteworthy that a transient spike in phospho-Raf was observed at 3 to 8 h. This event was

A

PMA: - + - + - +   - + - + - + - + - + - +

|WT2| WT3 WT5 |V3| V5 |V7| V9 |V10| V11

phenotype: sens sens sens   intermed res res res intermed res

B

RasGRP1

actin

WT2   V7
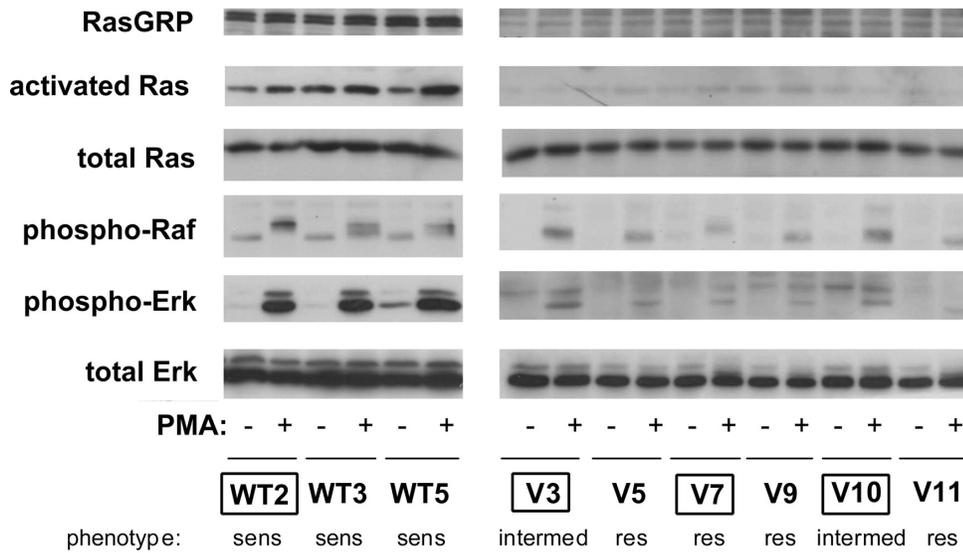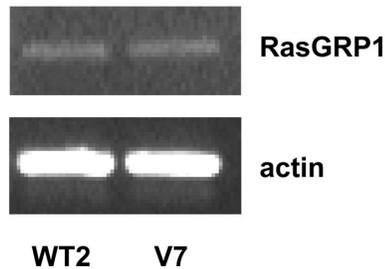
Figure 2-3. Expression of RasGRP in clonal EL4 cell lines.

A. The indicated cell lines were incubated in the absence and presence of 100 nM PMA for 15 min. Whole-cell extracts, equalized for protein, were immunoblotted for RasGRP, total Ras, phospho-Raf, phospho-Erk, and total Erk. In addition, a Ras pulldown assay was performed to detect GTP-bound activated Ras, as described under *Materials and Methods*. All incubations were done in the same experiment; blots for the two cell lines were exposed in parallel. The cell lines that are "boxed" are the ones used in Figure 2-1; the other cell lines serve as replicates within each phenotype ("sens," PMA-sensitive; "res," PMA-resistant; "intermed," intermediate phenotype.

B. Semiquantitative RT-PCR was performed for untreated WT2 and V7 cells, using primers for murine RasGRP1. Actin was also amplified as a control for loading. The products were imaged under UV light on an ethidium bromide gel.

not correlated with a further increase in phospho-Erk levels. The phospho-Raf bands seen at 3 to 8 h exhibited progressive upward gel mobility shifts, suggestive of additional phosphorylation events. In V7 cells (Fig. 2-4B), effects of PMA on Erk activation were minimal, as shown previously. It is noteworthy that a transient spike in Erk activation was observed 4 h after PMA addition. This event was correlated with a concomitant increase in phospho-Raf. Thus, PMA induces effects on Raf phosphorylation in both sensitive and resistant EL4 cells after several hours. RasGRP1 protein levels unexpectedly increased in V7 cells at 4 h and were maintained at this level for at least 48 h. Despite the increase, the level of RasGRP1 protein was always much lower than that seen in untreated WT2 cells. These data establish that PMA causes down-regulation of RasGRP1 in sensitive EL4 cells, and up-regulation in resistant EL4 cells. The role of RasGRP1 in acute Erk activation was further explored.

Manipulation of RasGRP1 Levels in EL4 Cells

To address the role of RasGRP1 in phorbol ester response, we used small interfering RNA (siRNA) to reduce RasGRP1 levels in PMA-sensitive cells expressing endogenous RasGRP1. As shown in Fig. 2-5A, siRNA against RasGRP1 was very effective in reducing RasGRP1 mRNA levels in WT2 cells. Accordingly, Ras-GRP1 protein levels were severely reduced after siRNA treatment (Fig. 2-5B). PMA-induced Ras and Erk activations are blocked in cells lacking RasGRP1. These data confirm that RasGRP1 is largely responsible for conferring PMA-induced Ras and Erk activation to sensitive EL4 cells.

A   **WT2 Cells**

RasGRP

phospho-Raf

phospho-Erk

total Erk

0   1   2   3   4   8 12 24 36 48   **PMA (hours)**

B   **V7 Cells**

RasGRP

phospho-Raf

phospho-Erk

total Erk

0   1   2   3   4   8 12 24 36 48   **PMA (hours)**
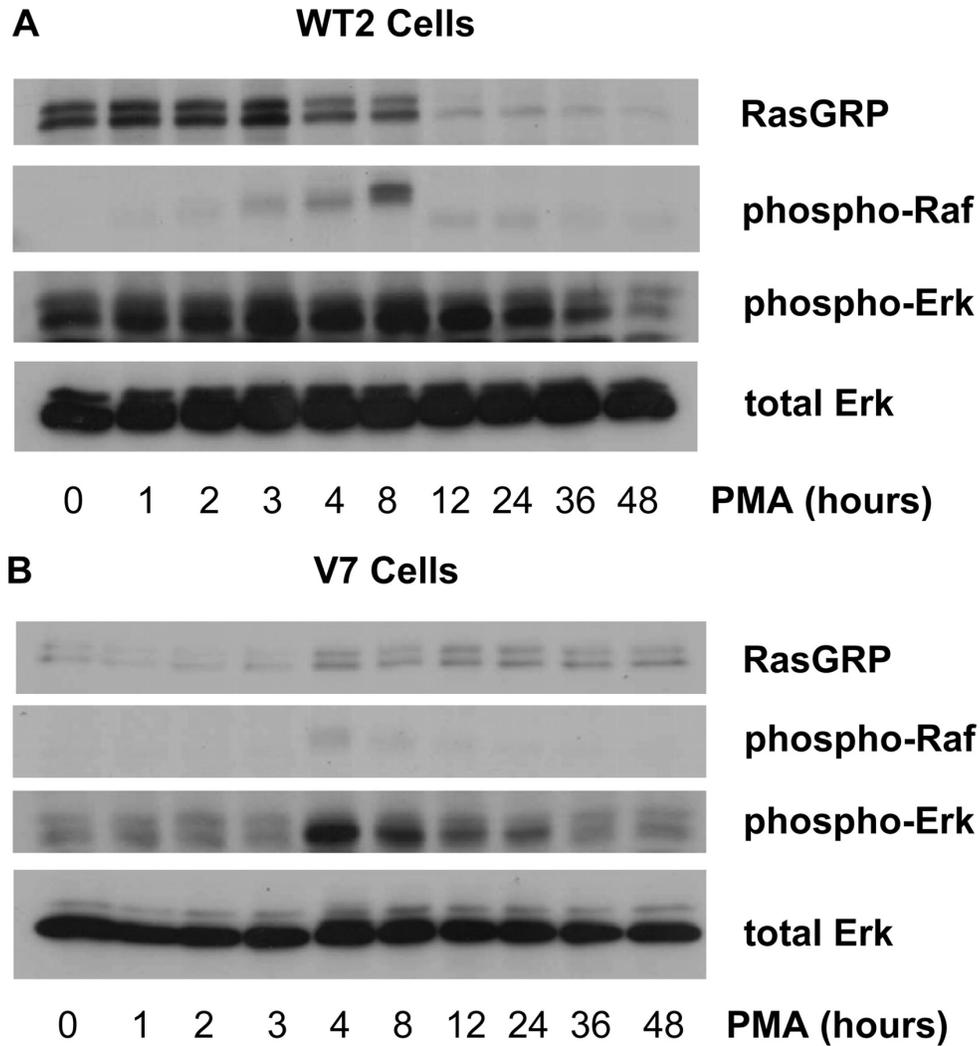
Figure 2-4. Long-term effects of PMA on RasGRP levels and on Raf and Erk activation.
WT2 (A) and V7 (B) cells were incubated for the indicated times with 100 nM PMA. Whole-cell extracts, equalized for protein, were immunoblotted for RasGRP, phospho-Raf, phospho-Erk, and total Erk. All incubations were done in the same experiment; the blots were exposed in parallel.
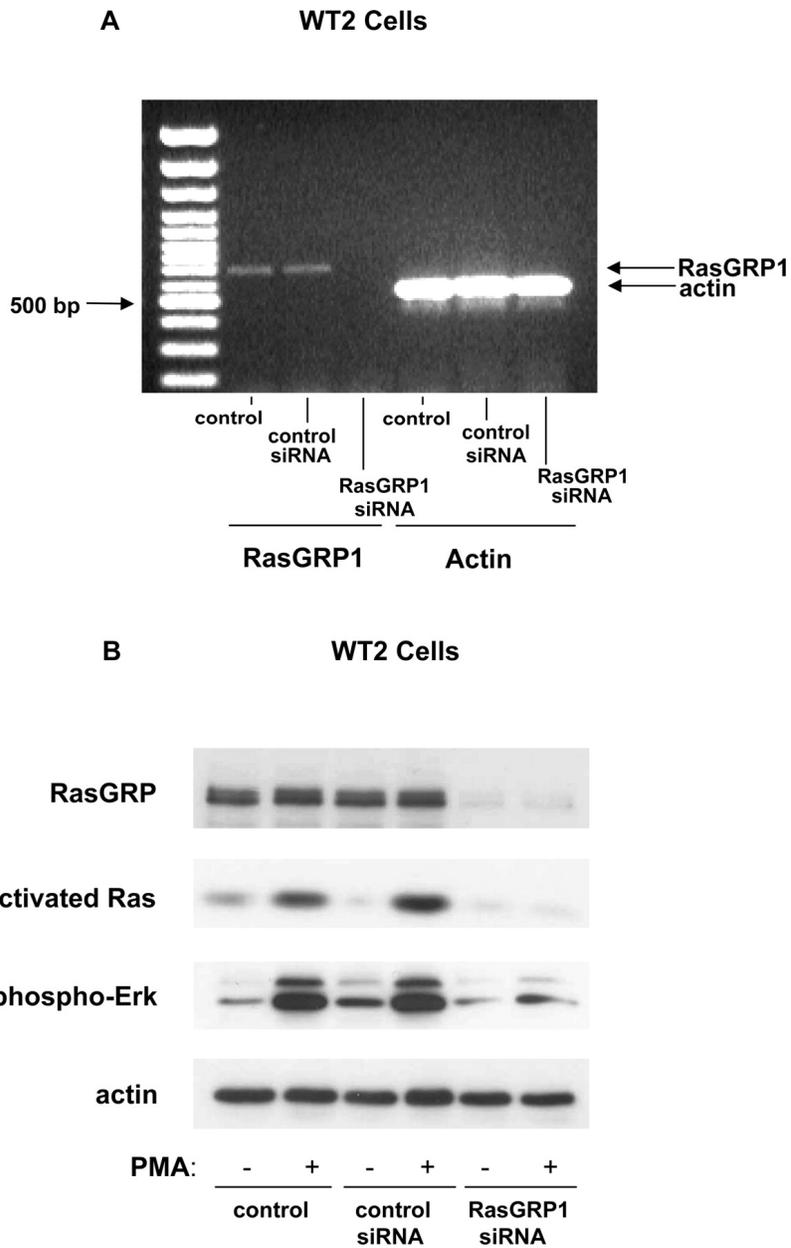
Figure 2-5. Effects of RasGRP knockdown on PMA-induced Ras and Erk activation.
A. WT2 cells were incubated in the absence of siRNA (control), or in the presence of either a control siRNA or RasGRP siRNA, as described in Methods. Message levels for RasGRP and actin were assessed by semi-quantitative RT-PCR. B. WT2 cells were treated as for in Panel A, and then incubated in the absence and presence of 100 nM PMA for 15 minutes. Levels of RasGRP, phospho-Erk, and actin were assessed in whole-cell extracts, equalized for protein. Activation of Ras (pulldown of GTP-bound Ras) was tested in the same experiment.

Effects of RasGRP Knockdown on Proliferation

As mentioned earlier, PMA-induced growth arrest is one of the hallmarks of the PMA-sensitive phenotype. PMA-sensitive cells (e.g., WT2) are unable to proliferate in the presence of PMA, whereas resistant cells (e.g., V7) continue to proliferate. Previous work in our laboratory showed that the cytostatic response of sensitive cells can be blocked by a pharmacologic inhibitor of MEK/Erk activation (Sansbury *et al.,* 1997). We therefore tested whether knockdown of RasGRP1 would have a similar effect. WT2 cells were incubated with and without siRNA for RasGRP1. Proliferation was analyzed over two days, in the absence and presence of 100 nM PMA. As shown in Fig. 2-6A, control WT2 cells were unable to proliferate in the presence of PMA. In contrast, cells treated with siRNA for RasGRP1 proliferate at similar rates with or without PMA (Fig. 2-6B). A control siRNA had no effect on PMA-induced growth arrest. Immunoblots revealed that RasGRP1 knockdown was maintained for at least 48 h after treatment with RasGRP siRNA (data not shown). Together, these results demonstrate that RasGRP1 was required for PMA-induced growth arrest.

Effects of RasGRP1 Knockdown on IL-2 Production

Another hallmark of the PMA-sensitive phenotype is production of IL-2 in response to PMA (Sansbury *et al.,* 1997). Therefore, the effects of RasGRP1 knockdown on this response were tested. The time course of IL-2 production is shown in Fig. 2-7A. WT2 cells secrete IL-2 when incubated with 100 nM PMA; IL-2 levels in the medium increase from 8 to 24 h after PMA addition. In contrast, V7 cells do not secrete IL-2 in response to PMA. A similar lack of response

A

WT2 Cells

B
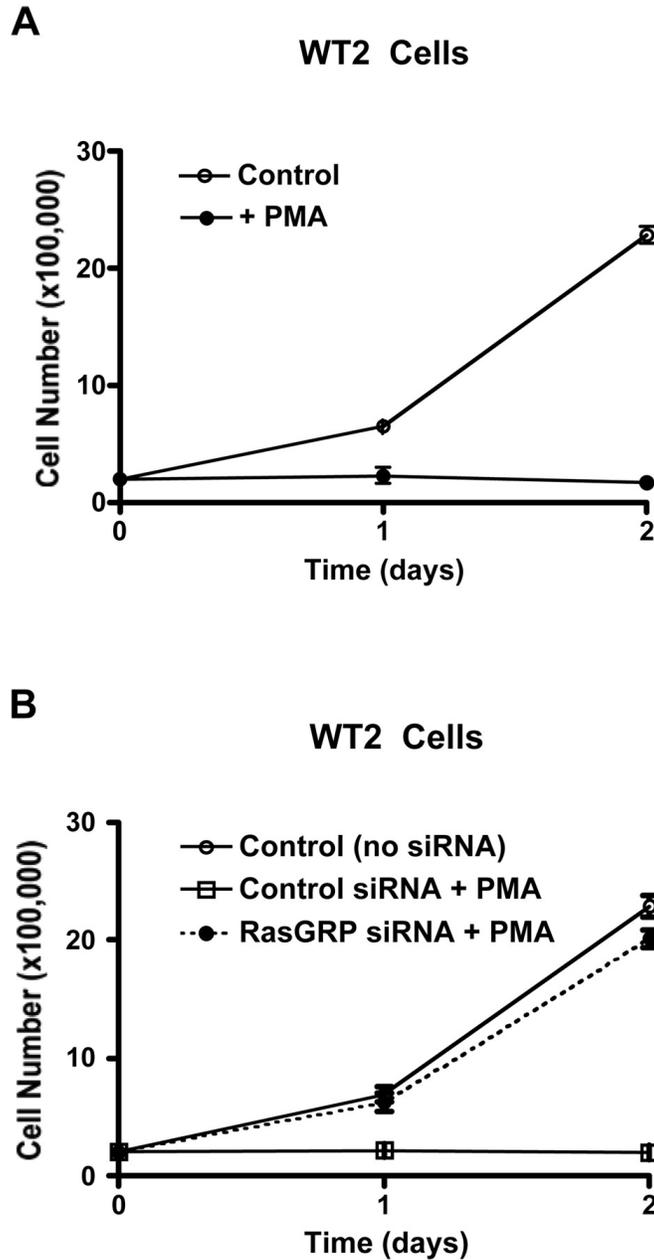
WT2 Cells

Figure 2-6. Effects of RasGRP knockdown on PMA-induced growth arrest.
WT2 cells were incubated without additions (A) and with either a control siRNA or RasGRP siRNA (B), as described under *Materials and Methods*. Cell proliferation was then assessed, as described under *Materials and Methods*, in the absence and presence of 100 nM PMA. Each data point represents the mean ± S.D. of values from triplicate samples of cells.

was observed in intermediate clones V3 and V10 (data not shown). Basal levels of IL-2 are higher in WT2 cells than in V7 cells (170 versus 44 pg/ml; $p < 0.004$ by two-tailed t test). As shown in Fig. 2-7B, the effects of RasGRP1 siRNA on PMA-induced IL-2 production were analyzed at 24 h in WT2 cells. Treatment with RasGRP1 siRNA blockd the ability of PMA to induce IL-2 production in these cells. In addition, the siRNA significantly reduced basal levels of IL-2 in WT2 cells ($p < 0.004$ by two-tailed t test). A control siRNA had no effect on IL-2 production. These data show that RasGRP1 was required for PMA-induced IL-2 production in EL4 cells.

2.5 DISCUSSION

In this study, we further explored the differences in phenotype between EL4 cells that are PMA-sensitive and PMA-resistant. We demonstrated that RasGRP1 protein was much more highly expressed in PMA-sensitive cells. Using siRNA knockdown strategy, we showed that RasGRP1, a phorbol ester and diglyceride receptor, was responsible for the "classic" features of the PMA-sensitive phenotype (IL-2 production and growth arrest), as well as for the high level of Erk activation seen in sensitive cells upon PMA treatment.

Our data establish that RasGRP1 played a key role in all phases of PMA response in EL4 cells. First, we demonstrated that PMA induced Ras activation most efficaciously in cells expressing RasGRP1. A previous study showed that Ras is activated in response to PMA treatment of sensitive EL4 cells (Rayter *et al.,* 1992). Our data extend this observation, using a newer technique to assess Ras activation. Furthermore, using additional EL4 cell lines, we

A

B
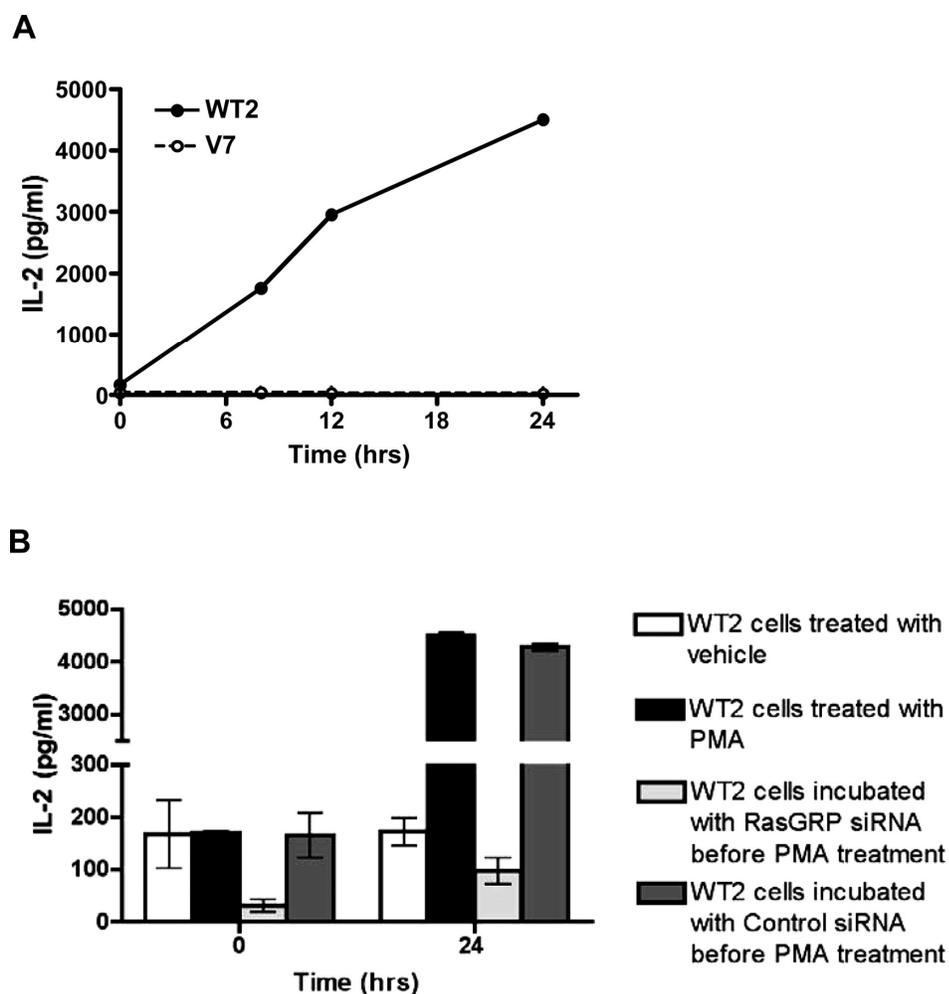
Figure 2-7. Effects of RasGRP knockdown on PMA-induced IL-2 production.

A. The time course of PMA-induced IL-2 production was assessed in WT2 and V7 cells. Cells were incubated with 100 nM PMA for the indicated times. IL-2 in the medium was quantified by enzyme-linked immunosorbent assay, as described under *Materials and Methods*. Each data point indicates the mean ± S.D. of values from duplicate wells of cells; the error bars are included within the data points.

B. WT2 cells were treated with or without control siRNA or siRNA for RasGRP1 as described under *Materials and Methods*. Cells were then incubated with and without 100 nM PMA for 24 h. "Control" refers to incubation with the vehicle (0.1% ethanol) for PMA addition. Each value represents the mean ± S.D. from duplicate wells of cells.

correlated the time course of Ras activation with that of Erk activation. Next, we demonstrated knockdown of RasGRP1 in PMA-sensitive cells resulted in loss of PMA-induced Ras activation, Erk activation, and IL-2 production. Thus, our data established that the signaling events that have historically defined "PMA sensitivity" in EL4 cells were all conferred by RasGRP1 expression.

RasGRP1 has been previously implicated in the IL-2 induction observed when primary T-lymphocytes are stimulated with PMA and calcium ionophore (Ebinu *et al.,* 2000). This previous study used an overexpression approach. Our study, using a knockdown approach, definitively established a requirement for RasGRP1 in PMA-induced IL-2 secretion in EL4 cells. It should be noted that EL4 cells differ from primary T cells in that they can achieve substantial IL-2 production in the presence of PMA alone (Sansbury *et al.,* 1997). However, the PMA-induced pathways (Ras, Erk, AP-1) seem to be the same in each case. IL-2 production is not believed to be responsible for PMA-induced cell arrest in EL4 cells; the magnitude and duration of Erk activation seem to induce this adverse effect via other downstream events (Desrivieres *et al.,* 1997; Werlen *et al.,* 2003).

It is noteworthy that Erks were activated to some extent in response to PMA in resistant and intermediate phenotype cells, in which PMA-induced Ras activation was barely detectable. Our results, therefore, suggest that there were alternative (non-Ras) pathways for PMA-induced Erk activation in these cells. The differences observed in Raf-1 phosphorylation status between the two cell lines, along with the effects of PMA on Raf-1 phosphorylation, might provide some insight into such pathways. Thus, these cell lines continue to present an intriguing paradox.

Although both sensitive and resistant EL4 cells express multiple PKC isoforms (Sansbury *et*

al., 1997; Resnick *et al.,* 1998), our data show that RasGRP1 was most critical for PMA-induced Ras activation. These data are consistent with the results of studies establishing that RasGRP isoforms, which are guanine nucleotide exchange factors, act as important receptors for phorbol esters and diglycerides in intact cells (Brose and Rosenmund, 2002; Roose *et al.,* 2005). This did not exclude a role for PKC isoforms in other PMA-mediated responses, including the low-level Erk activation seen in EL4 cells lacking RasGRP1. The recent demonstration of cross-talk between PKCs and RasGRP3 (Roose *et al.,* 2005; Zheng *et al.,* 2005) suggests further aspects for future study. In particular, the latter study showed that both RasGRP and PKCs were required for maximal Ras/Erk activation in T-lymphocyte cell lines (Roose *et al.,* 2005). It should be emphasized that, because RasGRP isoforms are selectively expressed in certain cells and tissues (Ebinu *et al.,* 1998; Tognon *et al.,* 1998), these proteins are not likely to be responsible for mediating PMA response in all cell types.

The mechanisms underlying the differences in RasGRP1 protein expression between EL4 cell lines remain to be elucidated. Our data indicate that mRNA transcripts for RasGRP1 were expressed at similar levels in both WT2 and V7 cells. Expression of RasGRP isoforms can be regulated at the post-transcriptional level. In particular, post-transcriptional events have been shown to disrupt expression of hRasGRP4 protein in some cell lines and human patient samples (Yang *et al.,* 2002). It is important to note that the observed loss of RasGRP1 expression in the PMA-resistant EL4 cell lines studied here did not occur in response to a genetic manipulation or a known selective pressure but was found as a naturally occurring variation within the "wild-type" (PMA-sensitive) stock in cell culture. This suggests the intriguing potential for gain

or loss of RasGRP1 under physiologic conditions. It is also interesting to note that although PMA causes the expected down-regulation of RasGRP protein in sensitive EL4 cells, there is a modest up-regulation in resistant cells (Fig. 2-4). The mechanism underlying this phenomenon, and its significance, remain to be explored. Another novel observation made in this experiment, which used a much longer time course than was used previously (Ku and Meier, 2000), concerns the delayed effects of PMA on Raf phosphorylation at 4 to 8 h. The pathway(s) responsible for these effects has not yet been determined.

Although PKC isozymes and RasGRP possess similar C1 domains, there are differences in their ligand recognition properties (Lorenzo *et al.,* 2000; Shao *et al.,* 2001; Reuther *et al.,* 2002; Rong *et al.,* 2002; Madani *et al.,* 2004; Pu *et al.,* 2005). These differences may be responsible for the distinct dose-response profiles observed for PMA-induced Erk activation in sensitive versus resistant EL4 cells (Ku and Meier, 2000), as well as for the differential effects of various PKC activators on other signaling events in these cells (Sansbury *et al.,* 1997). EL4 cell lines continue to provide a unique model system in which to examine the multiple signaling proteins involved in phorbol ester responses. In particular, the differences in endogenous RasGRP1 expression between EL4 cell lines lend themselves to further studies of the role of this signaling protein in phorbol ester and diglyceride responses.

CHAPTER THREE

REGULATORY EVENTS MODULATING PHORBOL ESTER-INDUCED RAF

ACTIVATION IN EL4 MURINE LYMPHOMA CELLS

3.1 SUMMARY

Raf kinase functions in the Ras pathway to transmit mitogenic, differentiative, and oncogenic signals to downstream kinases. Raf can be phosphorylated at multiple sites in ways that regulate its function. In this study, we used a series of EL4 lymphoma cell lines to examine signals regulating the profile of Raf phosphorylation in response to the tumor promoter phorbol 12-myristate 13-acetate (PMA). In EL4 cells, "PMA sensitivity" refers to the ability of PMA to induce growth arrest via Erk activation. We have previously shown that RasGRP, a phorbol ester receptor, confers strong Raf (Ser338)/Erk activation in the PMA-sensitive phenotype. However, even though both PMA-resistant (e.g., V7) and intermediate phenotype (e.g., V3) cells lack RasGRP, PMA-induced Raf/Erk activation is higher in V3 than in V7 cells. The current study tested the hypothesis that differences in the extent of PMA-induced Erk activation between cell lines (i.e., V7 vs. V3) are due to additional regulatory interactions. Multiple differences in both extent and duration of three types of Raf phosphorylation events, Ser338, Ser259, and Ser289/296/301, were observed between cell lines. We found that basal Akt phosphorylation is high in V7 cells, very low in WT2 cells, and at a moderate level in intermediate cells (V3). PMA-induced Raf (Ser338) and Erk activations are enhanced following inhibition of Raf (Ser259) phosphorylation by the Akt inhibitor LY294002 in all cell types tested (WT2, V3 and V7). The MEK inhibitor U0126 blocks PMA-induced Erk phosphorylation in WT2 cells, with suppression of Raf (Ser289/296/301) inhibitory phosphorylation and enhancement of Raf (Ser338) activating phosphorylation. In summary, multiple signaling events act in concert to regulate the extent and duration of PMA-induced Erk activation.

3.2 INTRODUCTION

Raf, named for *Rapidly Accelerated Fibrosarcoma*, was discovered as a retroviral oncogene, v-Raf (Rapp *et al.,* 1983) or v-MIL (Jansen *et al.,* 1983). Raf serine/threonine kinase belongs to the MAPKKK family (Klysik *et al.,* 2008). A-Raf, B-Raf, and Raf-1 (or c-Raf) are the three known isoforms of Raf in mammalian cells. While A- and B-Raf display tissue specific patterns of expression, Raf-1 is widely expressed. Raf kinase is involved in "normal" physiological processes such as cellular metabolism, cell cycle progression, and cell death, as well as in tumorigenesis. Raf-1 is frequently activated in tumors, either through overexpression or mutation (Mullen *et al.,* 2006). Thus, a comprehensive understanding of Raf regulation and its activation/ deactivation processes would be highly significant.

Raf-1 activation needs to be tightly regulated to control fundamental cellular processes. Studies focusing on Raf-1 phosphorylation document both positive and negative effects of Raf-1 phosphorylation. For example, p21 activated kinase (PAK) and Src mediate Raf-1 phosphorylation on positive regulatory sites and augment Raf-1 activity (Balan *et al.,* 2006). Negative regulation of Raf-1 has been reported, in that phosphorylation of Ser259 can inactivate Raf-1, probably by promoting the formation of a Raf autoinhibitory complex via association with 14-3-3 (Heckman *et al.,* 2005). The kinase responsible for Raf-1 Ser259 phosphorylation is a subject of considerable controversy, although several candidate kinases, including PKA, Akt, and AMP-activated kinase, have been proposed.

Through previous studies in our lab, we know that PMA-resistant and intermediate EL4 cells lack RasGRP expression and PMA-induced Ras activation. Nonetheless, the level of

Raf/Erk activation in intermediate cells is higher than that of PMA-resistant cells, which also lack RasGRP. We previously showed that the constitutive level of Akt activation is high in PMA-resistant cells, but low in PMA–sensitive cells (Knoepp, *et al.,* 1996). It has been reported that phospho-Akt can negatively regulate Raf-1 activation in some cell types (Chang *et al.,* 2003). We therefore hypothesized that the negative regulation mediated by different basal levels of phospho-Akt contributes to the distinct PMA-induced Raf/Erk activation profiles in different EL4 cell lines.

The extracellular signal-regulated kinases (Erks) are members of a subfamily of MAPKs activated via a cascade involving Ras, Raf, and MEK. Erk auto-regulation appears to be a key regulatory step of the Raf-1/Erk activation process. It has been reported that Erk-1 phosphorylates Raf-1 Ser289/296/301 and stabilizes the active form of Raf-1 by attenuating its inactivation, which contributes to the positive regulation of Raf/MEK/Erk cascade (Balan *et al.,* 2006). On the other hand, another report suggested that Raf Ser296 and 301 contribute to negative regulation of Raf-1 (Hekman *et al.,* 2005), and that phosphorylation of Ser338 blocks autoinhibition mediated by Erk (Tran *et al.,* 2005).

The elaborate network of Raf-interacting proteins, including PKCs and the inhibitory molecule RKIP, comprise key regulatory elements of the Raf/MAPK cascade (Klysik *et al.,* 2008). Raf also has the ability to interact with accessory molecules such as 14-3-3, Hsp90, and kinase suppressor of Ras (KSR). Binding of chaperonin 14-3-3 stabilizes Raf-1 in an inactive conformation (Bulow *et al.,* 2007), while HSP90 and KSR may serve to stabilize active Raf (Blagosklonny *et al.,* 2002). It has been shown that Raf-1 phosphorylated at the negative

regulatory site Ser259 can be dephosphorylated by protein phosphatase 1 (Kriegsheim *et al.,* 2006) and protein phosphatase 2A (PP2A), thus positively regulating Raf activation (Dougherty *et al.* 2005; Adams *et al.,* 2005). Protein phosphatase 5, a negative regulator of Raf-1 activation, dephosphorylates Ser338 (Kriegsheim *et al.,* 2006; Shah *et al.,* 2006).

The events that regulate Raf activation are only partially understood, but they almost certainly involve multiple phosphorylations and dephosphorylations that trigger conformational changes and exposure of the N-terminal regulatory domain of the protein (Klysik *et al.,* 2008). However, the functions of the phosphorylations and dephosphorylations of specific individual residues are not fully understood. Here we address these questions in a comprehensive manner by investigating the roles of multiple phosphorylation sites of Raf-1 in response to a single stimulus. Our results indicate that negative regulation of Raf-1 at Ser259 by PI3K/Akt and Erk-mediated feedback auto-inhibition of Raf-1 at Ser289/296/301 contribute to the different profiles of PMA-induced Raf and Erk activation in EL4 cell phenotypes.

3.3 METHODS

Cell Culture

EL4 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals). WT-derived (PMA-sensitive) clonal cell lines were maintained in suspension culture dishes; variant clones were maintained in standard tissue culture dishes.

Antibodies were obtained from the following sources: phospho-Erk, Promega (Madison, WI); Erk-1, Santa Cruz Biotechnology (Santa Cruz, CA); PTEN, p-PTEN, phospho-Akt, Akt, phospho-c-Raf (Ser259), phospho-c-Raf (Ser289/296/301), phospho-c-Raf (Ser338), and c-Raf, Cell Signaling Technology Inc. (Beverly, MA).

EL4 cells were treated with and without 100nM PMA as indicated. After treatment, cells were collected; adherent cells were harvested using a cell scraper. Cells were lysed (for components of lysis buffer, refer to Chapter 2) and extracts were sedimented by centrifugation at 10,000*g* for 10 min at 4°C to remove insoluble material. Samples equalized for protein (100 μg), as determined by Coomassie Protein Assay (Pierce), were separated by SDS-PAGE on 10% Laemmli gels, transferred to PVDF paper, and incubated with antibodies. Blots were developed using enhanced chemiluminescence reagents (Amersham).

3.4 RESULTS

PMA-induced Acute Raf Phosphorylation in Clonal EL4 Cell Lines

Time course experiments were performed with several EL4 cell lines to examine the extent of PMA-induced Raf phosphorylation (Fig. 3-1). As shown in Fig. 3-1 A, PMA induces strong Raf Ser338 phosphorylation in WT2 cells that begins within 5 minutes and is maximal at 30 minutes. In V7 cells, the weaker peak of Raf Ser338 phosphorylation induced by PMA occurs at 15 minutes and is attenuated within 30 minutes (Fig. 3-1 B).

Raf Ser259 is phosphorylated in the resting state in all cells tested (Fig. 3-1). Both basal and

PMA-induced Raf Ser259 phosphorylations are stronger in V7 than in WT2 cells. Raf Ser259 phosphorylation decreases after 15 minutes of PMA treatment in WT2 cells, in contrast to the decline after 30 minutes of PMA stimulation in V7 cells. Raf Ser289/296/301 phosphorylation is much more robust in WT2 than in V7 cells. Raf Ser289/296/301 phosphorylation starts within 5 minutes of PMA treatment, and is maintained at high level for at least 60 minutes in WT2 cells. In V7 cells, the phosphorylation is very weak but follows a similar time course. In both V3 and V10 cell lines, which are intermediate phenotypes, the levels of PMA-induced Raf Ser259, Ser289/296/301 and Ser338 phosphorylation are between those of WT2 and V7 cells (Fig. 3-1 C, D). The responses of V3 and V10 are not identical, but in both cases are "intermediate". Taken together, these data suggest that the patterns of PMA-induced phosphorylation of multiple Raf residues are distinct in the different EL4 cell phenotypes.

PMA-induced Long-term Raf Phosphorylation in Clonal EL4 Cell Lines

Since PMA-induced responses in EL4 cells extend beyond 60 minutes (Han *et al.,* 2007), we tested the long-term effects of PMA on Raf phosphorylation/dephosphorylation. Phosphorylation at Ser 259 occurs as Ser338 is being dephosphorylated in both WT2 and V7 cells (Fig. 3-2 A, B). In WT2 cells, PMA-induced Raf Ser338 phoshorylation is maintained for 4 hours at high intensity and then declines to basal levels at 8 hours after PMA addition (Fig. 3-2 A). In V7 cells, PMA-induced Raf Ser338 phoshorylation is very weak but also disappears after 8 hours of PMA stimulation (Fig. 3-2 B). The basal level of Raf Ser259 phoshorylation is higher in V7 than WT2 cells, which supports a negative regulatory effect of phospho-Ser259. Following an initial

A.

WT2 Cells

(PMA-sensitive phenotype)

p-Raf (Ser259)
p-Raf (Ser289/296/301)
p-Raf (Ser338)
c-Raf

0 5 15 30 60   PMA (minutes)

B.

V7 Cells

(PMA-resistant phenotype)

p-Raf (Ser259)
p-Raf (Ser289/296/301)
p-Raf (Ser338)
c-Raf

0 5 15 30 60   PMA (minutes)

C.

V3 Cells

(intermediate phenotype)

p-Raf (Ser259)
p-Raf (Ser289/296/301)
p-Raf (Ser338)
c-Raf

0 5 15 30 60   PMA (minutes)

D.

V10 Cells

(intermediate phenotype)

p-Raf (Ser259)
p-Raf (Ser289/296/301)
p-Raf (Ser338)
c-Raf

0 5 15 30 60   PMA (minutes)

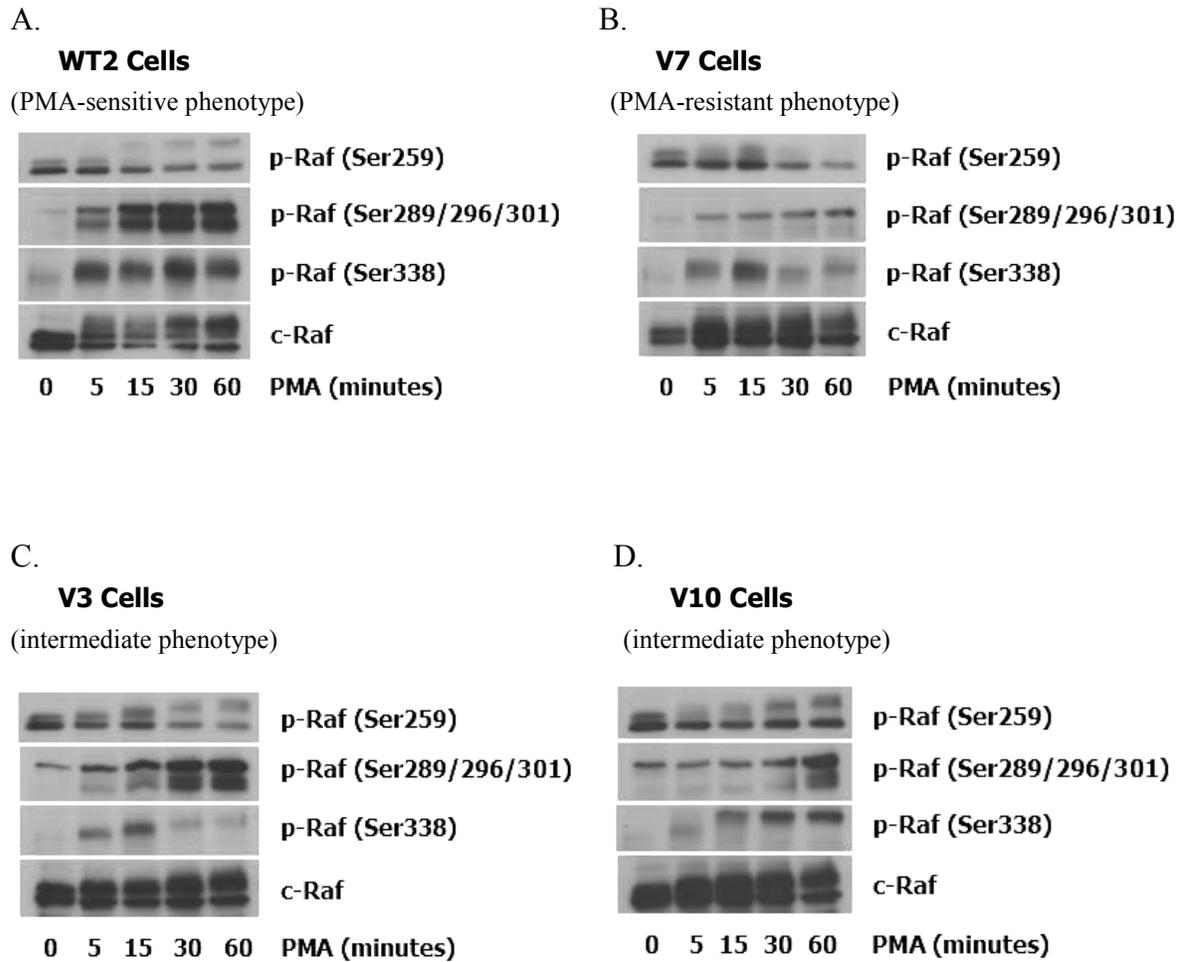**Figure 3-1. Time course of the effects of PMA on Raf phosphorylation in clonal EL4 cell lines.**

WT2 (A), V7 (B), V3 (C), and V10 (D) cells were treated with or without 100 nM PMA for the indicated times. Immunoblots were performed on whole-cell lysates for phospho-Raf (Ser259, Ser289/296/301, Ser338), and c-Raf. All incubations were done in the same experiment; blots for the four cell lines were exposed in parallel.

decrease in Raf Ser259 phoshorylation, it reappears after 3-4 hours in both WT2 and V7 cells and then gradually increases. Raf Ser289/296/301 is phosphorylated by 1 hour after PMA addition, and persists for 8 hours in both WT2 and V7 cells. The magnitude of this response is much higher in WT2 than V7 cells, consistant with the higher level of Erk activation observed in WT2 cells. These data establish that multiple residues are phosphorylated and dephosphorylated in a coordinated manner to regulate the Raf activation process.

In blots for total Raf, multiple species appear between 1 and 8 hours of PMA treatment. In order to differentiate the effects of phosphorylation of specific residues with respect to Raf "gel shifts", we prepared cell extracts treated with PMA for 3 hours (which show the full spectrum of bands), ran them on a single gel, and blotted for phospho-Raf (Ser259, Ser289/296/301, Ser338) and c-Raf. We find that Raf phosphorylated on Ser289/296/301 has the slowest migration on SDS-PAGE, and appears as the top two bands. Raf phosphorylated on Ser259 has the fastest mobility on gel, and appears as the bottom two bands. Raf phosphorylated on Ser338 is the single band in the middle (Fig. 3-2 C). Taken with the phosphorylation data, these results explain the differences in gel mobility of c-Raf between cell lines (e.g. Fig. 3-1).

Regulation of PMA-induced Raf/Erk Phosphorylation by PI3K/Akt Signaling Pathway

In order to test whether the PI3K/Akt signaling pathway is involved in regulation of the Raf/Erk cascade in EL4 cells, we examined basal Akt phosphorylation in representative EL4 cells. We find that there is strong constitutive Akt phosphorylation in PMA-resistant cells (V7), while basal phospho-Akt is low in PMA-sensitive cells (WT2), and is at a moderate level in

A.

WT2 Cells

phospho-Raf (Ser259)

phospho-Raf (Ser289/296/301)

phospho-Raf (Ser338)

c-Raf

0 1 2 3 4 8 12 24 36 48   PMA (100nM, hours)

B.

V7 Cells

phospho-Raf (Ser259)

phospho-Raf (Ser289/296/301)

phospho-Raf (Ser338)

c-Raf

0 1 2 3 4 8 12 24 36 48   PMA (100nM, hours)

C.

Inhibitory   Activation

Auto-regulatory

c-Raf   p-S259   p-S301   p-S338

S301
S338
S259

WT2 V7   WT2 V7   WT2 V7   WT2 V7   PMA (3 hours)

Figure 3-2. Long-term effects of PMA on Raf phosphorylation in clonal EL4 cell lines.
WT2 (A) and V7 (B) cells were incubated for the indicated times with 100 nM PMA. Whole-cell extracts, equalized for protein, were immunoblotted for phospho-Raf (Ser259, Ser289/296/301, Ser338), and c-Raf. C. WT2 and V7 cells were treated with PMA for 3 hours. Whole-cell extracts, equalized for protein, were run on the same gel, blotted for phospho-Raf (Ser259, Ser289/296/301, Ser338), and c-Raf to show the physical location of multiple Raf species. All incubations were done in the same experiment; the blots were exposed in parallel.
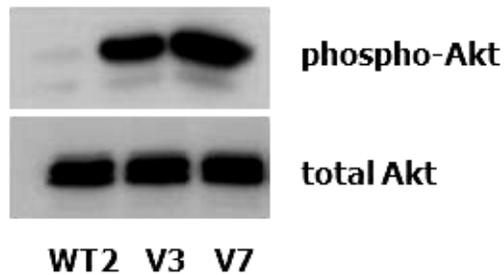
intermediate cells (V3) (Fig. 3-3 A).

To test whether constitutive Akt phosphorylation is affected by prolonged PMA treatment, we performed a time course examing phospho-Akt levels upon PMA stimulation (Fig. 3-3 B). The weak constitutive phospho-Akt signal decreased further upon PMA treatment in WT2 cells. PMA had no detectable effect on Akt phosphorylation in V7 cells.
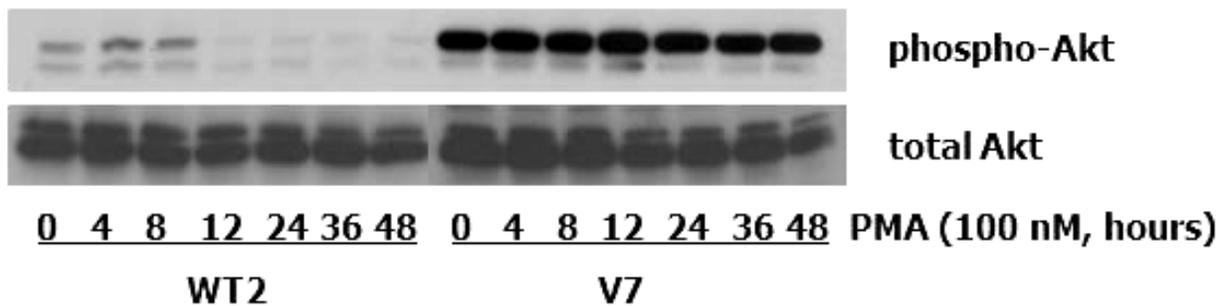
To detect whether constitutive Akt phosphorylation is mediated by the PI3K pathway, we treated V7 cells with LY294002, a PI3K inhibitor. The data show that incubation for two hours with 50μM LY294002 is sufficient to fully block constitutive Akt phosphorylation (Fig. 3-3 C). This condition was used in subsequent experiments. Thus, constitutive Akt phosphorylation in EL4 cells is mediated by PI3K and can be blocked by 2 hours treatment with 50μM LY294002.

It has been reported that a negative feedback loop involving Raf and Akt exists, in which Akt phosphorylates Raf Ser259 and thereby inhibits the Raf/Erk signaling pathway (Chang *et al.,* 2003). To test whether this situation exists in EL4 cells, we examined the effects of LY294002 on PMA-induced Raf/Erk activation. PMA treatment did not alter Akt phosphorylation after 15 minutes. As shown in Figure 3-4 A, negative regulation of Erk activation by PI3K/Akt exists in EL4 cells. There is enhanced Raf Ser338 and Erk activation in all of the EL4 cells tested when the PI3K/Akt pathway is blocked by LY294002, Raf and Erk are activated by PMA to a more similar level between resistant and intermediate cells after LY294002 treatment (Fig. 3-4 A, B, C), although the response is still not as strong as that seen in PMA-sensitive cells. To further examine this effect, we examined the time course of PMA-induced Raf (Ser259, Ser289/296/301, Ser338) and Erk phosphorylation with and without LY294002 treatment. As shown in Figure 3-5,

A.

phospho-Akt

total Akt

WT2 V3 V7

B.

phospho-Akt

total Akt

0 4 8 12 24 36 48   0 4 8 12 24 36 48   PMA (100 nM, hours)

WT2   V7

C.

V7 Cells

phospho-Akt

total Akt

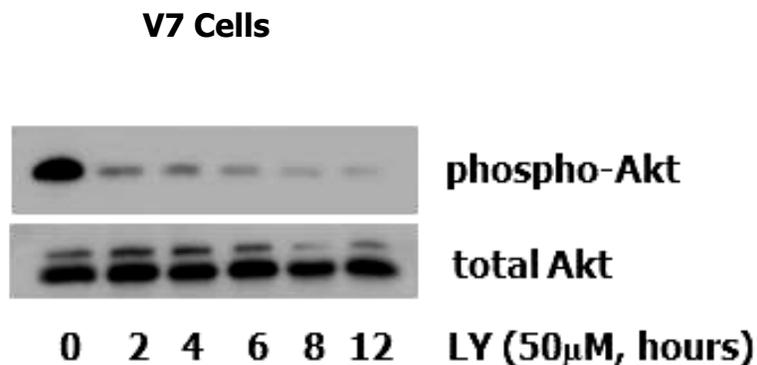0   2   4   6   8 12   LY (50μM, hours)

Figure 3-3. Constitutive Akt phosphorylation in clonal EL4 cell lines.

A. Basal Akt phosphorylation in WT2, V3, and V7 cells. whole cell extracts, equalized for protein, were immunoblotted for phospho-Akt and total Akt.

B. Time course of PMA on constitutive Akt phosphorylation in WT2 and V7 cells. WT2 and V7 cells were incubated with 1000 nM PMA for indicated times. Whole-cell extracts, equalized for protein, were immunoblotted for phospho-Akt and total Akt.

C. Time course of the effects of LY294002 on constitutive Akt phosphorylation in V7 cells. V7 cells were incubated with 50 μM LY294002 for indicated times. Whole-cell extracts, equalized for protein, were immunoblotted for phospho-Akt and total Akt.

A.
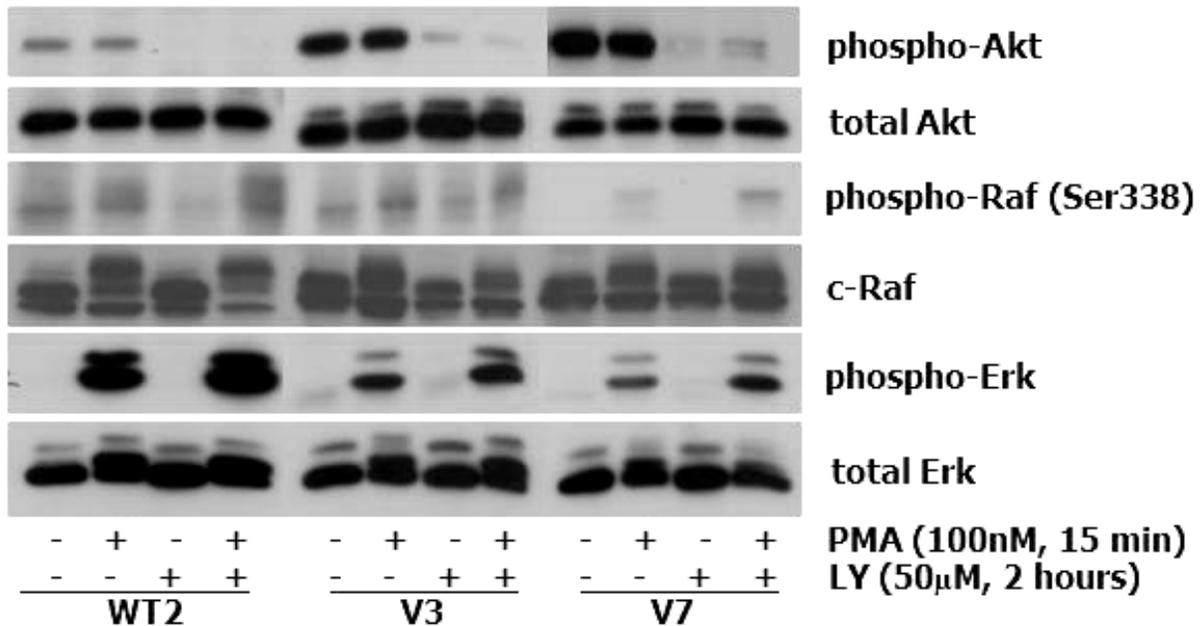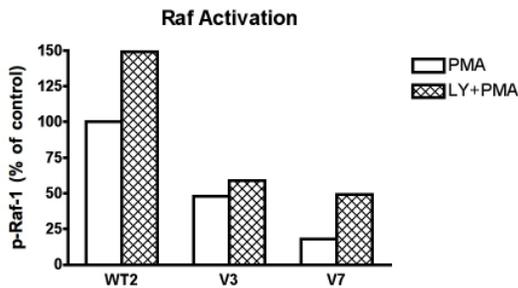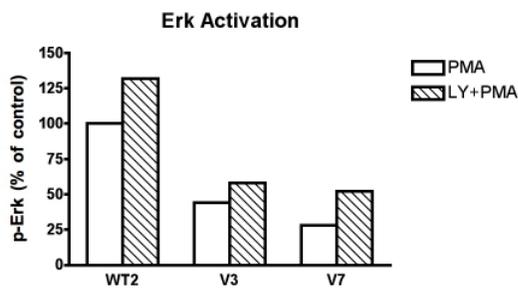
Figure 3-4. The effects of LY294002 on Raf and Erk activation in clonal EL4 cell lines.

A. Effects of LY294002 treatment on Akt phosphorylation and PMA-induced Raf and Erk phosphorylation in representative EL4 cells. The indicated cell lines were incubated in the absence and presence of 50 μM LY294002 for 2 hours, and then treated with or without PMA for 15 minutes. Whole-cell extracts, equalized for protein, were immunoblotted for phospho-Akt, total Akt, phospho-Raf (Ser338), c-Raf, phospho-Erk, and total Erk.

B. Data from panal A were qualified by densitometry and expressed as activated Raf (Ser338) in each cell line upon PMA treatment with/without LY294002 incubation. WT2 treated with PMA was defined as 100% for quantification.

C. Data from panal A were quantified by densitometry and expressed as activated Erk in each cell line upon PMA treatment with/without LY294002 incubation. WT2 treated with PMA was defined as 100% for quantification.

V7 Cells
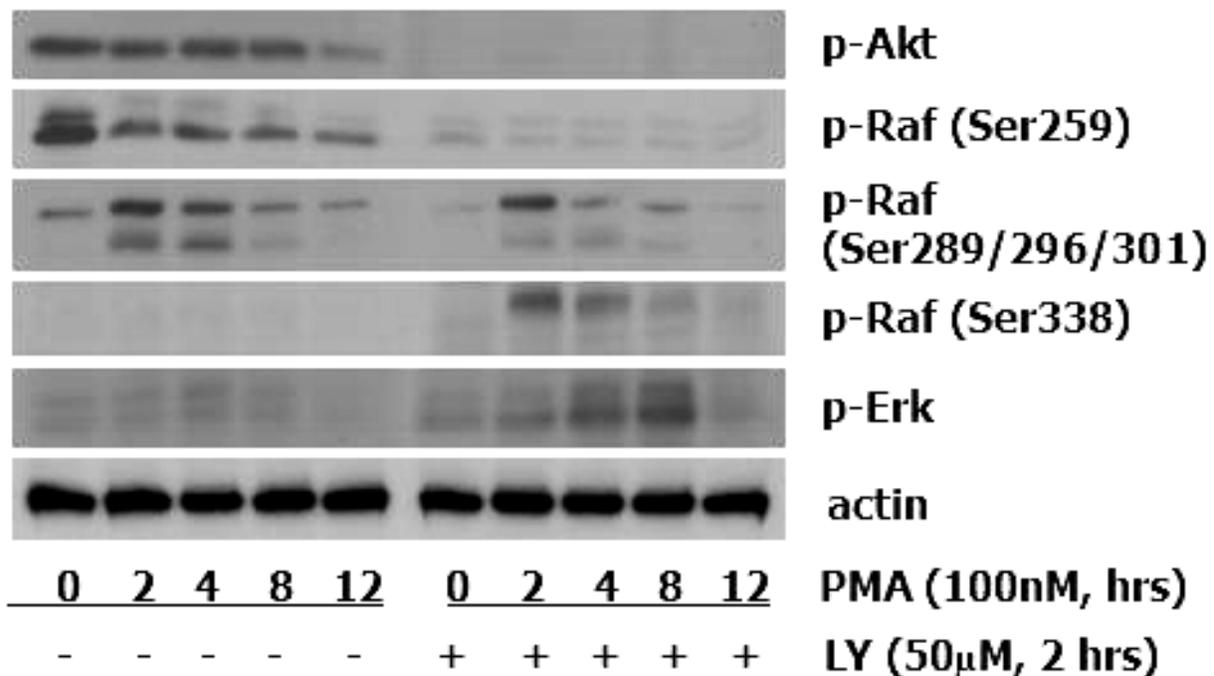
Figure 3-5. Time course of the effects of LY294002 on PMA-induced Raf and Erk phosphorylation in V7cells.
Time course of the effects of LY294002 on PMA-induced Raf and Erk phosphorylation in V7 cells. V7 cells were incubated in the absence and presence of 50 μM LY294002 for 2 hours, and then treated with PMA for the indicated time.   Whole-cell extracts, equalized for protein, were immunoblotted for phospho-Akt, phospho-Raf (Ser259, Ser289/296/301, Ser338), phospho-Erk, and total Erk.

phosphorylation of Raf Ser259 is blocked following the inhibition of phospho-Akt by LY294002,with concomitant enhancement of Raf Ser338 and Erk phosphorylation. There is attenuation of the lower band of phospho-Raf Ser289/296/301 upon LY294002 treatment, which is likely because this species is dually phosphorylated on Ser259 and Ser289/296/301. These results demonstrate that PI3K/Akt signaling pathway negatively regulates the Raf/Erk cascade by phosphorylation of Raf Ser259.

PTEN and Constitutive Akt Activation in PMA-resistant Cells

To test whether PTEN inactivation is responsible for the relatively high level of basal phospho-Akt in V7 cells, PTEN expression was analized by immunoblotting in representative EL4 cells. There is no significant difference between PMA-sensitive and -resistant EL4 cells with respect to total PTEN and phospho-PTEN. PMA does not affect PTEN phosphorylation within 15 minutes (Fig. 3-6). Thus, differences in PTEN expression are unlikely to be responsible for differences in constitutive Akt phosphorylation between EL4 cell lines.

Erk Feedback Regulation of PMA-induced Raf/Erk Signaling Cascade

In order to further test the role of feedback auto-regulation by Erk on the Raf/Erk pathway, we used U0126, a MEK inhibitor, to block Erk activation. We used WT2 cells, which have the strongest PMA-induced Erk activation, for this experiment to facilitate detection of U0126-induced changes. As shown in Figure 3-7 A, PMA-induced Erk activation can be completely blocked by incubation with 10μM U0126 for 2 hours. This condition was used in

subsequent experiments. We performed a long-term time course of PMA-induced Raf phosphorylation with and without U0126 treatment (Fig. 3-7 B). PMA-induced Erk activation is completely blocked by U0126. This effect is accompanied by the attenuation of Raf phosphorylation on Ser289/296/301 and by enhanced Raf Ser338 phosphorylation (both amplitude and duration). The phosphorylation of Raf Ser259 is not affected by U0126 treatment. The disappearance of the lower band in the presence of U0126 likely reflects the loss of dual phosphorylation on Ser289/296/301. These results confirm that Erk activation has an auto-inhibitory effect on Raf, via phosphorylation of Ser289/296/301.
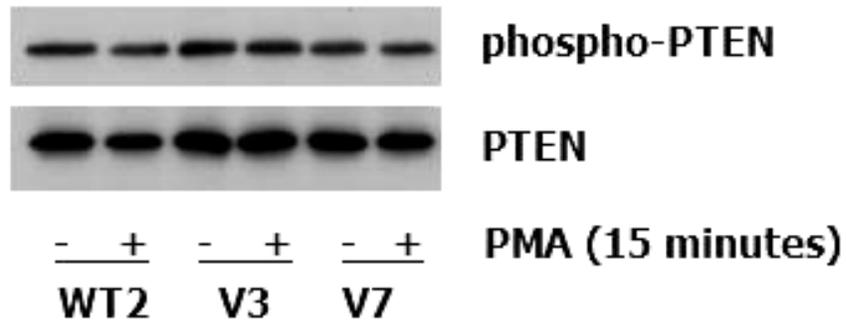
Figure 3-6. Basal PTEN and phospho-PTEN expression in clonal EL4 cell lines.
Cells were treated with or without 100nM PMA for 15 minutes. Immunoblots were performed on whole-cell lysates for PTEN and phospho-PTEN.

A.

WT2 Cells

phospho-Erk

total Erk

| - | + | - | + | + | PMA (100 nM, 15 minutes) |
| - | - | 1 | 1 | 2 | U0126 (10 µM, hours) |

B.

WT2 Cells

p-Raf (Ser259)

p-Raf (Ser289/296/301)

p-Raf (Ser338)

p-Erk

total Erk

| 0 | 1 | 2 | 4 | 8 | 12 | 0 | 1 | 2 | 4 | 8 | 12 | PMA (100nM, hrs) |
| - | - | - | - | - | - | + | + | + | + | + | + | U0126 (10µM, 2 hrs) |

Figure 3-7. The effects of U0126 on PMA-induced Raf and Erk phosphorylation in WT2 cells.

A. Effects of U0126 treatment on PMA-induced Erk phosphorylation in WT2 cells. WT2 cells were incubated in the absence and presence of 10 µM U0126 for the indicated time, and then treated with or without PMA for 15 minutes. Whole-cell extracts, equalized for protein, were immunoblotted for phospho-Erk and total Erk.

B. Time-course of the effects of U0126 on PMA-induced Raf and Erk phosphorylation in WT2 cells. WT2 cells were incubated in the absence and presence of 10 µM U0126 for 2 hours, and then treated with PMA for the indicated times. Whole-cell extracts, equalized for protein, were immunoblotted for phospho-Raf (Ser259, Ser289/296/301, Ser338), phospho-Erk and total Erk.

3.5 DISCUSSION

In this study, we further explored the signals that contribute to the different EL4 cell phenotypes. We demonstrate that negative regulation of Raf Ser259 by the PI3K/Akt pathway plays a role in the differences in PMA-induced Raf/Erk activation between PMA-resistant and intermediate cells. In addition, Erk mediated feedback auto-inhibition at Raf Ser289/296/301 contributes to the extent and duration of PMA-induced Raf Ser338 activation. This study illustrates that phosphorylation of different residues on Raf contribute to the Raf activation/deactivation process, and that multiple signals are involved in this scenario.

Despite intensive investigations, the mechanism of Raf regulation has still not been completely elucidated. Phosphorylation/dephosphorylation of Raf plays a crucial role in the Raf activation process. Phosphorylation of Raf Ser338 correlates with Ras-mediated stimulation and is required for Raf activation (Hekman *et al.,* 2005). The activity of Raf-1 is negatively regulated by phosphorylation of Ser259, and Raf-1 activation is accompanied by dephosphorylation of this site (Tran *et al.,* 2005). Several studies have shown that phosphorylation of Raf Ser259 by protein kinase A results in negative regulation of Raf-1 (Bulow *et al.,* 2007; Dumaz *et al.,* 2002; Dhillon *et al.,* 2002). Other investigations have suggested that the inhibitory cross-talk, mediated by phosphorylation of Raf Ser259 by PI3K/Akt, is required for the differentiation of vascular smooth muscle and intestinal epithelial cells (Reusch *et al.,* 2001; Laprise *et al.,* 2004). Our data support that the phosphorylation of Ser259, via the PI3K/Akt pathway, negatively regulates Raf Ser338 phosphorylation and subsequent activation of Raf-1. As shown in Figure 3-4, basal Akt activity and phosphorylation of Raf Ser259 can be blocked by the PI3K inhibitor LY294002,

resulting in enhanced Raf Ser338 and Erk activation. Raf and Erk were activated by PMA to a greater extent in resistant and intermediate cells in the presence of LY294002, although the extent was still not as strong as that seen in PMA-sensitive cells (Fig. 3-4 A, B, C). This is consistant with our previous finding that RasGRP expression is largely responsible for the high level of PMA-induced Erk activation in PMA-sensitive EL4 cells. It needs to be mentioned that Ser259 phosphorylation does not directly interfere with Raf-1 activity but rather regulates its activity through the binding competition between Ras and 14-3-3 (Light *et al.,* 2002). Together, these data indicate that the negative regulation mediated by different levels of basal phospho-Akt activity, at least partially, contribute to the distinct patterns of PMA-induced Raf/Erk activation between PMA-resistant and intermediate EL4 cells.

To test whether constitutive Akt phosphorylation is affected by PMA treatment in EL4 cells, we performed a long-term time course of Akt phosphorylation level upon PMA stimulation (Fig. 3-3 B). The weak basal phospho-Akt further decreased within 12 hour of PMA treatment in WT2 cells. This result could be consistent with our previous report that WT2 cells undergo growth arrest upon long-term PMA incubation (Sansbury *et al.,* 1997). It is worth further study to determine whether decreased phopho-Akt in WT2 cells is due to the onset of growth arrest, or whether the reduced phospho-Akt contributes to the growth arrest of WT2 cells.

The activation of PI3K is countered by phophoinositide phosphatases (Wymann *et al.,* 2003). Numerous human malignancies are associated with inactivating mutations in the gene Phosphatase and Tensin homologue deleted on chromosome 10 (PTEN), leading to deregulated hyperactivity of Akt (Blume-Jensen *et al.,* 2001). PTEN, a tumor suppressor gene, hydrolyses

PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, removing the 3-phosphate from PI3K lipid product. Our results shown that the expression of PTEN does not differ between different EL4 cell phenotypes (Fig. 3-6), and is therefore not likely to be responsible for differences in constitutive Akt activity between EL4 cells. The mechanism responsible for constitutive Akt phosphorylation in EL4 cells requires additional investigation.

It has been reported that Raf Ser296 and 301 contribute to negative regulation of Raf-1 (Hekman *et al.,* 2005), and that phosphorylation of Ser338 in response to Ras activation blocks the autoinhibition mediated by Erk (Tran *et al.,* 2005). However, Erk activation can also provide a novel mechanism for a positive feedback of Raf-1 regulation (Balan *et al.,* 2006). In our study, we find that simultaneous phosphorylations of Ser338 and Ser289/296/301 occur during the same period of time (Fig. 3-2 and 3-7). In other words, our results indicate that phosphorylation of Ser289/296/301 by Erk auto-regulation counteracts the phosphorylation of Ser 338, which may be a key mechanism for preventing the overamplification of a proliferative signal. A previous study indicated that in Swiss 3T3 cells, the MEK inhibitor PD098059 increases Raf-1 activation by PDGF but not by EGF (Alessi *et al.,* 1995). In this regard, it is possible that Erk feedback auto-inhibition is a mitogen-dependent phenomenon. Our findings add another layer of complexity to the Raf-1 regulation mechanism and suggest that varying Erk activation levels in the cell can modulate the output from Raf-1. It is reported that Erk2 has been positively associated with proliferation, while Erk1 may inhibit the effects of Erk2 (Pouyssegur *et al.,* 2002). It is therefore worth further investigation to determine whether the feedback inhibition of Raf is mediated by Erk1 instead of Erk2.

In summary, the work described in this chapter has provided an unusually detailed profile of the upstream phosphorylation events involved in PMA-mediated Erk activation.

CHAPTER FOUR

ROLE OF RAF KINASE INHIBITOR PROTEIN IN EL4 CELL PHENOTYPE

4.1 SUMMARY

Raf kinase inhibitory protein (RKIP; also known as phosphatidylethanolamine-binding protein or PEBP), an inhibitor of Raf-mediated activation of extracellular signal-regulated kinase (Erk), is expressed at a higher level in PMA-resistant V7 cells than in the PMA-sensitive WT2 and intermediate V3 cells. We examined the role of RKIP in EL4 cell phenotype. Immunoblotting was used to detect RKIP expression and Raf-1/Erk phosphorylation in WT2, V3 and V7 cells, which are representative PMA-sensitive, intermediate and –resistant EL4 cells. Proliferation, migration and invasion assays were performed in V7 cells treated with and without RKIP siRNA. The results show that RKIP inhibits Raf Ser338 phosphorylation and downstream Erk activation. PMA-induced Raf Ser338 and Erk phosphorylations are enhanced in V7 cells treated with RKIP siRNA. RKIP depletion by siRNA increases Raf Ser289/296/301 phosphorylation, reflecting enhanced Erk feedback phosphorylation. These results indicate that selective regulation of Raf-1 at Ser 338 and Ser 289/296/301 by RKIP limits the dynamic range of Erk MAPK activation in response to PMA. Down-regulation of RKIP in V7 cells does not affect cell proliferation, but is associated with decreased *in vitro* migration and invasion. We conclude that RKIP modulates Raf/MAPK activation in EL4 cells, and contributes to the enhanced migration and invasion potential of PMA-resistant EL4 cells.

4.2 INTRODUCTION

RKIP, also known as phosphatidylethanolamine-binding protein (Bernier *et al.,* 1986), is a ubiquitously expressed and highly conserved protein (Trakul *et al.,* 2005) whose function has

remained largely enigmatic. In recent years, there has been an increased interest in RKIP due to the discovery of its ability to influence intracellular signaling cascades, cell cycle regulation, differentiation, programmed cell death, and metastasis (Keller *et al.,* 2004; Trakul *et al.,* 2005).

RKIP has been shown to modulate the Raf/MEK/Erk cascade (Sasaki *et al.,* 2003; Matheny *et al.,* 2004; Wakioka *et al.,* 2001). Previous studies have suggested that RKIP inhibits activation of Raf-1 by blocking phosphorylation of Raf-1 by p21-activated kinase (PAK) and Src kinases (Trakul *et al.,* 2005). RKIP binds to Raf-1 by competitive interference with MEK binding to Raf (Yeung *et al.,* 2000; Zhu S. *et al.,* 2005), since the binding sites for MEK and Raf-1 overlap on RKIP (Klysik *et al.,* 2008; Trakul *et al.,* 2005). Thus, the current state of knowledge suggests that RKIP inhibits Raf-1 activation as well as the ability of Raf-1 to phosphorylate its substrate MEK, indicating that RKIP may regulate the Raf-1/MEK/Erk pathway at multiple levels (Park *et al.,* 2006). It was reported that protein kinase C, activated by either phorbol ester or epidermal growth factor (EGF), phosphorylates RKIP at Ser 153, and that this phosphorylation causes dissociation of RKIP from Raf-1 and subsequent activation of the Erk pathway (Klysik *et al.,* 2008; Corbit *et al.,* 2003). However, the role of RKIP in EL4 cells and other hematopoietic cells has not been examined previously.

RKIP is a modulator of cell signaling that functions as an endogenous inhibitor of multiple kinases at multiple levels. In addition to Raf-1, B-Raf and two other members of the MAPKKK family, TAK1 and NIK, are also targets of RKIP. Additional research indicates that RKIP also binds MEK and Erk (Klysik *et al.,* 2008). When phosphorylated by protein kinase C, RKIP binds to and inhibits GRK2 (G protein coupled receptor kinase-2), a serine/threonine kinase that acts as

a negative regulator of G-protein coupled receptors (Lorenz *et al.,* 2003; Klysik *et al.,* 2008). Grk2 also plays a role in cell locomotion in some cell types (Lijima *et al.,* 2002). For example, GRK2 loss is correlated with increased cell locomotion in leukocytes (Vroon *et al.,* 2004). RKIP can also target IKKs, upstream kinase activators of IκB kinase (Yeung *et al.,* 2001; Klysik *et al.,* 2008), and thus negatively regulate the NF-κB pathway (Yeung *et al.,* 2001). It has been reported that RKIP contributes to the monocytic differentiation process via inhibition of NF-κB signaling, independent from effects on the Ras/Raf/MEK/Erk pathway (Schuierer *et al.,* 2006). Thus, RKIP can influence NF-κB-regulated processes such as those that mediate production of cytokines, cytokine receptors, and cell adhesion molecules, as well as cancer and invasion.

RKIP can potentially regulate cell division through inhibition of Raf-1. RKIP depletion leads to a decrease in mitotic index, and MEK inhibition can rescue the effects of RKIP depletion (Eves *et al.,* 2006), which indicates that the anti-mitotic response is due to uncontrolled activation of Raf/MEK/Erk pathway. As a negative regulator that controls the amplitude and dose response of Raf-1 kinase activity rather than the absolute on or off state, RKIP moderates the extent of Erk activation. It is likely that RKIP plays a regulatory role in the G1-S and G2 stages of the cell cycle as well as in mitosis (Rosner *et al.,* 2007). RKIP can indirectly influence the Aurora B kinase and spindle checkpoints, and thus profoundly impact the fidelity of the cell cycle (Eves *et al.,* 2006).

Recently, RKIP has been identified as a metastasis suppressor gene; this function correlates with regulation of Erk activity (Fu *et al.,* 2003). The report by Fu indicates that cell lines derived from metastatic prostate cancers have decreased levels of RKIP mRNA and protein as compared

with primary tumor cell lines. A decrease in RKIP was also detected in melanoma and breast cancer. The increased metastasis potential elicited by RKIP depletion is due, at least in part, to elevated Erk activity (Schuierer *et al.,* 2004; Hagan *et al.,* 2005). Loss of RKIP expression could act as a marker of tumor relapse, distant metastasis and prognosis in colorectal cancer (Minoo *et al.,* 2007; Al-Mulla *et al.,* 2007) and prostate cancer (Fu et al., 2005). RKIP can reverse cancer cell resistance to chemotherapy in prostate and breast carcinoma cell lines (Chatterjee *et al.,* 2004). RKIP may serve as an immune surveillance gene, since its low expression or absence allows tumors to escape host immune cytotoxic effector cells (Baritaki *et al,* 2007). Furthermore, over-expression of RKIP in metastatic cancer cells can decrease their invasiveness. Treatment of breast and prostate cancer cells with chemotherapeutic agents induces RKIP expression and predisposes these cancer cells to apoptotic death (Chatterjee *et al.,* 2004; Jazirehi *et al.,* 2004).

On the other hand, there is substantial evidence that RKIP contributes to enhanced metastasis. It is reported that RKIP knockdown suppresses cell motility in Madin-Darby canine kidney (MDCK) epithelial cell monolayers, while RKIP overexpression promotes cell migration (Bement *et al.,* 2005). RKIP expression was found to be higher in a tumorigenic and metastatic murine fibrosarcoma cell line than in the weakly tumorigenic and nonmetastatic parental cell line from which it was derived (Hayashi *et al.,* 2005). It has been reported that RKIP is involved in the positive regulation of epithelial cell migration. There is no report regarding the function of RKIP in hematopoietic cells so far to our knowledge.

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that localizes to focal contact sites and has been linked to the survival, proliferation, and motility signals of cells. Many

stimuli can activate the catalytic activity of FAK, including integrin clustering, growth factors, reagents that stimulate G-protein coupled receptors, and mechanical stimuli (Hanada *et al.,* 2005). Binding of integrins to extracellular matrix proteins results in FAK phosphorylation (Sieg *et al.,* 2000; Schaller, 2001). It is increasingly recognized that over-expression of FAK is linked to the invasive properties of cancer cells (Hunger-Glaser *et al.,* 2004).

Elucidating the mechanism of RKIP action is important both for a complete understanding of Raf regulation and generation of potential therapeutic reagents. The purpose of the study described in this chapter was to test how RKIP regulates PMA-induced Raf-1/Erk phosphorylation, and whether RKIP is involved in the enhanced metastasis potential of PMA-resistant EL4 cells. We hypothesized that RKIP plays a role in regulating migration and invasion of EL4 cells. The following were examined: 1) RKIP expression in representative EL4 cell lines; 2) the effects of RKIP on Erk activation, including phosphorylation of specific residues of Raf-1; and 3) the contributions of RKIP to *in vitro* migration and invasion of V7 cells. Our findings support a role for RKIP in the regulation of V7 cell metastasis.

4.3 METHODS

Cell Culture

EL4 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, or Summit Biotechnology, Fort Collins, CO). WT2 cells were maintained in suspension culture dishes (Corning) to discourage selection for adherent cells; other clones were maintained in standard tissue culture dishes.

Immunoblotting

Antibodies were obtained from the following sources: phospho-Erk, Promega (Madison, WI); Erk-1, Santa Cruz Biotechnology (Santa Cruz, CA); FAK, phospho-FAK (Tyr397), RKIP and phospho-c-Raf (Ser259), phospho-c-Raf (Ser289/296/301), phospho-c-Raf (Ser338), c-Raf, Cell Signaling Technology Inc. (Danvers, MA). EL4 cells were treated with and without PMA as described. Equalized protein of whole cell lysates were separated by SDS-PAGE on 10% Laemmli gels, transferred to polyvinylidene difluoride paper, incubated with antibodies, and developed using enhanced chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

siRNA Experiments

RKIP siRNA, control siRNA, siRNA transfection reagent, and transfection medium were obtained from Santa Cruz Biotechnology. Incubation with siRNA was carriered at as described in Chapter two. Protein levels for RKIP, phospho-Raf (Ser259, Ser289/296/301, Ser338), c-Raf, phospho-Erk, and total Erk (immunoblotting) were assessed in cell extracts using the methods described above.

Cell Proliferation Assays

For cell proliferation assays, growing cells ($\geq$95% viability) were seeded in 24-well tissue culture plates at 2×10^5 cells/well with 2 ml of complete medium (including serum). Cells were incubated with 100 nM PMA or 0.1% ethanol (vehicle) at 37°C for varying times in complete

medium. Cell number was determined by mixing cells with 0.02% trypan blue in phosphate buffered saline (PBS) and counting dye-excluding cells using a hemocytometer.

Cell Migration Assay

Migration was assayed using collagen IV-coated inserts (8-µm pore size, BD Falcon) in 6-well plates. 2 ml of RPMI containing 2×10^5 cells with indicated treatment were added to the upper chamber. The lower chamber contains 2.5 ml of RPMI with 10% FBS. The inserts were incubated overnight at 37 °C. The non-migrating cells on the upper surface of the membrane were then removed with a cotton swab. The cells that were able to migrate through the pores onto the lower surface of the membrane were fixed, stained with crystal blue (Scholar Chemistry, West Henrietta, NY), and counted.

Cell Invasion Assay

In vitro cell invasion was determined using a modified Boyden chamber method. EL4 cells $(2 \times 10^5$ cells) with the indicated treatments were added to the upper level of the chamber in serum-free media. To the lower wells, media with 10% fetal bovine serum (FBS) were added. Invasion was measured as the ability of cells to pass through Matrigel, an artificial basement membrane matrix. The membranes were fixed and stained with crystal blue. Cells that had invaded into the lower wells were counted using a microscope.

Proliferation data were expressed as mean $\pm$ SD, and were assessed with analysis of variance (ANOVA). Migration and invasion data were analysed with Chi-squared test.

4.4 RESULTS

RKIP Expression in EL4 Phenotypes

First, we tested basal levels of RKIP expression by immunoblotting in representative EL4 cell lines. The basal level of RKIP is higher in V7 cells as compared to WT2 and V3 cells (Fig. 4-1, A).

To examine whether RKIP expression was affected by PMA treatment, we treated V7 cells with PMA for varying times and then immunoblotted for RKIP. Consistent with Figure 4-1 A, basal RKIP expression in V7 cells is the highest among the EL4 clones tested. RKIP protein levels were slightly enhanced upon PMA treatment compared, with basal level, in all tested EL4 phenotypes; the enhancement follows the same pattern as of downstream Raf Ser338 and Erk activation (Han *et al.,* 2007) (Fig. 4-1, B). However, since the increase in RKIP is observed within 5 minutes, it is not likely to reflect an increase in protein expression. Rather it could reflect release of pre-existing RKIP from a Triton-X-insoluble compartment (i.e., "translocation"). Alternatively, PMA-induced RKIP phosphorylation could enhance recognition of the protein by anti-RKIP in immunoblotting. Previous work in our lab showed that Raf/Erk is activated earlier by PMA in PMA-sensitive WT2 cells than PMA-resistant V7 cells (Han *et al.,* 2007). Here we consistently find earlier enhancement of RKIP protein by PMA in WT2 cells

than in V7 cells (Fig. 4-1, B). RKIP begins to increase after 5 minutes of PMA treatment in WT2 cells, while the PMA-induced increase in RKIP protein is detected after 15 minutes of PMA treatment in V7 cells despite the high basal RKIP expression (Fig. 4-1, B). In both V3 and V10 cell lines, the increase in RKIP is detected after 5 minutes of PMA treatment (Fig. 4-1, B), which correlates with the pattern of Raf/Erk activation. RKIP returned toward basal level by 60 minutes in all three cell lines. Taken together, these data suggest that PMA can alter RKIP localization or antibody recognition in EL4 cells, and the pattern coincides with PMA-induced Raf/Erk activation. Changes in RKIP localization in response to PMA could conceivably be a consequence of PKC-mediated phosphorylation (Trakul *et al.,* 2005).

Effect of RKIP Expression on PMA-induced Raf/Erk Phosphorylation in V7 Cells

Protein kinases achieve cell signaling through phosphorylation of specific sites on target molecules. Because RKIP selectively regulates Raf-1 activation (Trakul *et al.,* 2005), and Raf-1 is a key mediator of the Erk MAPK cascade, we explored the effects of RKIP on the phosphorylation specific residues on Raf in more detail. To determine the role of RKIP, we tested the effects of RKIP depletion on PMA-induced Raf phosphorylation and Erk activation in V7 cells.

First, we tested the effectiveness of RKIP siRNA. V7 cells treated with RKIP siRNA demonstrated attenuated RKIP expression, while cells incubated with control siRNA showed no difference as compared with untreated V7 cells (Fig. 4-2, A).
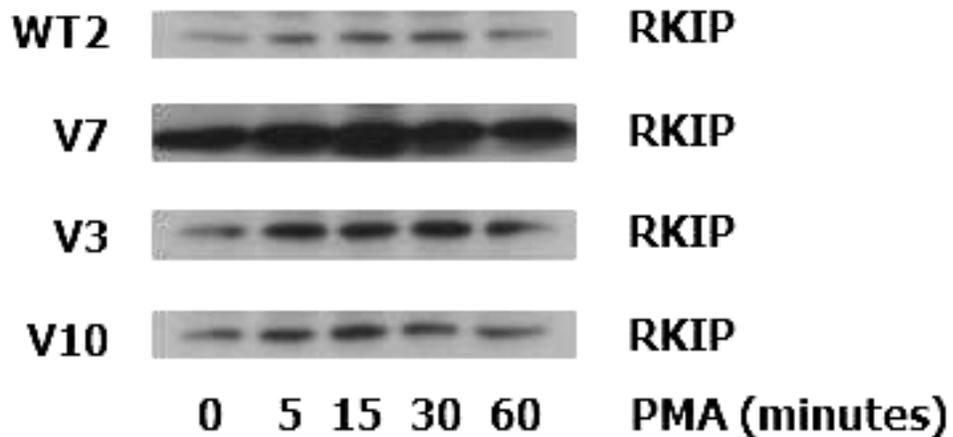
A.
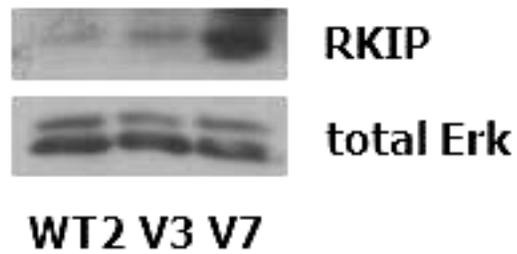
B.

Figure 4-1. Basal and PMA-induced RKIP protein levels in clonal EL4 cell lines.

A. Basal protein levels for RKIP were assessed in WT2, V3 and V7 cells by immunoblotting of whole-cell extracts, equalized for protein.

B. WT2, V7, V3, and V10 cells were treated with or without 100 nM PMA for the indicated times. Immunoblots were performed on whole-cell lysates for RKIP. Immunoblots were run together and exposed in parallel.

RKIP siRNA treatment potentiated PMA-induced Raf-1/Erk activation in V7 cells, as shown by increased Raf-1 Ser338 and Erk phosphorylation (Fig. 4-2, B). We are not surprised to find enhanced phosphorylation of Raf Ser289/296/301 as well, considering that Ser289/296/301 phosphorylation is mediated by Erk feedback phosphorylation. The phosphorylation of Raf Ser259 was not affected by RKIP (Fig. 4-2, B). Thus, RKIP modulates Raf signaling both directly and indirectly via Erk feedback, limiting the response of the cell to the PMA stimulus.
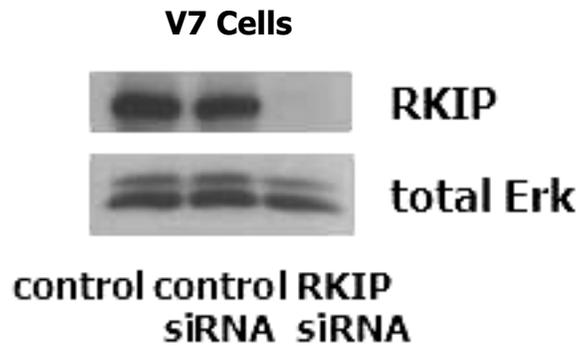
Effect of RKIP Knockdown on FAK and Phospho-FAK Expression in V7 Cells

Since RKIP can be involved in cell adhesion, and FAK expression varies between PMA-sensitive and –resistant EL4 cells (unpublished data), we asked whether RKIP might influence FAK levels or activity. First, we tested basal FAK and phospho-FAK (Tyr397) expression in WT2 and V7 cells. As shown in Figure 4-3 A, V7 cells express FAK and have basal phospho-FAK (Tyr397), while WT2 cells do not express FAK. To study whether RKIP functions through the FAK pathway, FAK and phospho-FAK (Tyr397) levels were tested in untreated V7 cells, control siRNA treated or RKIP siRNA treated V7 cells. Our results indicate that RKIP down-regulation does not affect FAK or phospho-FAK (Tyr397) levels in V7 cells (Fig. 4-3 B).

RKIP Expression and In Vitro Proliferation, Migration and Invasion of V7 Cells

To test for effects of RKIP on V7 cell motility, we modulated RKIP expression in V7 cells by incubating V7 cells with control siRNA or RKIP siRNA. Proliferation, migration, and
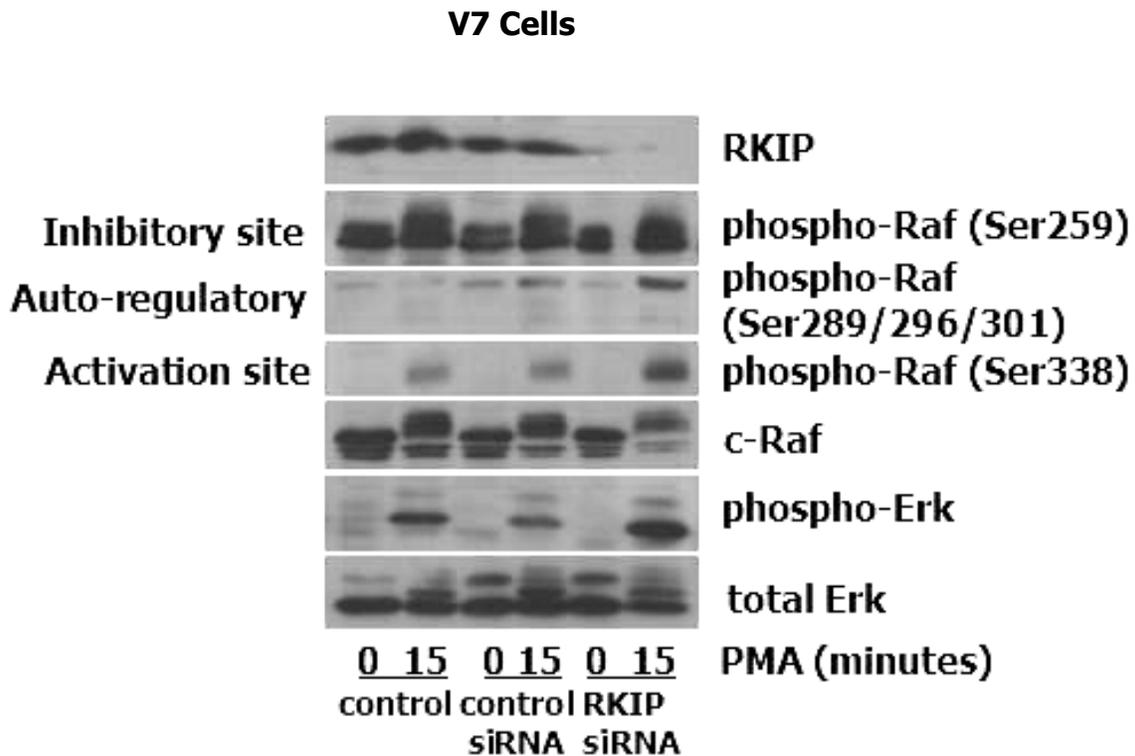
A.

V7 Cells

RKIP

total Erk

control control RKIP
siRNA siRNA

B.

V7 Cells

RKIP

Inhibitory site — phospho-Raf (Ser259)

Auto-regulatory — phospho-Raf (Ser289/296/301)

Activation site — phospho-Raf (Ser338)

c-Raf

phospho-Erk

total Erk

0 15 0 15 0 15 PMA (minutes)
control control RKIP
siRNA siRNA

Figure 4-2. Effects of RKIP knockdown on PMA-induced Raf and Erk phosphorylation in V7 cells.

A. V7 cells were incubated in the absence of siRNA (control) or in the presence of either a control siRNA or RKIP siRNA, as described under *Materials and Methods*. The protein level for RKIP was assessed by immunoblot of whole-cell extracts, equalized for protein.

B. V7 cells were treated as in panal A, then incubated in the absence and presence of 100 nM PMA for 15 min. Levels of RKIP, phospho-Raf (Ser259, Ser289/296/301, Ser338), c-Raf, activated Erk (phospho-Erk), and total Erk (loading control) were assessed by immunoblotting of whole-cell extracts, equalized for protein.
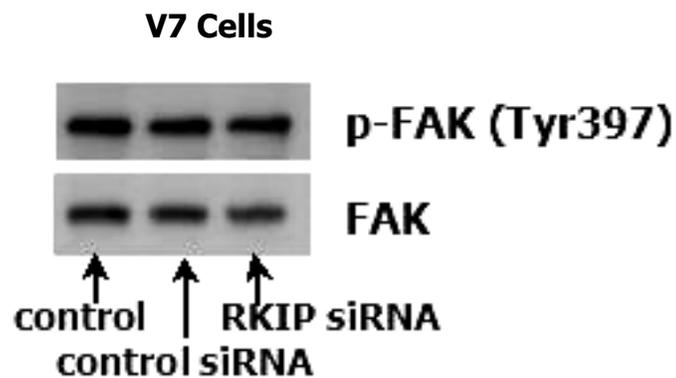
A.

B.

Figure 4-3. Effects of RKIP knockdown on FAK and phospho-FAK levels in V7 cells.

A. Basal FAK and phospho-FAK (Tyr397) expression in WT2 and V7 cells, was assessed by immunoblotting of whole-cell extracts from untreated cells.

B. V7 cells were incubated in the absence of siRNA (control) or in the presence of either a control siRNA or RKIP siRNA, as described under *Materials and Methods*. Protein levels for FAK and phospho-FAK (Tyr397) were assessed by Western blot of whole-cell extracts, equalized for protein.

invasion assays were then performed.

There were no statistically significant differences between *in vitro* proliferation rates in untransfected V7 cells, control siRNA treated and RKIP siRNA treated V7 cells (Fig. 4-4, A). These data suggest that modulation of RKIP expression levels has no effect on proliferation rate in V7 cells.

In contrast, silencing of RKIP has a significant effect on cell migration. Since V7 cells only have trivial migration and invasion without FBS, we used 10% FBS to induce migration and invasion in the following experiments. Decreasing RKIP expression in V7 cells by RKIP siRNA is associated with a 43% decrease in *in vitro* migration ability compared with untransfected V7 cells, while the control siRNA treated group shows no significant effect (Fig. 4-4, B).

Invasion is one of the key components of the metastatic cascade. Accordingly, to examine whether RKIP expression is associated with cancer cell invasiveness, we measured the invasive ability of V7 cells that were untreated, incubated with control siRNA, and incubated with RKIP siRNA using an *in vitro* invasion assay. Decreasing RKIP expression in V7 cells by RKIP siRNA was associated with a 29% decrease in *in vitro* invasive ability compared with that of untreated V7 cells, while control siRNA has no significant effect (Fig. 4-4, C).

In summary, down-regulation of RKIP expression in V7 cells impaired serum-induced migration and invasion, suggesting that RKIP expression is associated with the enhanced metastasis potential of V7 cells.

A.
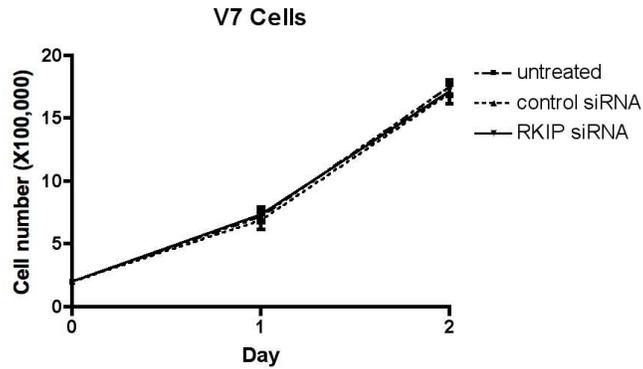
B.
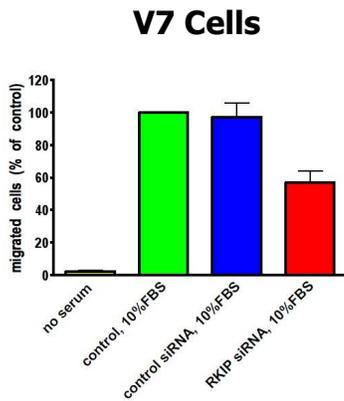
C.
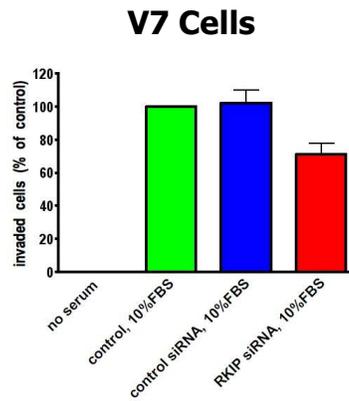
Figure 4-4. Effects of RKIP knockdown on proliferation, migration, and invasion in V7 cells.
V7 cells were incubated without additions, and in the presence and absence of either a control siRNA or RKIP siRNA, as described under *Materials and Methods*. Cell proliferation (A), migration (B), and invasion (C) were then assessed, as described in *Materials and Methods*. Each data point represents the mean ± S.D. of three independent experiments.

4.5 DISCUSSION

In the current study, we have demonstrated that the basal level of RKIP expression is higher in V7, which is an EL4 cell line with enhanced metastasis potential, than in WT2 and V3 cells. Knockdown experiments indicate that RKIP regulates Raf-1 phosphorylation in a residue specific manner, in that phosphorylation of Ser338 and Ser289/296/301 are modulated by RKIP, while Ser259 is not.   Furthermore, we have identified a potential role for RKIP in enhancing metastasis in EL4 murine lymphoma cell model. Specifically, our results show that RKIP expression is associated with enhanced *in vitro* migration and invasion of V7 cells.

It has been reported that cell lines derived from metastatic prostate cancers have decreased levels of RKIP mRNA and protein as compared with primary tumor cell lines (Fu *et al.,* 2003). A decrease in RKIP level was also detected in melanoma and breast cancer (Schuierer *et al.,* 2004; Hagan *et al.,* 2005). However, RKIP expression was found to be higher in a tumorigenic and metastatic murine fibrosarcoma cell line than in the weakly tumorigenic and nonmetastatic parental cell line from which it was derived (Hayashi *et al.,* 2005). Despite the controversy, in the current study we find that the basal level of RKIP expression is higher in V7 than in WT2 and V3 cells (Fig. 4-1, A), which is in consistent with the greater metastasis capacity of V7 cells (Knoepp *et al.,* manuscript in preparation). The expression of RKIP inversely correlates with PMA-induced Raf-1 and Erk activity in EL4 cells, providing an additional modulatory influence (in addition to RasGRP) on these processes. The mechanism responsible for the differences in RKIP expression between EL4 cell lines needs further study.

RKIP functions as a negative modulator that controls the amplitude of Raf kinase activity

rather than the absolute on or off state (Rosner *et al.,* 2007). In mammalian cells, RKIP inhibits Raf-1 signaling to Erk, suppressing Raf-1-induced transformation (Yeung *et al.,* 2001). Signaling downstream of MEK is attenuated when RKIP blocks the interaction between Raf and MEK (Klysik *et al.,* 2008). In this study, we effectively suppressed RKIP expression in V7 cells by RKIP siRNA (Fig. 4-2, A), and then studied the PMA-induced Raf/Erk phosphorylation status (Fig. 4-2, B). Down-regulation of RKIP potentiated PMA-induced Raf-1/Erk activation in V7 cells, as shown by the increased Raf-1 Ser338 and Erk phosphorylation. Activation of Raf-1 is dependent on phosphorylation at Ser 338 by Ras (Trakul *et al.,* 2006). Thus, inhibition of this phosphorylation event by RKIP would impair activation of Raf-1 and downstream MEK/Erk.

Our results also indicate enhanced PMA-induced phosphorylation of Raf Ser289/296/301 after incubation with RKIP siRNA, which suggests enhanced Erk feedback phosphorylation. Thus, RKIP modulates Raf signaling both directly and indirectly via Erk feedback, in both cases limiting the response of the cell to PMA stimuli. The phosphorylation of Raf Ser259 was not affected by RKIP (Fig. 4-2, B), which is consistent with Ser259 being the site regulated by the PI3K/Akt cascade. These data support a residue-specific regulation of Raf by RKIP. The effect of RKIP depletion on the potentiation of PMA-induced Erk activation is consistent with results obtained by others (Matheny *et al.,* 2004; Sasaki *et al.,* 2003; Wakioka *et al.,* 2001). Thus RKIP, acting as an inhibitor of the Raf-1/MAPK signaling cascade, plays a key role in regulating the Raf-1/Erk pathway.

The development of malignancy is often associated with perturbation of cell proliferation, migration and invasion. Molecular pathways involved in the detachment, migration of malignant

cells from the primary tumor site, and invasive colonization of distant organs are poorly understood. The search for genes that influence metastatic processes is a very important part of the overall effort to find cures for cancer. A decrease in RKIP was detected in prostate cancer, melanoma and breast cancer, where the increased metastasis potential elicited by RKIP depletion is due, at least in part, to elevated Erk activity (Fu *et al.,* 2003; Schuierer *et al.,* 2004; Hagan *et al.,* 2005). In contrary to the enhanced invasiveness and metastasis by hyperactivated Raf/MEK/Erk signaling pathway in these cellular model systems, strong Erk activation correlates with PMA-induced cell death (Sansbury *et al.,* 1997) and a less aggressive tumor phenotype (Han *et al.,* 2007) in EL4 lymphoma cells. It is not surprising to find that RKIP functions differently in different cellular systems, considering the multiple cellular responses to different amplitudes of Erk activation.

From previous studies (Sansbury *et al.,* 1997), we know that PMA induces growth arrest and cell death in WT2 cells through robust Erk activation. The decreased migration and invasion potential by RKIP siRNA transfection in EL4 cells is not related to the inhibitory effects of RKIP on the Raf/Erk pathway, since U0126 treatment did not significantly recover the effects of RKIP siRNA on decreasing migration and invasion potential (data not shown). It is possible that RKIP affects Raf/Erk in addition to other oncogenic signaling pathways to promote tumor migration and invasion. In this case blocking the Erk pathway alone could not significantly recover the decreased metastasis potential caused by RKIP siRNA. A more intriguing interpretation could be that RKIP, in addition to the regulation of Erk pathway, fulfills another yet unknown function that regulates metastasis, and that the RKIP targets relevant for metastasis need further

evaluation.

RKIP downregulation does not decrease the migration and invasion rates to the level seen without serum (Fig. 4-4 B, C). This is consistant with that fact that other signaling pathways can contribute to migration and invasion, such as PI3K/Akt and FAK.

RKIP is a key regulator of cell motility. It has been reported that RKIP is involved in the positive regulation of epithelial cell migration, since RKIP-overexpressing MDCK cells grow like fibroblasts without cell-cell contact, and transform into highly migratory cells with long protrusions (Zhu *et al.,* 2005). RKIP might represent as a general upstream regulator of cell motility. It is reported that RKIP knockdown suppresses cell motility, while RKIP overexpression promotes cell migration. RKIP overexpression changes the basic features of epithelial cell growth, causing MDCK cells to lose cell-cell adhesions and generally resemble other more motile cell types (Bement *et al.,* 2005). The role of cell anchorage in migration has attracted much attention. Tumor cells frequently over-express FAK, suggesting a positive correlation between FAK expression and malignancy. FAK is a tyrosine kinase that integrates signals from adhesion molecules and growth factors. Binding of integrins to extracellular matrix proteins results in FAK phosphorylation. FAK can activate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, resulting in cell migration. FAK is necessary for growth factor-stimulated motility of fibroblasts, and enhances migration when introduced into fibroblasts lacking FAK. The differential roles of FAK in regulating cell circle progression versus migration appear to involve complexes with different effectors. Confirming work done previously in this lab, we show that V7 cells, which cause more *in vivo* metastasis than WT2 cells, have FAK expression and

constitutive FAK (Tyr397) phosphorylation, while WT2 cells do not (Fig. 4-3 A). Constitutive FAK and phospho-FAK (Tyr397) expression in V7 cells was not affected by RKIP siRNA transfection (Fig. 4-3 B). However, FAK may play a permissive role in RKIP-mediated migration. It may be also worthwhile to test whether RKIP promotes migration of EL4 cells by interacting with other cell locomotion receptors, such as GRK2 (Klysik *et al.*, 2008; Lorenz *et al.,* 2003; Vroon *et al.,* 2004) and Rock-α kinase (Ehrenreiter *et al.,* 2005).

In summary, our results suggest that RKIP regulates Raf-1 phosphorylation in a residue specific manner. Furthermore, we have identified a role for RKIP in enhancing migration and invasion in EL4 cells.

CHAPTER FIVE

GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 GENERAL CONCLUSIONS

The EL4 murine lymphoma cell line, in its variant forms, serves as a particularly useful model system in which to examine molecular mechanisms of malignant transformation. In particular, the EL4 cell line is unique in its utility as a model system in which to identify signaling pathways involved in PMA response. EL4 cells exist in phorbol ester-sensitive, –resistant, and intermediate variants. Erk activation is required for PMA-induced toxicity and cell death in EL4 cells. Previous studies showed that the tumorigenicity of V7 (PMA-resistant) is much more significant than that of WT2 (PMA-sensitive) cells, as reflected by more liver and other organ metastasis *in vivo* and a shortened life span. The research described in this dissertation contributed to the understanding of the molecular differences underlying these variations in cell phenotypes.

First, we explored the differences in phenotype between EL4 cells that are PMA-sensitive and PMA-resistant. We demonstrated that RasGRP1 protein is much more highly expressed in PMA-sensitive cells. Using siRNA knockdown strategy, we showed that RasGRP1, a phorbol ester and diglyceride receptor, is responsible for the "classic" features of the PMA-sensitive phenotype (IL-2 production and growth arrest), as well as for the very high level of Erk activation seen in sensitive cells upon PMA treatment.

Second, this work illustrated that phosphorylation of different residues on Raf all contribute to the Raf activation/deactivation process, and that multiple signals are involved in this scenario. We demonstrate that the negative regulation of Raf Ser259 by the PI3K/Akt plays a role in the differences in of PMA-induced Raf/Erk activation between PMA-resistant and intermediate cells.

Erk mediated feedback auto-inhibition at Raf Ser289/296/301 contributes to the extent and duration of PMA-induced Raf Ser338 activation.

Third, we showed that RKIP regulates Raf-1 phosphorylation in a residue-specific manner, in that phosphorylation of Ser338 and Ser289/296/301 is modulated by RKIP, while Ser259 is not. Furthermore, we have identified a potential metastasis enhancing role of RKIP in EL4 murine lymphoma cell model. Specifically, our results show that RKIP expression is associated with enhanced migration and invasion of V7 cells.

5.2 FUTURE DIRECTIONS

Our data indicate that mRNA transcripts for RasGRP1 are expressed at similar levels in both WT2 and V7 cells, while RasGRP1 protein expression is very different between EL4 cell lines. The differences in mRNA levels need to be examined more quantitatively, since subsequent gene microarray data from our lab suggest that RasGRP1 mRNA is several fold lower in V7 than in WT2 cells (Chahal, unpublished data). Nontheless, expression of RasGRP isoforms may be regulated at the post-transcriptional level. The underlying mechanism is worthy of future studies, since RasGRP1 has such a profound effect on cell phenotype.

It is also interesting to note that although PMA causes the expected down-regulation of RasGRP protein in sensitive EL4 cells, there is a modest up-regulation in resistant cells (Fig. 2-4). The mechanism underlying this phenomenon, and its significance, remain to be explored.

It is noteworthy that Erks were activated to some extent in response to PMA in resistant and intermediate phenotype cells, in which RasGRP expression is weak and PMA-induced Ras

activation is barely detectable. These results, therefore, suggest that there are alternative (non-Ras) pathways for PMA-induced Erk activation in these cells, which deserve further investigation.

We find that there is strong constitutive Akt phosphorylation in PMA-resistant cells (V7), while basal phospho-Akt is low in PMA-sensitive cells (WT2), and at a moderate level in intermediate cells (V3) (Fig. 3-3 A). As shown in figure 4-3 A, V7 cells have basal FAK and phospho-FAK (Tyr397) expression, while WT2 cells do not. It has been reported that FAK can activate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Whether FAK act as upstream signal contributing to constitutive Akt phosphorylation in EL4 cells awaits further investigation.

The next "hot" area is how hyperphosphorylated/desensitized Raf-1 is recycled to a signaling-competent state under normal and disease conditions. The mechanisms responsible for Raf-1 dephosphorylation are poorly understood, although the common view is that multiple phosphatases involved. Even less is known concerning the mechanisms that regulate the activity of these phosphatases. Clearly such information is very important, as many of the phosphatases, which have been identified so far, also play important roles as tumor suppressor genes.

Drugs targeting the Erk pathway at the level of Raf may be particularly useful because Raf is the key activator of the Erk pathway, whereas other upstream targets such as growth factor ligands, receptor tyrosine kinases, or even Ras have other potential effectors. Raf kinase inhibitors are promising anticancer agents because they may be effective not only in tumors with constitutive active or aberrant Ras or Raf signaling, but also in those with deregulated signal transduction due to overexpression or overactivation of upstream growth factors or their

receptors. Raf-1 has at least thirteen regulatory phosphorylation residues with either stimulatory or inhibitory function, which makes inhibition of Raf a complicated affair, except that most kinase inhibitors act at the catalytic site. The existence of integration of multiple signaling pathways and feedback responses may provide some explanation to the ongoing challenge of understanding how cells respond differently to the same factor under varying cellular conditions.

In the current study, we have demonstrated that the basal level of RKIP expression is higher in V7, which is an EL4 phenotype with enhanced metastasis potential, than in WT2 and V3 cells. The mechanism responsible for the differences in RKIP expression (e.g., pre- or post-translational) between EL4 cell lines needs further analysis.

Future studies may provide tools to target the metastasis enhancing property of RKIP in malignancies with high basal RKIP expression. Drugs that reduce RKIP expression or activity may provide a potent means of treatment for cancers with high basal RKIP level. A small molecule, locostatin, has already been shown to abrogate RKIP's ability to inhibit Raf-1 (Zhu *et al.,* 2005), which would be particularly useful for the control of metastatic cells that display enhanced steady state levels of RKIP.

In summary, the results of this study support our hypothesis that RasGRP1 confers PMA-sensitive responses; regulation of Raf-1 activation by PI3K/Akt pathway contributes to the characteristic of intermediate phenotypes; and RKIP is responsible for the enhanced metastasis potential of PMA-resistant cells.

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