IDENTIFICATION OF AN *IN VITRO* BINDING PROTEIN FOR AND THE *IN VIVO* PHOSPHORYLATION SITES OF THE RAS SUPPRESSOR RSU-1

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IDENTIFICATION OF AN *IN VITRO* BINDING PROTEIN FOR

AND

THE *IN VIVO* PHOSPHORYLATION SITES OF

THE RAS SUPPRESSOR RSU-1

by

Gerard W. Dougherty

Dissertation submitted to the Faculty of the Program in Molecular Pathobiology of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, 2000.

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"Identification of a C-terminal Binding Protein to Rsu-1 by Far Western Cloning"

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ABSTRACT

Title of Dissertation: Identification of an *in vitro* binding protein for and the *in vivo* phosphorylation sites of the Ras Suppressor Rsu-1.

Gerard W. Dougherty

Doctor of Philosophy, 2000

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The Ras Suppressor Rsu-1 is a 33 kDa protein originally discovered as a cDNA that suppressed v-Ras transformation of NIH-3T3 fibroblasts. Rsu-1 overexpression in several cell lines (NIH-3T3, U251, PC12, MCF7) increased ERK activity and levels of $p21^{CIP}$, and inhibited growth rate and decreased JNK activity. Stabilization of p53 and inhibition of CDK2 and cyclin D –associated kinase activity has also been observed in MCF7 cells stably transfected with Rsu-1. In this study we attempted to identify the binding proteins to Rsu-1 by the Far Western Cloning method. Preliminary experiments with a fusion protein containing the glutathione-S-transferase (GST) tag and the carboxy-terminus of Rsu-1 showed binding to a protein of approximately 30 kDa both in solution and immobilized on nitrocellulose. A K562 human leukemic cell library was screened with ^{32}P-labeled GST-C-terminal

Rsu-1. Results indicate that one subclone from approximately one million plaques screened bound to the Rsu-1 protein *in vitro*. This subclone, designated PI-4, expressed a protein of approximately 30 kDa. Sequence analysis of this subclone suggests homology to an as yet uncharacterized gene sequence on the long arm of chromosome 21 and also the WAS protein. Additional studies were done to ascertain the role of Rsu-1 phosphorylation on its biological activity. The Rsu-1 sequence contains several consensus phosphorylation sites for protein kinase C (PKC), protein kinase A (PKA) and casein kinase II. Previous experiments showed that Rsu-1 is phosphorylated *in vivo* in response to growth factor and TPA, a known activator of PKC. Phosphoamino acid analysis of Rsu-1 suggests that *in vivo* phosphorylation occurs on serine residues. In this study site-directed mutagenesis of individual Rsu-1 consensus PKC sites revealed that two serine residues (serine-4 and serine-163) are phosphorylated *in vivo* in response to TPA. This is supported by evidence that Rsu-1 phosphorylation can be specifically blocked by the PKC inhibitor bisindolylmaleimide I (BIM), but not by inhibitors of tyrosine kinase, MEK and PI3K. Stable transfectants of non-phosphorylatable Rsu-1 mutants in MCF7 were isolated and tested for growth rate and effect on apoptosis. Previous studies with Rsu-1 have demonstrated an enhancement of apoptosis in response to TNF-α and staurosporine in MCF7 cells, accompanied by stabilization of p53 protein levels. Results indicate that non-phosphorylatable mutants of Rsu-1 still exhibit growth inhibition but do not enhance apoptosis in MCF7 cells. Together these results support the hypothesis that PKC or a PKC-dependent serine kinase mediates the proapoptotic effect but not growth inhibitory effect of Rsu-1.

IDENTIFICATION OF A C-TERMINAL BINDING PROTEIN TO RSU-1 BY FAR WESTERN CLONING

and

IDENTIFICATION OF THE *IN VIVO* PHOSPHORYLATION SITES OF THE RAS SUPPRESSOR RSU-1

By

Gerard W. Dougherty

Dissertation submitted to the Faculty of the Department of Pathology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2000

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LIST OF ABBREVIATIONS

ERK: Extracelluar Regulated Kinse

JNK: c-Jun N-terminal Kinase

CDK: Cyclin Dependent Kinase

GST: Glutathione-S-Transferase

PKC: Protein Kinase C

PKA: Protein Kinase A

CKII: Casein Kinase 2

TPA: 12-*O*-Tetradeconylphorbol-13-acetate

BIM: Bisindolylmaleimide I

MEK: MAPK / ERK Kinase

TNF: Tumor Necrosis Factor

PARP: Poly ADP-Ribose Polymerase

PI3K: Phophoinositide-3-Kinase

LRR: Leucine Rich Repeat

GTP: Guanine Triphosphate

GDP: Guanine Diphosphate

GEF: Guanine Exchange Factor

GAP: GTP Activating Protein

NF1: Neurofibromatosis 1

MAPK: Mitogen Activated Protein Kinase

SOS: Son Of Sevenless

NGF: Nerve Growth Factor

EGF: Epidermal Growth Factor

PDGF: Platelet – Derived Growth Factor

ROCK: Rho-Associated Kinase

DAG: Diacylglycerol

WAS: Wiskott Aldrich Syndrome

SUR-8: Suppressor of Ras - 8

SLK: Synthetic Lethal Kinase

DTT: Dithiotritiol

BSA: Bovine Serum Albumin

DMEM: Dulbecco Minimal Essential Media

SDS: sodium dodecyl sulfate

KSR: kinase suppressor of Ras

PBS: phosphate buffered saline

HMW: high molecular weight

HA: hemagluttinin

PAGE: polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

ATP: Adenosine Triphosphate

PTEN: Phosphatase and Tensin homolog deleted on chromosome Ten

IPTG: Isopropyl-*B*-D-thiogalactoside

INTRODUCTION

Our studies have focused on the biochemical and biological properties of the Rsp-1 / Rsu-1 gene. The Rsu-1 gene was originally cloned as a cDNA that suppressed Ki-*ras* transformation in NIH-3T3 fibroblasts but not transformation by v-*mos* or v-*src* (Cutler, 1992). Rsu-1 is a single copy gene that is well conserved among human, murine and nematode species, and its RNA is ubiquitously expressed in higher eukaryotes. The Rsu-1 gene encodes a 277 amino acid protein of approximately 33kD. Interestingly, the amino-terminal portion of Rsu-1 contains seven adjacent leucine-rich repeats (LRR)(figure 1). A related LRR region is present in three proteins reported to interact with *ras*: yeast adenylyl cyclase, SUR-8 and most recently Ce-FLI-1 (Colicelli, 1990; Sieburth, 1998; Goshima, 1999). Leucine-rich repeats are not exclusive to binding Ras, however, and represent a general protein structure found in prokaryotic, plant and mammalian proteins that likely mediate binding to a wide variety of proteins (reviewed in Kobe, 1995 and Kajava, 1998). The carboxy-terminus of Rsu-1 appears unique since no homology has been found to other protein sequences in the current databases. In contrast to the hydrophobic LRR region of Rsu-1, the carboxy-terminus of the Rsu-1 protein is highly charged and contains a high percentage of polar and basic amino acids such as lysine, arginine, and threonine (figure 1).

Based on the ability of Rsu-1 to suppress Ki-*ras* transformation of NIH-3T3 fibroblasts, experiments have addressed the relation of Rsu-1 to the Ras signal transduction pathway (Masuelli, 1996). The *ras* genes were originally discovered as highly oncogenic retroviruses that caused sarcomas in rats and mice (reviewed in

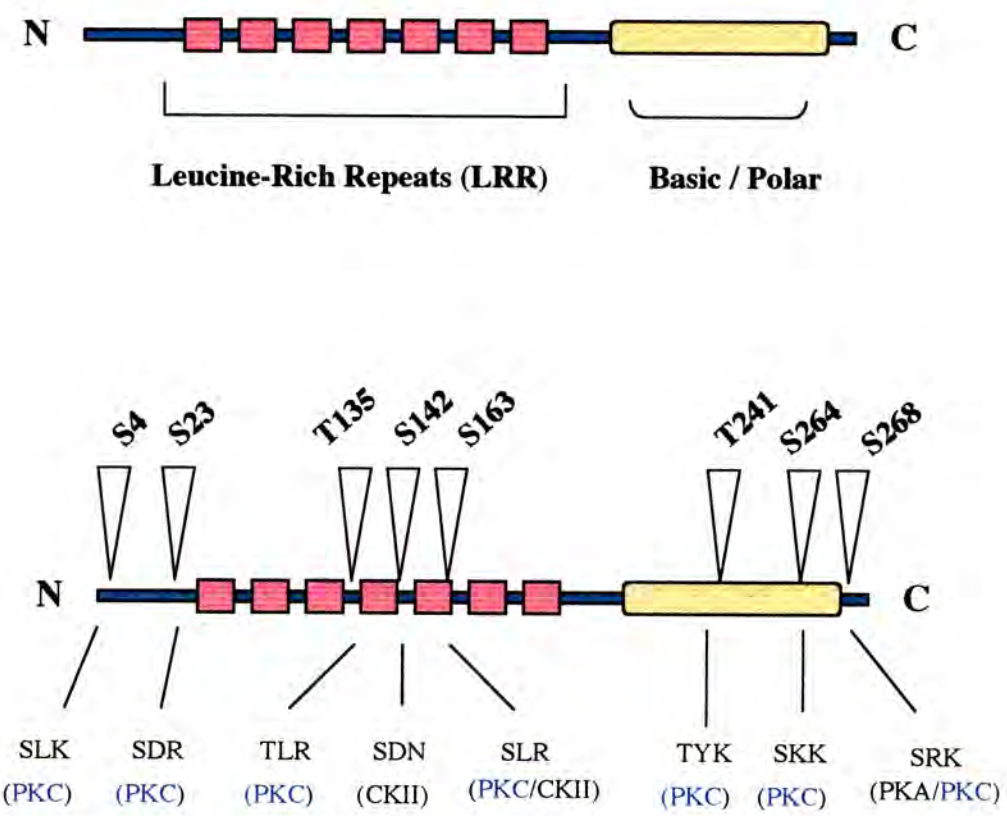

Figure 1. The Structural and Molecular Features of Rsu-1. Top: the protein domains of Rsu-1. A series of seven leucine-rich repeats (LRR) span the first two-thirds of the molecule, followed by a C-terminal region rich in basic and polar residues, such as lysine and arginine. Bottom: the major post-translational modification of Rsu-1 is predicted to be phosphorylation, due to several consensus phosphorylation sites throughout the molecule. PKC = Protein Kinase C, PKA = Protein Kinase A, CKII = Casein Kinase II. The three letters above each enzyme site represent the consensus sequence for that enzyme. PKC sites are highlighted in blue.

Lowy, 1993). In 1982 several groups identified the human homologue of *ras* and found that it was mutated in several human tumor cell lines but not in normal human cells (Der, 1982; Shih, 1982; Pulciani, 1982; Shimizu, 1983). This prompted the search for mutated *ras* genes in other human cancers, and it was found that *ras* had activating mutations in a high percentage of certain cancers, such as pancreatic (90%), colon (50%), thyroid (50%) and myeloid leukemia (50%) (reviewed in Bos, 1989). This strongly suggested that aberrant Ras or Ras-dependent signaling is an important component of carcinogenesis (Bos, 1989; Lowy, 1993).

Other genes that suppress Ras transformation have been discovered. Rev-1 / Rap-1 was also discovered as a gene that suppressed K-*ras* transformation in an expression cloning assay (Kitayama, 1989; Noda, 1989; Zhang, 1990), and RasGAP can suppress transformation by both c-*ras* and the *src* oncogenes (DeClue, 1991; Nori, 1991; Zhang, 1990). The neurofibromatosis protein NF-1 contains a GAP-related domain which has RasGAP activity (Ballester, 1990; DeClue, 1991; Martin, 1990; Xu, 1990), and can suppress oncogenic Ras transformation (Nakafuka, 1993).

More recently other proteins such as MSX-2, Tel, and Lumican have been discovered to suppress Ras transformation (Takahashi, 1997; Rompaey, 2000; Yoshioka, 2000). MSX-2 is a homeobox-containing gene that is implicated in development, specifically organogenesis and cell differentiation (Foerst – Potts, 1997). The Tel gene is a member of the ETS family of transcription factors that is the target of a chromosomal translocation in myeloid leukemia (Buijs, 2000). Interestingly, Lumican is a leucine-rich repeat protein and a component of the extracellular matrix, and has recently been shown to function in the assembly of the

collagen network in connective tissue (Svenson, 2000). Characterization of proteins that can suppress Ras activity might provide insights to the mechanism of Ras-dependent transformation as well as the role of Ras and Ras-dependent signaling in carcinogenesis.

Mammalian *ras* genes belong to the superfamily of small GTPases including Rho, Rac, Rab, Arf and Ran (reviewed in Neer, 1995). These GTP-binding proteins or "G" proteins cycle between an active GTP-bound state and inactive GDP-bound state. This is regulated by guanine nucleotide exchange factors (GEFs), which accelerate loading of GTP onto the G-protein active site, and GTPase activating proteins (GAPs) which promote hydrolysis of the guanine nucleotide and promote an inactive state of the G protein. The *ras* subfamily has three members (H-, K-, and N-*ras*) which are highly conserved but show variable homology at their C-terminal 25 amino acids, which contain the CAAX motif required for plasma membrane association (reviewed in Shields, 2000).

Studies in the powerful genetic models of the fruit fly *D. melanogaster* and the nematode *C. elegans* have demonstrated that *ras* is required for eye development and vulval differentiation, respectively (Therrien, 1995; Kornfeld, 1995; Sundaram, 1995). However, the function of Ras in mammals is undoubtedly more diverse (reviewed in McCormick, 1997). To date several mammalian Ras-effector molecules have been identified which bind Ras-GTP in its effector-loop domain: Raf-1 (Moodie, 1993; Van Aelst, 1993; Vojtek, 1993), MEKK1 (Lange-Carter, 1994; Russell, 1995), PKC-ζ (Diaz-Meco, 1994; Berra, 1995), the GAP proteins p120 (Trahey, 1987; Adari, 1988; Vogel, 1988) and NF1 (Martin, 1990; Ballester,

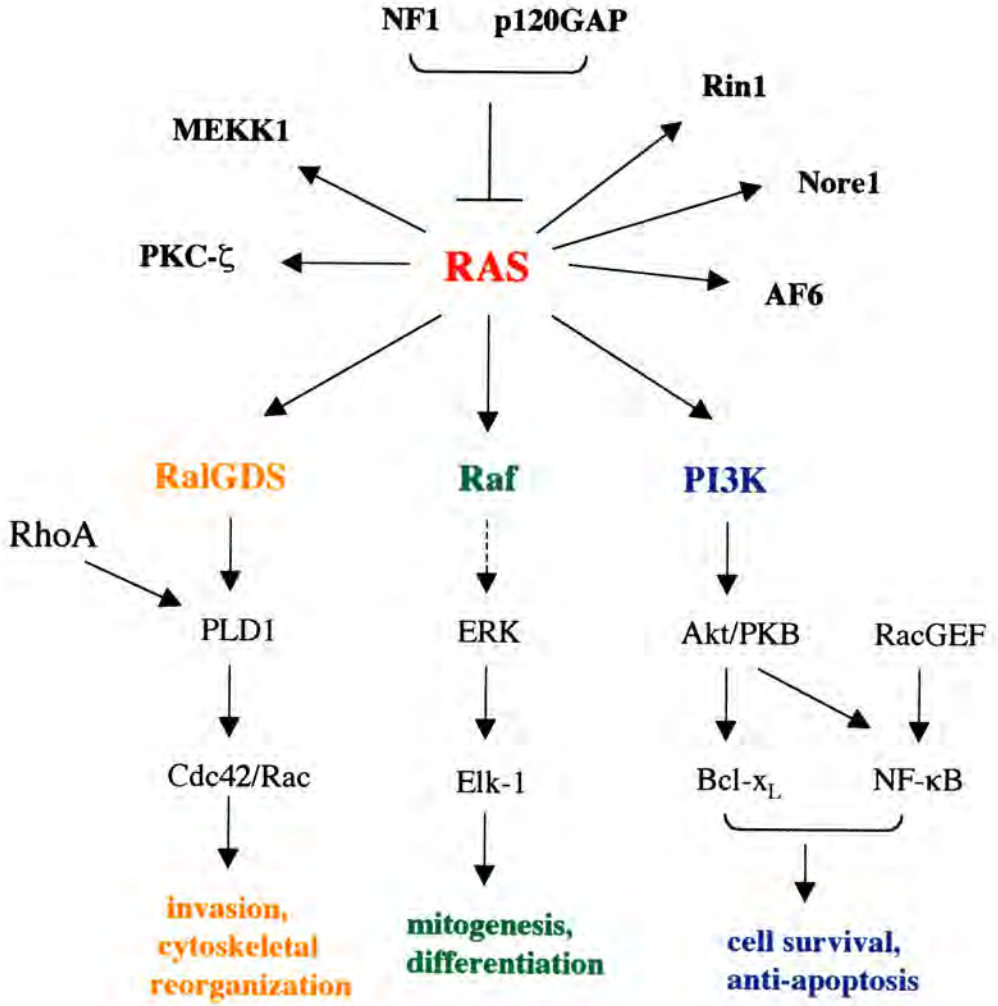

Figure 2. RalGDS, Raf and PI3K represent the best known effector pathways of Ras. RalGDS is a GDP-GTP exchange factors (GEF) which can activate Cdc42/Rac in addition to RhoA. This is mediated by Phopholipase D1, which converts phosphatidylcholine (PC) to phosphatidic acid (PA). Ras activation of the Raf serine/threonine kinases and activation of the MAPKs remains an important component of Ras signaling, leading to activation of Elk-1 and other transcription factors. PI3K activation leads to elevated activity of Akt/PKB and the promotion of cell survival. PI3K can also activate the RacGTPase, which is an important mediator of oncogenic Ras transformation. Other candidate effector molecules of Ras include the GAP effectors NF1 and p120 GAP, which promote inactive Ras, PKC-ζ, MEKK1, Rin1 Abl-interacting protein, Nore1 novel protein, and AF-6, a chromosomal translocation protein associated with human acute leukemias (adapted from Shields, 2000).

1990; Xu, 1990), the p110 subunit of PI-3 kinase (Rodriguez-Viciana, 1994 #), the GEF protein Ral (Hofer, 1994; Kikuchi, 1994; Spaargaren, 1994; Kikuchi, 1996), Rin1 (Han, 1995) and more recently AF-6 (Kuriyama, 1996) and Nore1 (Vavvas, 1998). The Ras protein mediates at least in part three distinct cellular events: mitogenesis and differentiation, via the Raf-MAP kinase pathway (reviewed in Shields, 2000), Rho-mediated actin cytoskeleton activation and transformation (reviewed in Zohn, 1998 and Van Aelst, 1997), and apoptosis (reviewed in Downward, 1998).

The most well studied effector pathway of Ras involves activation of the Raf-MAP kinase pathway (reviewed by Shields, 2000). Ras is normally associated with the inner face of the plasma membrane by C-terminal modifications, including farnesylation (Willingham, 1980; Grand, 1987). Activation of a receptor tyrosine kinase (RTK) or other stimuli recruits the Ras GEF protein SOS to the plasma membrane, where it catalyzes the exchange of GTP for GDP on Ras. GTP-bound Ras is active and binds cytoplasmic Raf-1, which in turn becomes activated and initiates a signaling cascade involving the protein kinases MAPKK (also known as MEK) and terminal MAPK (also known as ERK) (reviewed in Lopez-Ilasaca, 1998). ERK, once activated, can migrate into the nucleus and there phosphorylate and activate transcription factors essential for entry into the S phase of the cell cycle, namely c-Jun, ATF2, and Elk-1 (Robinson, 1997). From studies of growth factor signaling and the pheromone response in budding yeast, the MAP kinase pathway has been established as a major mechanism for controlling transcription in eukaryotes (reviewed by Hunter, 2000).

Initial studies with Rsu-1 focused on Ras-dependent signal transduction pathways. It was found that ectopic expression of Rsu-1 in PC12 and NIH-3T3 cells resulted in increased signal transduction to Raf and ERK-2 kinase, and decreased Ras-dependent signal transduction to c-jun N-terminal kinase (JNK) (Masuelli, 1996). Additionally, Rsu-1 expression inhibited RasGAP activity, which resulted in increased Ras-GTP, as well as elevated levels of SOS, Ras-GAP, and both non- and phosphorylated forms of Ras-GAP-associated p190. This data suggested that Rsu-1 might suppress Ras-mediated transformation at the level of a Ras-GAP dependent process or JNK activation.

Subsequent studies addressed the effect of Rsu-1 on differentiation using the PC12 cell line, which is a model for Ras-dependent neuronal differentiation (Masuelli, 1999). In response to either NGF treatment or Ki-MSV infection, it was found that neurite extension, which is a Ras-Raf-ERK dependent process, was enhanced by Rsu-1 expression. The activation of ERK2 activity in response to NGF was also elevated compared to control cells. Additionally, ectopic expression of Rsu-1 resulted in accumulation of $p21^{CIP}$ and growth inhibition in PC12 cells. The $p21^{CIP}$ protein acts as a negative regulator of the cell cycle (reviewed in Gartel, 1999) and was first cloned as a CDK (cyclin-dependent kinase) -binding protein (Harper, 1993). It was shown that $p21^{CIP}$ prevents G_1 / S cell cycle progression by inhibiting cyclin / CDK complexes (Xiong, 1993; Harper, 1995). Cyclin / CDK complexes are positive regulators of the cell cycle (reviewed in Morgan, 1995). The G_1 cyclins (cyclins D1, D2, D3 and E) and CDKs (CDK2, CDK3, CDK4 and CDK6) are all effectively inhibited by $p21^{CIP}$ (Harper, 1995).

It has been shown that active Rho can suppresses activation of $p21^{CIP}$ and lead to Ras-dependent induction of DNA synthesis (Olson, 1998). Activated Ras induces DNA synthesis in p21 $^{-/-}$ mouse embryo fibroblasts in which Rho activity is blocked (C3 exoenzyme, L63RhoA), suggesting that an important function of Rho signaling in transformation is to suppress $p21^{CIP}$ induction by Ras (Olson, 1998). This data suggested that Rsu-1 might prevent Ras-dependent transformation and enhance Ras-dependent differentiation by blocking Rho-mediated signaling and allowing accumulation of $p21^{CIP}$. More recent studies have examined the role of Rsu-1 in the MCF7 breast cancer cell line (Vasaturo, 2000). Based on the previous findings that Rsu-1 blocked JNK activity in PC12 and NIH-3T3 cell lines and led to elevated levels of $p21^{CIP}$, studies focused on Rho activity and downstream effects on the cell cycle.

The Rho family of proteins are GTPases whose major function is to regulate the assembly and organization of the actin cytoskeleton (reviewed in Hall, 1998). Architectural control of the cytoskeleton appears to be mediated in particular by the c-jun N-terminal kinase (JNK) and p38 mitogen-activated kinase pathways (Coso, 1995; Minden, 1995). Interestingly, activated JNK is reported to be required for Ras transformation (Clark, 1996), and its activation is dependent on the small G proteins Cdc42 and Rac (Coso, 1995; Minden, 1995; Olson, 1995). The dominant negative forms of Rho and Rac effectively inhibit Ras-dependent transformation, which suggests that the Cdc42 / Rac – JNK pathway plays an essential role for Ras-dependent transformation (Khosravi-Far, 1995; Prendergast, 1995; Qui, 1995).

Similar to other cell lines tested, Rsu-1 expression in MCF7 elevated ERK2 activity and decreased JNK activity. To address the possibility that Rsu-1 may suppress Ras-transformation at the level of PI-3 kinase, Rac, or Rho, several assays were performed. AKT kinase activity, which is PI-3 kinase dependent, as well as PAK activity, the Rac activated kinase, were not significantly altered by Rsu-1 expression as compared to control cell lines. However, the Rho kinase ROCK was reduced in the presence of Rsu-1 expression. This result suggested that Rho or Rho effector molecules might mediate the block to Ras transformation by Rsu-1. Additionally, Rsu-1 expression in MCF7 cells increased levels of $p21^{CIP}$ and decreased cyclin D associated CDK activity. Furthermore, c-myc activity, which is associated with mitogenesis and is deregulated in many types of cancers, was reduced approximately 40% compared to control cells in the presence of Rsu-1 expression. Previous experiments have shown c-myc expression to be inhibited in PC12 Rsu-1 clones (Cutler, unpublished data). Very recent studies indicate that Rsu-1 increases levels of $p21^{CIP}$ via stabilization of p53, and that Rsu-1 can enhance apoptosis in MCF7 cells in response to TNF-α and staurosporine (Vasaturo, submitted).

Evidence to this point suggested that Rsu-1 might inhibit growth and block Ras-dependent transformation through a Rho- and / or JNK-dependent process. Conceivably, Rsu-1 could promote growth arrest in G_1 via $p21^{CIP}$, and in the presence of strong Ras signaling induce senescence or apoptosis as a "safety" mechanism. Considering the role of Rsu-1 as an inhibitor of Ras-dependent transformation, more direct evidence suggests Rsu-1 may act as a tumor suppressor gene in certain

cancers. Tumor suppressor genes encode proteins whose loss of function in the cell contributes to the initiation and progression of carcinogenesis (reviewed in Levine, 1995; Ellisen, 1998; Kinzler, 1998). The Rsu-1 gene has been mapped to chromosome 10p13 (Tsuda, 1995). Based on evidence from numerous studies that two distinct regions of chromosome10p are commonly deleted in gliomas (Fults, 1990; Ransom, 1992; Ye, 1993), tumor cell lines including gliomas were screened for the expression of Rsu-1 RNA and protein.

The U251 glioblastoma cell line, which has only one copy of chromosome 10, does not express detectable levels of Rsu-1 protein (Tsuda, 1995). Following transfection of Rsu-1 into the U251 cell line, transfectants showed a reduction in both anchorage dependent and anchorage independent growth, and injection of these Rsu-1-U251 transfectants into athymic nude mice did not result in tumor formation (Tsuda, 1995). With an indication that Rsu-1 could reduce the growth rate of a glioblastoma cell line and block its tumorigenicity *in vivo*, and the fact that gliomas undergo loss of heterozygosity of chromosome 10p, specific mutations of the Rsu-1 open reading frame were examined in glioblastoma tumor samples.

A truncated Rsu-1 product was detected in 30% of high-grade gliomas and 2/3 oligodendrogliomas (Chunduru, 2000). The mutant form of Rsu-1 in these tumors has an internal 133-bp deletion corresponding to the deletion of a single exon. This results in translation of approximately the first two-thirds of the Rsu-1 protein with a nonfunctional carboxyl-terminus encoded by an alternative reading frame. The mutant Rsu-1 was cloned from glioma samples and tested for expression. This truncated form of Rsu-1 was not detected by Western blotting. Subsequent

pulse-chase experiments demonstrated that the Rsu-1 clone was translated but highly unstable, with a half-life of approximately 1 hour, in contrast to wild type Rsu-1 which is stable > 6 hours (Chunduru, 2000). More recently two artificial C-terminal Rsu-1 truncation constructs were tested and did not express detectable protein by Western blotting (Cutler, unpublished data). This suggests that the truncated alternatively-spliced product of Rsu-1 is unstable in cells and that the C-terminus is required for stability of the Rsu-1 protein.

This thesis outlines two studies that have extended the investigation of Rsu-1 as an inhibitor of cell growth. The first study presents the use of a cloning assay to identify binding proteins to Rsu-1, known as Far Western Cloning. The second project aim addressed the role of Rsu-1 phosphorylation on its biological activity. Based on previous observations that Rsu-1 was phosphorylated by specific stimuli, a second project tested which amino acids of Rsu-1 are phosphorylated *in vivo* and how phosphorylation affects Rsu-1 activity.

As the field of molecular biology shifts from genomics to proteomics, the determination of protein-protein interactions will remain essential to the characterization of individual proteins and the signaling networks they influence within the cell. Several approaches have been used to demonstrate such interactions, such as coimmunoprecipitation, yeast two-hybrid analysis, and expression library screening. Far Western Cloning is a well-known technique to identify and clone binding proteins (Takayama, 1997). In one of the earliest uses of this method, two binding partners to the tumor suppressor gene Rb were identified, RBAP-1 and RBAP-2 (Defeo-Jones, 1991). Over one hundred proteins have been discovered

using this method, including proteins involved in apoptosis (Macgregor, 1990), growth and differentiation (Blackwood, 1991), and even HIV replication (Nelbock, 1990). These earlier screening assays used cDNA libraries such as λgt11, the prototype expression library and cloning vector (Sambrook, 1989). Commercial technology has improved the choice and quality of expression libraries available today, including refined techniques such as phage display, which make this *in vitro* technique a reliable and proven means of identifying protein-protein interactions.

In Far Western Cloning a soluble protein probe is used to detect potential binding proteins expressed from a cDNA library and immobilized on nitrocellulose. The protein probe may be detected by antibody or radiolabeling. cDNAs encoding potential ligands are isolated by plaque purification (i.e. successive rounds of plating phage picks on agar). The phage cDNA insert is subcloned into a bacterial plasmid, which allows the cDNA (corresponding to the expressed binding protein) to be identified by sequencing. Other experiments, such as *in vitro* expression and binding, can also be done with the plasmid DNA. Chapter 2 of this thesis presents the results of Far Western Cloning using the C-terminus of the Rsu-1 protein.

In addition to the structural LRR domain and charged C-terminal domain of Rsu-1, its sequence contains several consensus phosphorylation sites for Protein Kinase C (PKC), Casein Kinase II (CK2), and Protein Kinase A (PKA). This suggested the major post-translational modification of Rsu-1 might be phosphorylation. Previous experiments in the laboratory have determined that Rsu-1 is phosphorylated *in vivo* in response to nerve growth factor (NGF) and epidermal growth factor (EGF), and also in response to the phorbol ester 12-O-

tetradecanoylphorbol-13-acetate (TPA). Additionally this phosphorylation is enhanced by an activated form of Ras and blocked by dominant negative (N17) Ras, and phosphorylation can also be specifically blocked by the PKC inhibitor bis-indomaleimide (BIM). These results support a hypothesis that Rsu-1 does function as a phosphoprotein, and that PKC or a PKC-dependent enzyme likely mediates this event.

PKC proteins are serine / threonine kinases that have now been implicated in several cellular functions, including mitogenesis, apoptosis, platelet activation, actin cytoskeletal activation, and modulaton of ion channels and secretion (reviewed in Toker, 1998). To date at least 12 isoforms of PKC have been identified, and they are classified in one of four categories: conventional PKCs (α, β, and γ) which are regulated by DAG, phosphatidylserine (PdSr), and Ca^{2+}; novel PKCs (δ, ε, η, and θ) which are regulated by DAG and PdSr; atypical PKCs (ζ, ι, and λ) which are stimulated by PdSr but not DAG or Ca^{2+}; and a group of more recently discovered enzymes that show homology to PKC, which include PKCμ / PKD and PRK / PKN (reviewed in Toker, 1998).

PKCs function primarily to relay signals initiated by phospholipid hydrolysis. PKC was originally discovered as a calcium activated enzyme, and later studies found that the metabolite diacylglycerol (DAG) potently regulated the activity of PKC (Inoue, 1977; Kishimoto, 1980). Tumor-promoting reagents such as phorbol ester were later discovered to activate PKC (Castagna, 1981), which led to studies on its role as an effector of cellular growth and regulation. Activation of G protein coupled receptors, tyrosine kinase receptors, and nonreceptor tyrosine kinases can

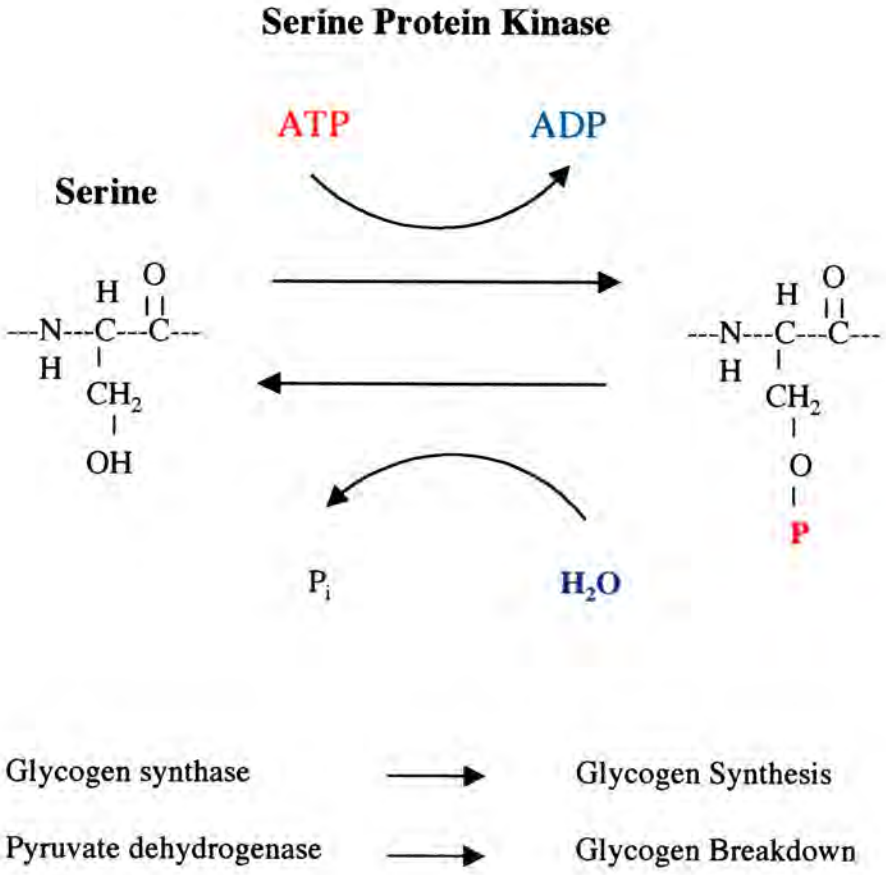

Figure 3. Kinase-Phosphatase Reactions Mediate a Multitude of Cellular Events. Kinases transfer the γ-phosphate moiety of ATP to the hydroxyl group of a serine, threonine or tyrosine on the target substrate. This reaction is rapidly reversible by the action of a phosphatase, which hydrolyzes water to release the phosphoryl group. The first kinase-phosphatase reactions elucidated involved glucose and glycogen metabolism. Several catabolic and anabolic pathways are regulated by kinases and phosphatases, as well as signal transduction of stimuli to regulate growth, differentiation and apoptosis.

lead to PKC activation by stimulation of either phospholipase C isoforms to yield DAG, or phospholipase D to yield phosphatidic acid and then DAG. Diacylglycerol is the key "on" switch for most PKCs, and it is now appreciated that phorbol esters like TPA activate PKCs in the same manner as DAG – binding to the C1 domain of PKC and relieving autoinhibition, allowing the phosphorylation of relevant substrates (reviewed in Toker, 1998).

Eukaryotic cells employ different types of covalent modifications to regulate the activity of a protein. These modifications include glycosylation, hydroxylation and fatty acid acylation, which are somewhat permanent, and the more transient modifications such as methylation, adenylation, and phosphorylation (reviewed in Edelman, 1987). Phosphorylation is by far the most common covalent modification to proteins as a means of regulation (reviewed in Edelman, 1987 and Hunter, 1995). The phosphorylation of proteins is catalyzed by enzymes called protein kinases, which transfer the gamma phosphoryl group of adenosine triphosphate (ATP) to the side chain hydroxyl groups of serine, threonine, or tyrosine residues (Figure 3). Less commonly the side chains of histidine, lysine, arginine and aspartic acid are targeted by kinases. This modification of the protein can be reversed by a second hydrolytic reaction catalyzed by protein phosphatases. Phosphorylation represents an elegant and powerful method to regulate proteins. First, phosphorylation of proteins can be achieved quickly and efficiently, which allows tight control of the activity of the protein. Second, the addition of the phosphoryl group itself alters the three-

dimensional structure of the protein and therefore its biological properties. The phosphoryl groups have a high charge density and tend to form salt bridges with arginine residues of interacting proteins. Phosphorylation generally targets amino acids distant from the catalytic site itself, inducing a change in the protein's conformation and subsequently altering the substrate's intrinsic properties (reviewed in Edelman, 1987 and Hunter, 1995).

The first kinase- phosphatase system involved in metabolic regulation was elucidated almost fifty years ago by Krebs and Fischer with the finding that interconversion of the two forms of glycogen phosphorylase mediated glycogen metabolism (reviewed in Krebs, 1979). Initially believed to be a limited form of bioregulation, other important discoveries like the observation that histones and other nuclear proteins undergo phosphorylation-dephosphorylation soon suggested its importance as a mechanism of cellular communication (reviewed in Edelman, 1987). Today it is clear that kinase-phosphatase reactions are involved in many metabolic pathways, including glycogen and glucose metabolism, cholesterol and triglyceride metabolism, neurotransmitter biosynthesis, as well as signal transduction from growth factors and other stimuli (reviewed in Hunter, 1995). Particularly relevant to this discussion, it is now appreciated that growth factor signaling (EGF, PDGF, and NGF) may be mediated principally or exclusively by changes in the state of phosphorylation (reviewed in Hunter, 2000).

Therefore, the second project outlined in this thesis has been to identify and test individual consensus phosphorylation sites of the Rsu-1 sequence as *in vivo* sites of phosphorylation. A series of Rsu-1 constructs, in which the PKC-consensus serine

or threonine residues were changed to a non-phosphorylatable form, either glycine or alanine, were created by site-directed mutagenesis. These mutants were transiently transfected into Cos-1 cells and tested for *in vivo* phosphorylation in response to TPA. Non-phosphorylatable mutants were then tested for biological effect, such as anchorage-independent growth, anchorage-dependent growth, and apoptosis. The downstream biochemical response of known effector pathways such as ERK, p53, and $p21^{CIP}$ was also compared in cells expressing non-phosphorylatable Rsu-1 and wild type Rsu-1. Chapter 3 of this thesis presents the identification of *in vivo* Rsu-1 phosphorylation sites. Together these studies attempt to more specifically determine the role of Rsu-1 as a Ras-dependent effector molecule, by identification of binding proteins to Rsu-1 and also the effect of phosphorylation on Rsu-1 activity.

Identification of a Binding Protein to C-terminal Rsu-1 by Far Western Cloning

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Running Title: C-terminal Rsu-1 Binds a 30kD protein *In Vitro*

SUMMARY

The Ras Suppressor Rsu-1 is a 33kD protein originally discovered as a cDNA that suppressed v-Ras transformation of NIH-3T3 fibroblasts. This finding, in addition to the homology of Rsu-1 to other Ras-binding proteins, suggested that Rsu-1 interacts with Ras or Ras effector molecules to block the transformation phenotype. Several reports have demonstrated the effect of Rsu-1 on the Ras / MAPK signal transduction pathway in NIH-3T3, U251, PC12 and most recently MCF7 cell lines. Ras overexpression increased ERK activity and levels of $p21^{CIP}$, and inhibited growth rate and decreased JNK activity of these cell lines. In this study we attempted to identify the binding proteins to the carboxy (COOH) region of Rsu-1 by the Far Western Cloning method. Preliminary experiments with a fusion protein containing the glutathione-S-transferase (GST) tag and the carboxy-terminus of Rsu-1 showed binding to a protein of approximately 30 kDa both in solution and immobilized on nitrocellulose. A K562 human leukemic cell library was screened with ^{32}P-labeled GST-COOH-terminal Rsu-1. Results indicate that one subclone from approximately one million plaques screened bound to the Rsu-1 protein *in vitro*. This subclone expressed a T7-fusion protein of approximately 100 kDa. Sequence analysis of this subclone suggests strong homology to a novel sequence on chromosome 21, and lesser homology to the Wiskott-Aldrich Syndrome (WAS) protein.

INTRODUCTION

Our laboratory has focused on the molecular and biological properties of the Rsu-1 gene. Rsu-1 was originally cloned as a cDNA that suppressed v-Ras transformation in NIH-3T3 fibroblasts (Cutler, 1992). Rsu-1 is well conserved between human and *C.elegans* (58% genetic identity and 76% amino acid similarity), and its RNA is ubiquitously expressed in higher eukaryotes. The amino-terminal portion of Rsu-1 is composed of leucine-rich repeats (LRR), a motif found in a wide variety of proteins, and which likely mediate protein-protein interactions (reviewed in Kobe, 1995 and Kajava, 1998). The Rsu-1 LRR region shows homology to three proteins known to interact with Ras: yeast adenylyl cyclase, SUR-8 and most recently Ce-FLI-1 (Colicelli, 1990; Sieburth, 1998; Goshima, 1999).

To further characterize the role of Rsu-1 in Ras-initiated signal transduction, the signaling pathway from Ras to "downstream" effector pathways was investigated. Initial experiments demonstrated that Rsu-1 expression in NIH-3T3 cells and PC12 cells inhibited RasGAP activity, which results in an increase in Ras-GTP levels, and also a reduction in JNK activity and enhancement of ERK activation (Masuelli, 1996). Further studies with the cell line PC12, which is a model for NGF- and Ras dependent differentiation (Bar-Sagi, 1985; Noda, 1985; Wood, 1992), demonstrated that Rsu-1 expression enhanced differentiation in these cells (Masuelli, 1999), which is characterized by neurite outgrowth and sustained ERK activity (Cowly, 1994). Additionally, Rsu-1 expression in PC12 cells enhanced differentiation via $p21^{CIP}$– dependent inhibition of cell cycle progression through G_1 (Masuelli, 1999).

Most recently, Rsu-1 has been studied in cell cycle regulation and the EGF signaling pathway in MCF7 breast carcinoma cells. MCF7 cells stably transfected with Rsu-1 enhanced the stability of p53, resulting in elevation of $p21^{CIP}$ levels, growth inhibition in G_1, and inhibition of anchorage-independent growth (Vasaturo, 2000, Vasaturo, submitted). Additionally, these transfectants showed decreased Rho-associated kinase (ROK) and cyclin dependent kinase 2 (CDK2) activity, and similar to previous cell lines tested, there was increased ERK activation and decreased JNK activation correlated with Rsu-1 expression. Together this data suggested that Rsu-1 acts in a Ras-dependent fashion to promote the differentiation pathway while blocking the transformation pathway. The possibility that Rsu-1 might interact with Ras or a Ras-effector molecule is supported by evidence that Raf-1 associates with the full length GST - Rsu-1 fusion protein *in vitro* (Masuelli, 1996).

Recently, two Ras-related GTPases, Rad and Ran, have been been used as protein probes to identify binding proteins by Far Western Cloning (Zhu, 1996; Coutavas, 1993). Far Western Cloning, also known as expression library screening or interaction cloning, has been well documented as a technique to identify and clone binding partners (Takayama, 1997). Over the past decade nearly 100 proteins have been discovered through this method, which have utilized charged motifs or peptides to efficiently bind ligands *in vitro*. For example, the retinoblastoma protein (Rb), a tumor suppressor gene and regulator of the cell cycle, was used as a probe to clone two binding partners, RBAP1 and RBAP-2 (Defeo-Jones, 1991). Proteins involved in apoptosis, growth and differentiation, and even HIV replication (Macgregor, 1990; Blackwood, 1991; Nelbock, 1990) have been identified in this manner. Essentially a

protein probe is used to screen an expression library (**Fig. 1**), and potential binding proteins to the probe can be recovered by cre-lox recombination and tested for *in vitro* expression and binding (**Fig. 2**).

In contrast to the hydrophobic leucine-rich repeat region of the N-terminus, Rsu-1 contains a highly charged carboxy-terminal region. This C-terminal region (amino acids 236 – 277) contains nearly 70% basic and polar amino acids, such as lysine / arginine and tyrosine / serine / asparagine. Based on the previous success of Far Western Cloning using protein probes that are highly charged and / or polar, we screened potential binding proteins to Rsu-1 using the C-terminal region expressed as a Glutathione-S-Transferase (GST) fusion protein. Several studies have used ^{32}P labeling of the protein probe as the method of detection for binding. In the case of Rad and Ran binding, the proteins were labeled with ^{32}P utilizing endogenous phosphorylation sites for Protein Kinase A (Zhu, 1996; Coutavas, 1993). Our studies have shown that Protein Kinase C efficiently phosphorylates the C-terminal region *in vitro*, which contains two consensus PKC and one consensus cAMP/PKA site. In this report we demonstrate the identification of a binding protein to C-terminal Rsu-1 by this method.

EXPERIMENTAL PROCEDURES

Preparation of the Rsu-1 Protein Probe. The C-terminal Rsu-1 probe contains the last forty one amino acids of Rsu-1 inserted in frame with glutathione transferase into the plasmid pGEX-2T and was described previously (Masuelli, 1996). Expression of this plasmid produces a 30 kDa fusion protein which contains the glutathione-S-transferase tag (GST). The fusion protein was grown in *E. coli* and induced with 0.5 mM isopropyl-*B*-D-thiogalactoside (IPTG). The protein was purified in one of two ways. Bacteria were resuspended in NETN lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40), sonicated (microtip, 10 – 20 seconds), and affinity-purified with Glutathione Sepharose beads (Pharmacia). Alternatively, bacteria were lysed in B-Per Reagent (Pierce), collected over GST columns, eluted and dialyzed (Slide-A-Lyzer, Pierce). Protein was quantitated by electrophoresis on 12% Tris-Gly gels (Novex) and staining with Gel Code Blue Stain reagent (Pierce). The control protein GST-2T and full length Rsu-1 fusion protein GST-2T-4 were prepared in a similar manner.

Radiolabeling of the Protein Probe. Approximately 20 μg of the C-terminal Rsu-1 probe GST-2T-4Δ was labeled with 0.25 – 0.50 mCi of γ-^{32}P-ATP, using purified rat brain Protein Kinase C (PKC) enzyme (Boehringer Manheim or Promega) in the following phosphorylation buffer: 20 mM Tris-Cl, pH 7.5 with 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT and 100 μg / ml phosphatidylserine (Sigma), with or without 0.25% bovine serum albumin (BSA). The labeled protein was washed several times in 100 mM Tris-Cl, pH 8.6, eluted with 500 mM glutathione,

and purified over NAP-10 columns (Pharmacia). Fractions of GST-2T-4Δ were collected and aliquots were counted by scintillation for specific activity and quantitated by autoradiography. Radiolabeled GST-2T-4Δ was diluted in binding buffer (10 mM Tris-Cl, pH 7.5, 250 mM NaCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 5% dry milk, and 50-fold Molar excess glutathione-S-transferase control protein) for Far Western cloning. The specific activity of the probe reached $\geq 2.25 \times 10^5$ cpm / μg for each experiment, and the hybridization solution $\geq 10^4$ cpm / ml was prepared following protocol recommendations (Takayama, 1997).

Coimmunoprecipitation of Potential Ligands from ^{32}P-labeled Cell Extracts. PC12 cells were labeled with ^{32}P-orthophosphate and incubated with GST-2T-4Δ, GST-2T-4 or the control GST-2T. Briefly, cells were preincubated in phosphate – free media (Dulbecco's Minimal Essential Media – DMEM) plus 1% Fetal Bovine Serum (FBS) and 25 mM HEPES. Cells were labeled with ^{32}P for 4 hours (0.5 mCi / ml), stimulated with NGF (200 ng / ml) for 15 minutes, and harvested and lysed in RIPA buffer (1% NP-40, 0.5% DOC, 150 mM NaCl, 1% SDS, 25 mM HEPES) plus the protease inhibitors aprotinin, pepstatin, and AEBSF. Lysates were precleared with the control protein GST-2T for 30 minutes and coimmunoprecipitated with either GST-2T-4Δ or GST-2T for 2 hours at 4°C. Coimmunoprecipitates were collected, solubilized in SDS gel loading buffer, and electrophoresed in a 10% Tricine Gel (Novex). The gel was transferred to nitrocellulose, exposed to film (Kodak) and examined by autoradiography.

Far Western Blotting of Whole Cell Lysates. To test whether radiolabeled GST-2T-4Δ could bind a potential ligand immobilized on nitrocellulose, whole cell

lysates of PC12, NIH-3T3 and U251 glioblastoma cell lines were screened. Briefly, confluent cell lines were harvested, resuspended in 1X phosphate-buffered saline (PBS), and centrifuged. Cells were lysed in RIPA buffer plus the protease inhibitors aprotinin, pepstatin and AEBSF. Lysates were normalized for protein concentration using BCA Protein Reagent (Pierce) and electrophoresed on a 10% Tricine gel (Novex). Proteins were then transferred to nitrocellulose and probed with radiolabeled GST-2T-4Δ, prepared as described above. The filter was blocked, incubated with GST-2T-4Δ and washed identically to conditions described for the Far Western Cloning Assay.

Far Western Cloning Assay. The K562 leukemic cell line cDNA library in the λ-SCREEN vector (Novagen) was screened with radiolabeled GST-2T-4Δ. Approximately 20,000 - 50,000 plaques / plate were plated in BL21(DE3)pLysE for primary screening. At 5 – 7 hours the plaques were incubated with nitrocellulose filters (Schleicher & Schuell) treated with 10 mM IPTG. Filters were lifted and washed in buffer containing 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 10 mM β-mercaptoethanol. Filters were blocked overnight at 4°C in buffer containing 10 mM Tris-Cl, pH 7.5, 250 mM NaCl, 5% dry milk and 10 mM β-mercaptoethanol.and then probed according to a procedure described previously (Zhu, 1997). Following incubation in binding buffer for 6 – 24 hours, the filters were washed in buffer containing 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.01% Tween 20 and 10 mM β-mercaptoethanol. Filters were immediately exposed to film (Kodak, X-Omat AR) at –80°C for 12 – 24 hours and then developed. Positive plaques were identified by ability to bind the labeled probe and

isolated as agar plugs using 1ml pipets. The agar plugs were stored in standard media (SM) and mixed with plating bacteria BL21(DE3)pLysE for secondary and tertiary platings as described above to isolate pure plaques.

Subcloning Reactive Phage Inserts. The cDNA inserts of plaques purified from tertiary screenings were subcloned into BM25.8 bacteria by plating on LB / carbenicllin plates, following manufacturer's instructions (Novagen). BM25.8 bacteria contain endogenous *cre* recombinase activity and plasmids with loxP sites, which allows site-specific recombination (cre-lox mediated) of cDNA inserts from the phage vector to the pSCREEN bacterial plasmid. Subclones were selected and grown in minimal media and plasmid DNA was isolated by Wizard Mini-Preps (Promega). Due to high endogenous nuclease activity of BM25.8 bacteria, the subcloned DNA was transformed into X_L-blue or Nova Blue competent bacteria for further analysis. Subclone DNA was purified by boiling mini-prep and digested with the restriction enzymes EcoRI and HindIII (Boehringer Manheim) to check the presence of inserts.

***In Vitro* Binding of Expressed Subclones to C-terminal Rsu-1.** Subclones were tested for stable expression of insert DNA. Recombinant subclones express a fusion protein containing the N-terminal T7 tag plus the potential ligand. Subclones were transformed into the bacterial expression host NovaBlue(DE3), grown in liquid culture and induced with 0.4 mM IPTG, and the fusion proteins were purified according to manufacturer's instructions (Novagen). Samples from expressed subclones were electrophoresed on 4 – 20% Tris-Gly gradient gels and transferred to nitrocellulose. Expressed inserts were identified by Western blotting with

monoclonal antibody to the N-terminal T7 tag (Novagen). Subclones that stably expressed a T7-reactive insert were tested further for *in vitro* binding to GST-2T-4Δ. Subclone lysates were co-precipitated with the GST-Rsu-1 probe or the control fusion protein GST-2T and electrophoresed on 10% Tris-Gly gels. Briefly, 250 µl of induced bacterial lysates that expressed the subclone insert were mixed with 25 µl of GST-2T or GST-2T-4Δ for 1 hour at 4°C. The lysates were centrifuged, the supernatant was removed and the GST pellets were resuspended in 50 µl 1X Tris-Gly loading buffer for electrophoresis. The samples were transferred to nitrocellulose and probed with monoclonal antibody to the T7 tag (Novagen).

Sequence Analysis of Subclones. Approximately 500 ng of subclone DNA was used for automated sequencing (ABI PRISM Dye Terminator Kit) according to manufacturer's instructions (Perkin Elmer), using primers specific for the pSCREEN vector (see appendix). Following PCR-based sequencing reactions, the DNA was purified to remove excess dye terminator using Auto Seq G-50 columns (Amersham Pharmacia Biotech). Sequence data was compared to GenBank using BLAST searches for identification (www.ncbi.nlm.nih.gov)

RESULTS

A series of GST-Rsu-1 fusion proteins were available for screening potential binding proteins (**Fig 3a**) which could be conveniently purified from *E. coli* (**Fig. 3b**). Initial experiments addressed whether the C-terminus of Rsu-1 could bind proteins in a GST pull-down assay in cell lines previously characterized with Rsu-1. The rat pheochromocytoma cell line PC12 was screened with the control (2T), full length (2T-4) and C-terminal Rsu-1 (2T-4Δ). PC12 cells were labeled with ^{32}P and treated with or without Nerve Growth Factor (NGF), which induces differentiation in this cell line. Results indicated that a protein of 30 kDa coimmunoprecipitated with 2T-4Δ in the presence of NGF, but not with the control or full length protein (**Fig 4a**). This experiment was repeated in NIH-3T3 and U251 glioblastoma cell lines using ^{35}S methionine to label cellular lysates. Results showed that 2T-4Δ bound a 30 kDa protein as well as a 70 kDa protein in both cell lines (data not shown). Together, this data suggested that the C-terminal Rsu-1 probe 2T-4Δ could bind a potential ligand efficiently in solution.

We chose the Far Western Cloning method to identify binding proteins specific to the carboxy-terminus of Rsu-1, due to its highly charged and polar composition. Additionally, the carboxy region of Rsu-1 contains three potential phosphorylation sites identified by PROSITE (two PKC sites , T241 and S264, and one cAMP/PKA site, S268), which affords a convenient method to label and detect the C-terminal probe. Experiments were done to determine if 2T-4Δ could be efficiently labeled *in vitro* and used in the Far Western Assay. PKC and PKA were compared for specific labeling of C-terminal Rsu-1. Experiments with PKA enzyme revealed poor labeling

of GST-2T-4Δ and nonspecific labeling of the control protein GST-2T (**Fig. 4b**). In contrast, PKC enzyme specifically labeled GST-2T-4Δ to a high degree, but not the control protein GST-2T (**Fig. 4c**). Accordingly, PKC labeling was chosen as the method for detection of GST-2T-4Δ, and further experiments demonstrated that the probe could be recovered to sufficient purity and specific activity (**Fig. 5a, 5b**). To test whether PKC-labeled 2T-4Δ could bind proteins immobilized on nitrocellulose, we screened whole cell lysates of NIH-3T3, U251 and PC12 cells. Results showed that a protein of approximately 30 kDa reacted with labeled 2T-4Δ in U251 lysates (**Fig. 5c**). This suggested the protein probe could bind potential binding proteins fixed to membrane, and gave sufficient indication to screen an expression library by Far Western Cloning.

Since previous analysis demonstrated that Rsu-1 is expressed in the K562 leukemic cell line and a high quality library of this cell line was commercially available (Novagen), this cDNA expression library was chosen for screening potential binding proteins to C-terminal Rsu-1. Approximately 9×10^5 plaques from three separate experiments were screened with radioactively labeled 2T-4Δ, and a total of thirty-four subclones were recovered for further analysis. Subclones were analyzed to determine the size of the cDNA insert (**Fig 6a**) and tested for stable protein expression *in vitro* (**Fig 6b**). Subclones that did not stably express an IPTG-inducible protein were eliminated as false positives. Subclones that did express proteins and that correlated with the cDNA insert size were then selected for *in vitro* binding to the 2T control and C-terminal 2T-4Δ fusion proteins. Results show that one subclone, designated P1-4, bound to 2T-4Δ *in vitro* (**Fig. 7a**). This clone has an

insert size of 800 bp which encodes approximately 30 kDa of protein sequence and expresses a fusion protein of approximately 100 kDa.

Sequence of approximately the first 200 nucleotides (excluding the T7 vector sequence) of subclone P1-4 shows homology to several sequences in the database by BLAST analysis (**Fig. 7b**). The strongest homology is represented by an as yet unidentified sequence on the short arm of chromosome 21. Interestingly, the sequence also shows similarity to the Wiskott-Aldrich Syndrome Protein (WASP), which has been previously shown to bind the Rho-family protein Cdc42 (Miki, 1996; Aspenstrom, 1996; Kolluri, 1996).

DISCUSSION

In this report we have identified *in vitro* a potential binding protein to the carboxy-terminus of Rsu-1 by Far Western Cloning. At this time the identity of the binding protein is unknown. The limited sequence of subclone P1-4 suggests strong homology to a region on chromosome 21 that has not yet been characterized. Further experiments will be required to clone the full sequence of the gene encoding this protein and demonstrate that this protein binds Rsu-1 *in vivo*. If binding is demonstrated by coimmunoprecipitation, then experiments will address the physiologic relationship of Rsu-1 to this unknown protein.

Additionally, this sequence also shows homology to the WAS protein (WASP). WASP associates with the Rho-family protein Cdc42 (Miki, 1996; Aspenstrom, 1996; Kolluri, 1996) and promotes actin polymerization via the Arp2/3 complex (Machesky, 1998; Machesky, 1999; Winter, 1999; Machesky, 1999). It is reasonable to speculate that Rsu-1 might bind a Rho-associated molecule such as WASP. Rho-family proteins are critical regulators of oncogenic Ras transformation (reviewed in Zohn,1998), and Rsu-1 has been shown to decrease Rho-dependent Rho-alpha kinase (ROK) activity and JNK activation (Vasaturo, 2000). Evidence also suggests that Ras-dependent transformation requires JNK activation, which is mediated at least partially by Cdc42 / Rac (Clark, 1996; Coso, 1995; Minden, 1995; Olson, 1995).

Rsu-1 has also been shown to affect actin cytoskeletal structure (Masuelli, 1996). NIH-3T3 Rsu-1 transfectants assume a flattened and enlarged morphology compared to control NIH-3T3 cells, and staining with phalloidin demonstrates a general decrease in actin organization with an accumulation in actin stress fibers.

Morphological changes have also been observed in U251 glioblastoma cell line transfected with Rsu-1, which form tightly adherent colonies of reduced growth rate, in comparison to the U251 control (Tsuda, 1995). Interestingly, our studies have identified mutations of the Rsu-1 gene in a certain percentage of gliomas (Chunduru, submitted). This mutation results in the translation of a prematurely truncated Rsu-1 protein which lacks the carboxy-terminus. This protein is highly unstable as detected by pulse-chase experiments, suggesting that the C-terminus is required for stability of the Rsu-1 protein. Hence, the C-terminal region of Rsu-1 might be the critical link to cytoskeletal regulation (by binding a Rho – associated molecule such as WASP) and Ras – dependent transformation and tumorigenesis.

Previous experiments in the laboratory have identified other potential binding proteins to Rsu-1. Using the full length Rsu-1 probe GST-2T-4, binding to purified Raf-1 was detected by immunoblotting in a pull down assay (Masuelli, 1996). Additionally, the protein Kinase Suppressor of Ras (KSR) was identified as a co-immunoprecipitating protein from Cos cells using anti-HA antibody to full length HA-Rsu-1 (Cutler, unpublished results). Interestingly, a recent study reported a complex of proteins associated with KSR, including an unidentified protein of 33 kD, the same molecular weight as Rsu-1. KSR and Raf-1 are structurally related - both proteins contain a serine / threonine kinase domain and a cysteine-rich zinc finger domain which binds phorbol esters and diglycerides, suggesting homology to the PKC family of enzymes (Downward, 1995).

Interestingly, the leucine-rich repeat (LRR) protein SUR-8, identified in a genetic screen in *C. elegans* as a suppressor of Ras-dependent vulval differentiation

(Sieburth, 1998), has been found to bind both Ras-GTP and Raf, and to enhance MAP kinase activation (Li, 2000). Several reports support a model where SUR-8, KSR, Raf-1, MEK and ERK form a complex linking receptor tyrosine kinases (RTKs) and Ras activation to downstream MAP kinase signaling (Sternberg, 1998; Joneson, 1998). Ectopic expression of Rsu-1 in a variety of cell lines results in enhanced ERK activation (Masuelli, 1996; Masuelli, 1999; Vasaturo, 2000). Other studies are currently underway to determine interactions specific to the LRR region of Rsu-1 using the yeast two-hybrid system. To date no association or binding to Ras has been detected using Ras and Rsu-1 constructs in the two-hybrid system.

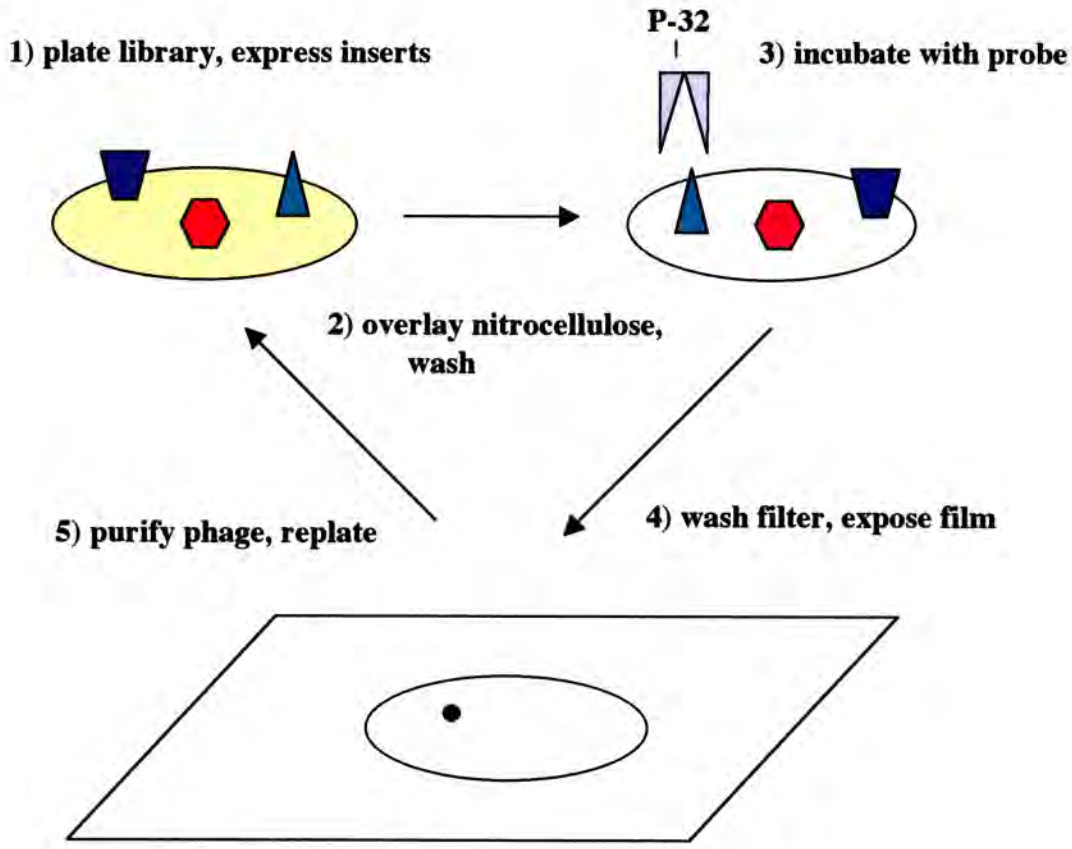


Figure 1. Far Western Cloning Assay. The method chosen to identify C-terminal Rsu-1 binding proteins was Far Western Cloning, also known as interaction cloning or expression library screening. Essentially, a protein probe (in this case, GST-COOH-terminal Rsu-1) is used to detect potential binding proteins expressed from a cDNA library and immobilized on nitrocellulose. The protein probe is radioactively labeled (P^{32}) to detect binding. cDNAs encoding potential ligands are isolated by plaque purification (i.e. successive rounds of plating phage picks on agar). The viral cDNA insert is subcloned by cre-lox recombination for further analysis

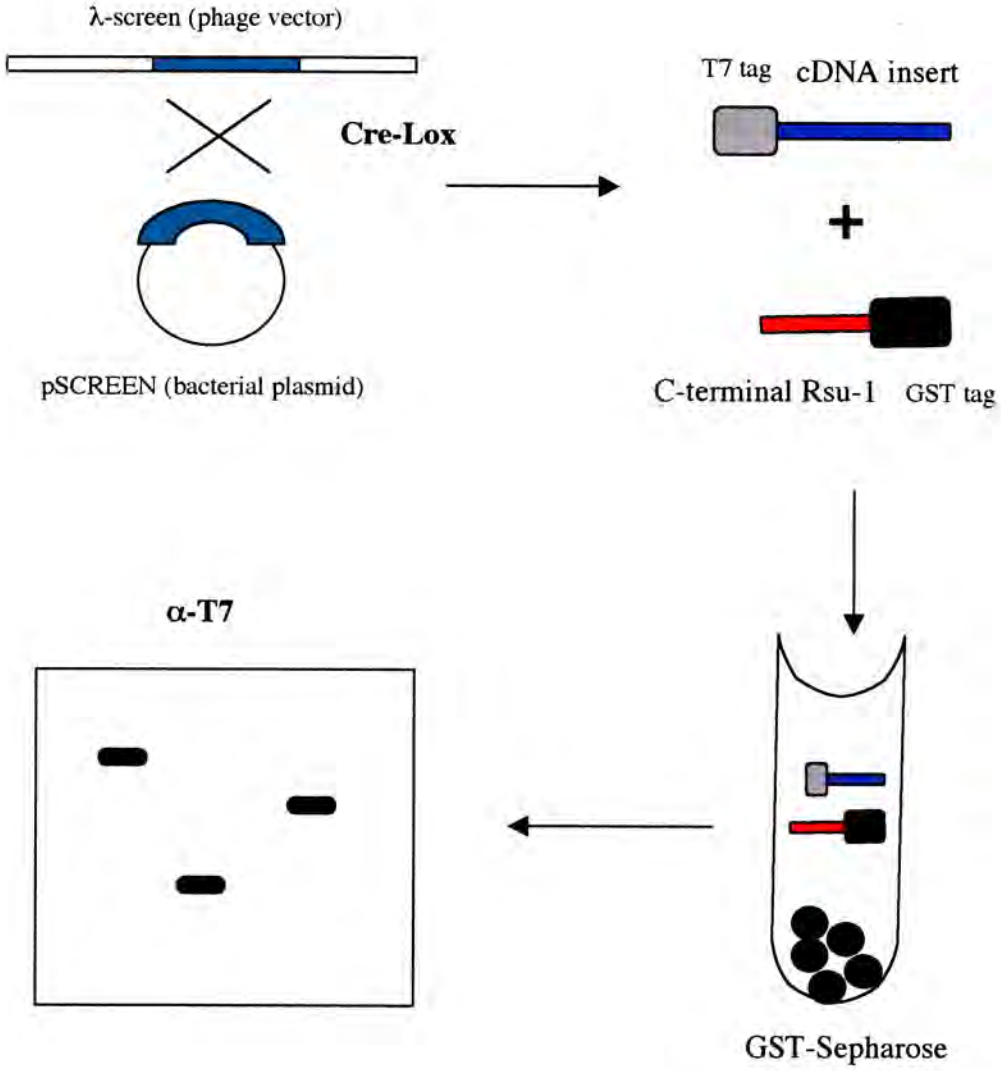


Figure 2. Scheme for Identification of Potential Binding Proteins to C-terminal Rsu-1. Potential binding proteins can be recovered from the library phage vector by cre-lox recombination. cDNAs containing an N-terminal T7 tag can be expressed and tested for binding *in vitro* to the C-terminal Rsu-1 probe 2T-4Δ. Interacting proteins can be "pulled down" with 2T-4Δ in the presence of GST-Sepharose beads, which have high affinity to the GST tag of 2T-4Δ. Potential binding proteins can be detected on nitrocellulose by immunoblotting with antibody specific to the T7 tag of the vector-encoded sequence.

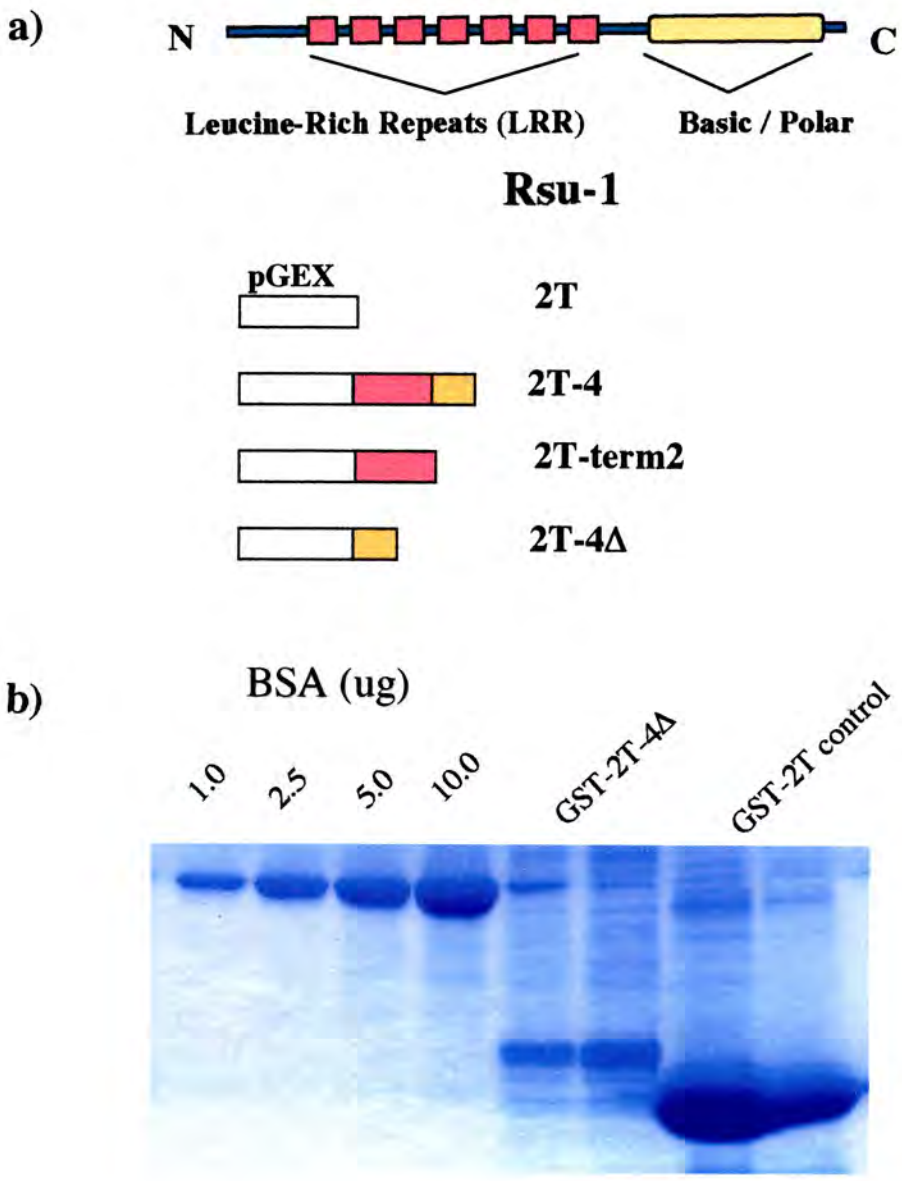


Figure 3. The C-terminal Rsu-1 Probe. a) previously constructed GST fusion protein vectors encoding full length, amino-terminal or carboxy-terminal Rsu-1 were available as probes for binding studies. For this study the COOH-terminal Rsu-1 probe 2T-4Δ and the control glutathione-S-transferase 2T were used. b) fusion proteins were grown in *E. coli*, induced with IPTG and purified as described. Aliquots of the fusion proteins (10 – 25 μl) were quantitated by comparison to varying amounts of BSA. Protein samples were mixed with Tris-Gly loading buffer, boiled for several minutes and then electrophoresed on 12% Tris-Gly gels. The gel was stained with Gel Code Reagent as described, rinsed with deionized water and dried by vacuum. Average yield of 2T-4Δ was 100 ng / μl.

Figure 4. a) coimmunoprecipitation of potential ligands from cell lysates. PC12 cells were metabolically labeled with ^{32}P-orthophosphate for 4 hours (500 μCi per plate), stimulated with NGF (200 ng / ml) for 15 minutes, washed with PBS and lysed in NP40 / DOC buffer. Lysates were precleared with the control fusion protein 2T and then coimmunoprecipitated with either 2T, 2T-4 or 2T-4Δ. The GST-Sepharose beads were washed 3X with NP40 / DOC buffer and resuspended in 1X Tricine buffer. Protein samples were briefly boiled and electrophoresed on 10% Tricine gel and then transferred to nitrocellulose. Film was exposed to the gel overnight (–80°C) and developed. **b) *in vitro* labeling of 2T-4Δ by PKA.** Reactions were done in the following buffer: MES, pH 6.9 (50mM), $MgCl_2$ (10mM), EDTA (0.5mM), γ-ATP (150mM), DTT (1mM) and BSA (0.25%). Reactions were started by adding PKA dilutions (0, 150, or 300 mU) to approximately 1 μg of 2T-4Δ and incubating for 15 minutes at 37°C. GST-Sepharose beads were centrifuged (2 minutes x 5000 rpm) and washed once in phosphorylation buffer. 2T-4Δ samples were resuspended in 50 μl Tris-Gly buffer, electrophoresed and developed by autoradiography. **c) *in vitro* labeling of 2T-4Δ by PKC.** Protein samples were prewashed in PKC buffer (Tris-Cl, pH 7.5, 20mM; $MgCl_2$, 10mM; and $CaCl_2$, 0.5mM) and labeled in the following buffer: Tris-Cl, pH 7.5, 20mM; $MgCl_2$, 10mM; $CaCl_2$, 0.5mM; 0.25% BSA, and phosphatidylserine, 100 μg / ml. Dilutions of PKC enzyme (0, 0.1mU, 0.2mU or 0.4mU) were added to start the reaction and incubated for 15 minutes at 37°C. Protein samples were washed and electrophoresed similar to the procedure of PKA labeling.

Figure 4

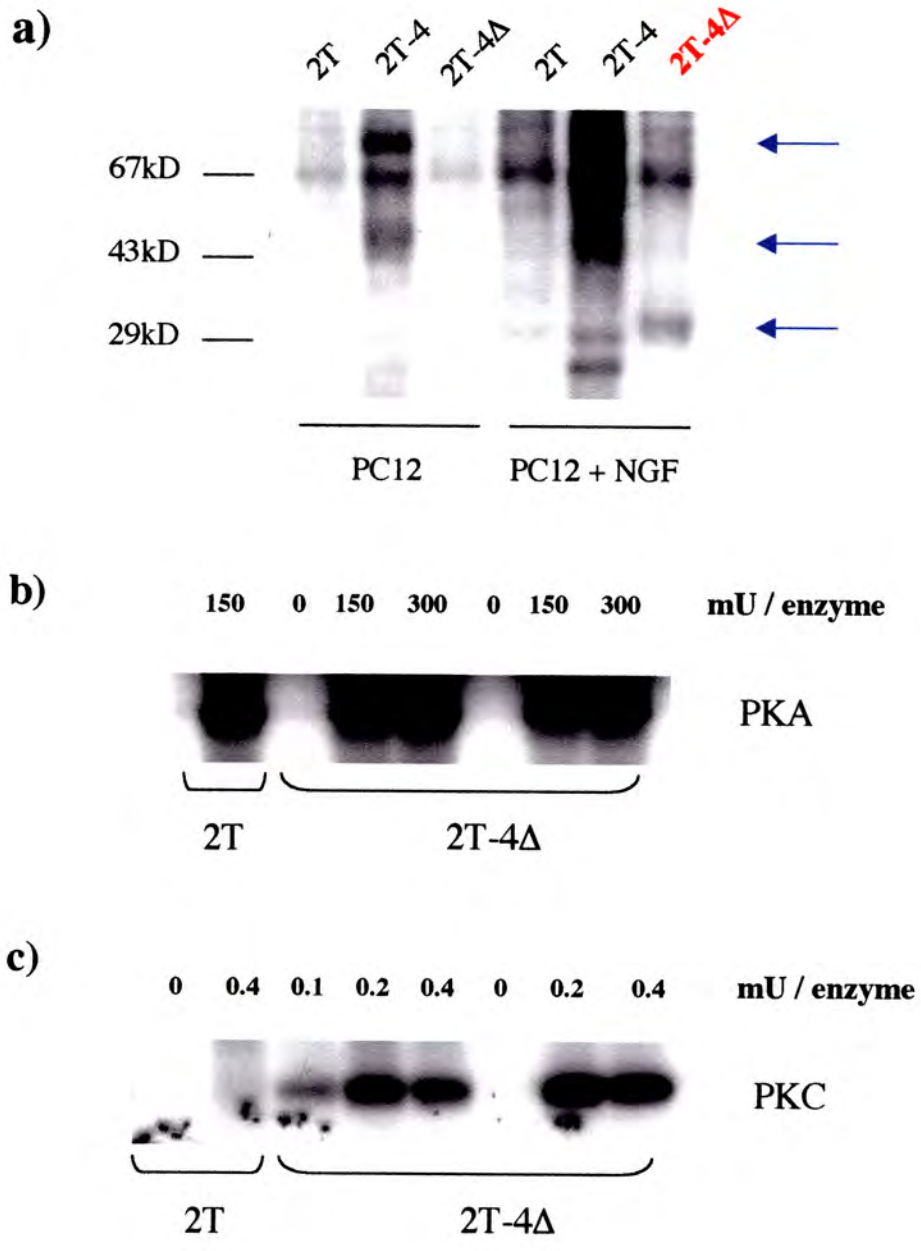

a)
2T
2T-4
2T-4Δ
2T
2T-4
2T-4Δ
67kD
43kD
29kD
PC12
PC12 + NGF
b)
150
0
150
300
0
150
300
mU / enzyme
PKA
2T
2T-4Δ
c)
0
0.4
0.1
0.2
0.4
0
0.2
0.4
mU / enzyme
PKC
2T
2T-4Δ

Figure 5. a) elution of radiolabeled 2T-4Δ for Far Western Screening. After radiolabeling 2T-4Δ, the GST-Sepharose beads were washed 2X with 0.5ml PKC wash buffer (20mM Tris-HCl, pH 7.5) and then incubated in GST elution buffer (100mM glutathione) to elute the 2T and 2T-4Δ proteins from the beads. Two separate elutions (1 hour and overnight) in 300 µl of elution buffer were done at 4°C on a rocker. 2.5 µl aliquots were taken from each elution as well as both washes and the original elution sample and added to 45 µl of 1X Tris-Gly buffer and electrophoresed as described above. b) **recovery of radiolabeled 2T-4Δ from NAP-10 filtration columns.** NAP-10 columns were equilibrated before use by adding buffer (10mM Tris-Cl, pH 7.5 / 250mM NaCl). The reaction mixture (about 600 µl) was poured directly over the column bed and 400 µl of equilibration buffer was added to bring the sample volume to 1.0ml. An additional 2.0ml of equilibration buffer was added to the column and 0.5ml fractions of the eluted protein was collected. 2.5 µl aliquots were added to 40 µl 1X Tris-Gly buffer for electrophoresis. c) **Far Western Screening of whole cell lysates.** A nitrocellulose filter containing lysates from NIH-3T3, U251 and PC12 cells normalized for protein concentration was incubated with 5 ml of solution containing the radiolabeled probe 2T-4Δ. The reaction was sealed in a plastic bag and incubated for approximately 15 hours at 4°C. The blot was washed 3X with buffer as described in the Far Western Assay, air-dried, and exposed to film for approximately 24 hours before it was developed.

Figure 5

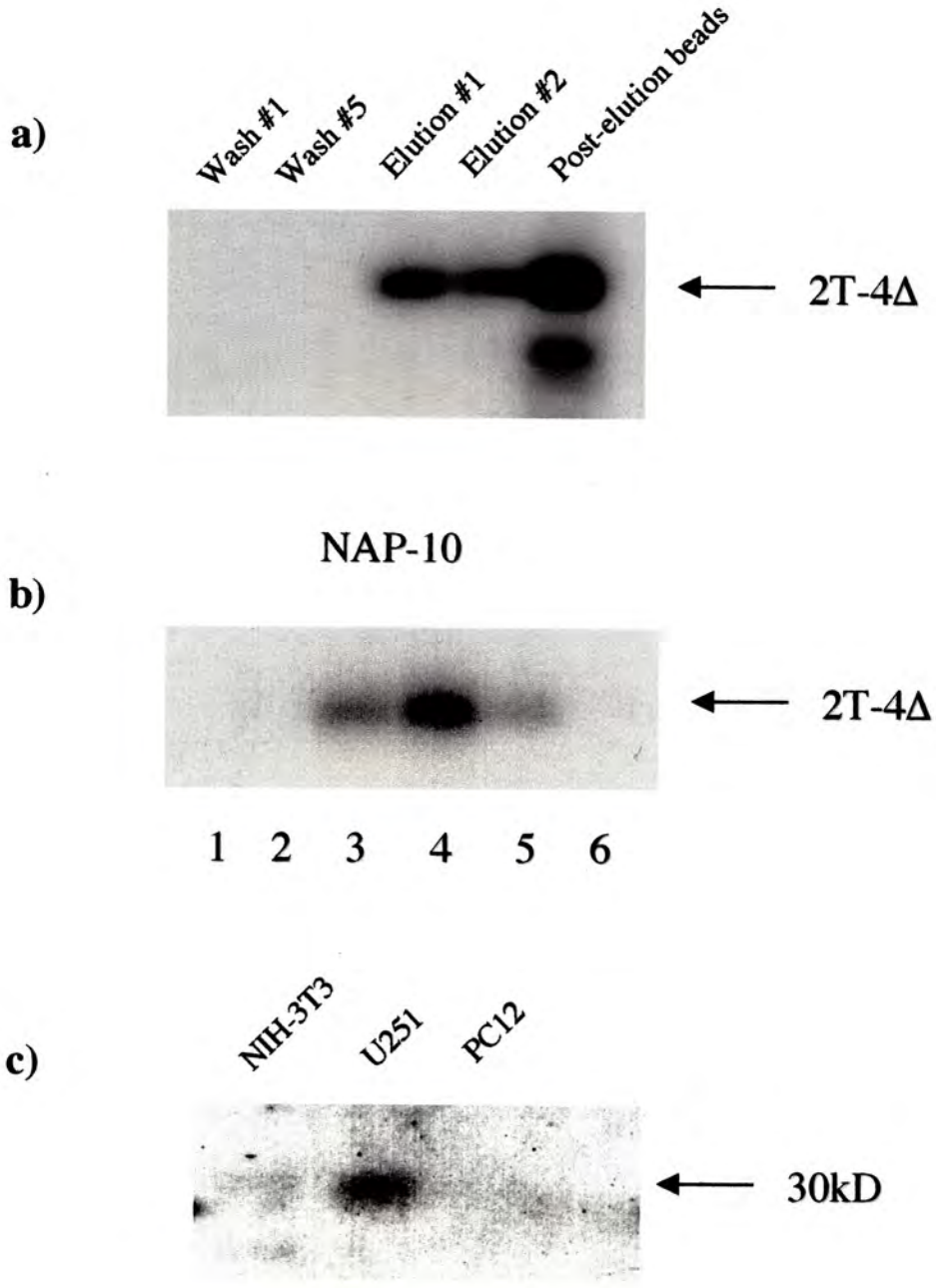
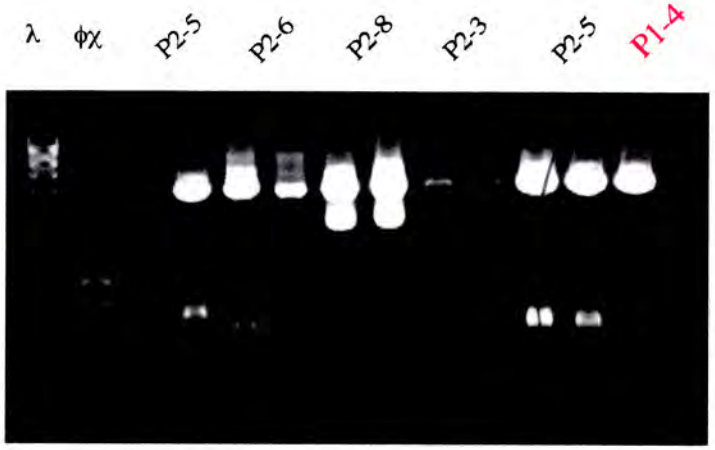

a)
Wash #1
Wash #5
Elution #1
Elution #2
Post-elution beads
2T-4Δ
NAP-10
b)
2T-4Δ
1 2 3 4 5 6
c)
NIH-3T3
U251
PC12
30kD

Figure 6. a) EcoRI / HindIII digestion of subclones recovered from Far Western Assay. After subclone DNA was purified by boiling mini-prep procedure, 10 µl of subclone DNA was digested with 10 µl of digestion mix containing the restriction enzymes EcoRI and HindIII. Samples were digested for 2 hours at 37°C and electrophoresed on a 1% agarose gel. 10 µl of the markers λ-HindIII and Phi-X / HaeIII were run with the digested samples. b) ***in vitro*** **expression of subclones.** Cultures of each subclone were grown in LB media plus ampicillin until the OD_{600} reached approximately 0.6. Protein expression was induced with 0.4mM IPTG for 3 hours, and then induced and uninduced cultures were harvested in the following manner: 10 µl lysozyme (100 µg / ml) and 100 µl 1% Triton X-100 were added to each culture, the tubes were placed on ice, sonicated 2X for 15 seconds and then centrifuged at 11,000 rpm x 15 min. (Sorvall, ST-Micro). 25 µl supernatant from both induced and uninduced cultures was mixed with 25 µl 2X Tris-Gly buffer and boiled briefly before electrophoresed on 12% Tris-Gly gels. 2.5 µl of the T7 control protein was run with 5 µl of the HMW protein marker on each gel.

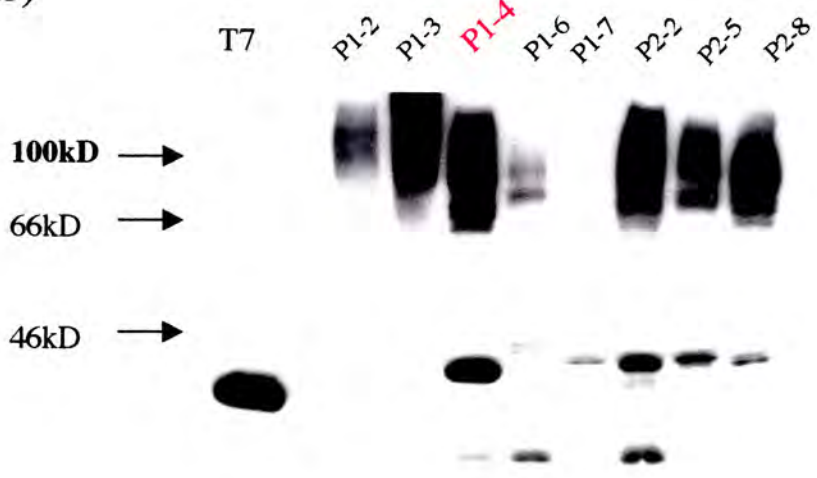
Figure 6

a)



EcoRI / HindIII

b)

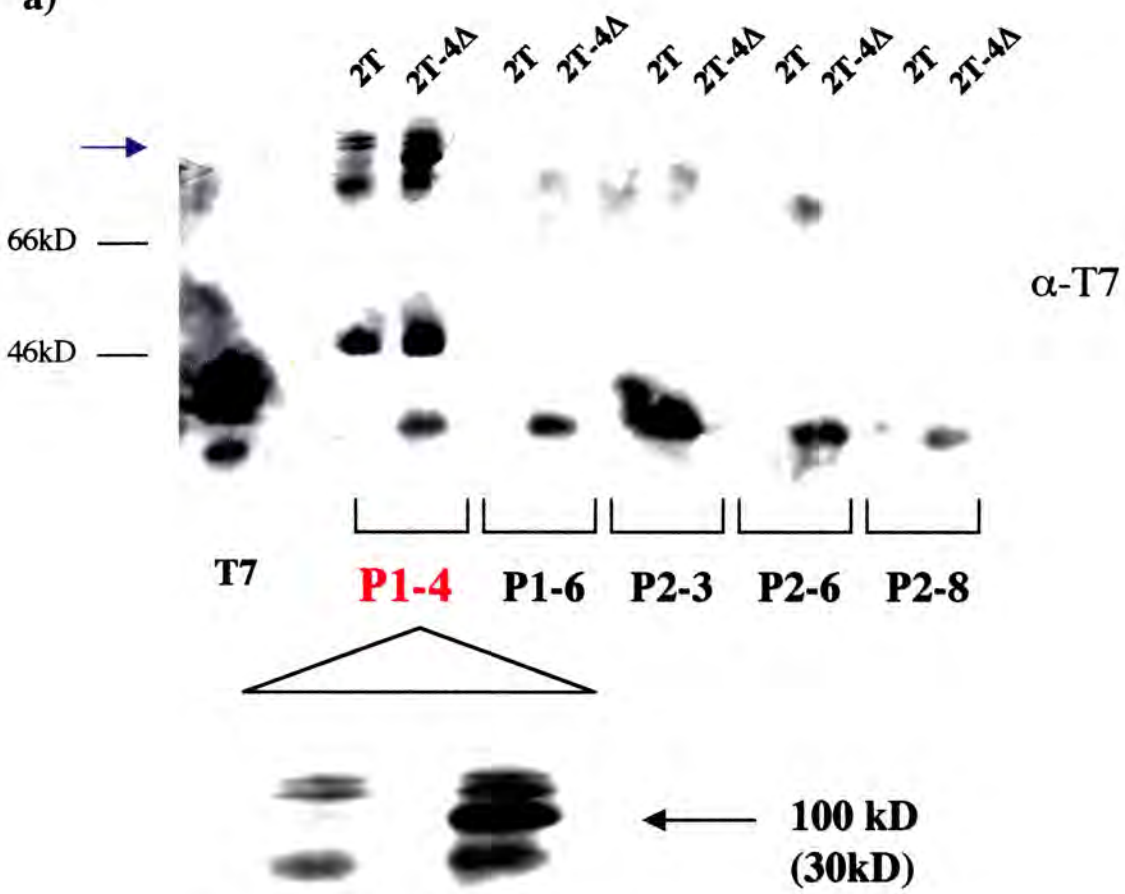


α-T7

Figure 7. a) in vitro binding assay of subclones to 2T-4Δ. Subclone cultures were grown in LB / ampicillin and induced with 0.4mM IPTG as described above. Approximately 250 µl of induced lysate from each subclone was incubated with 25 µl (1-2 µg) of the 2T and 2T-4Δ fusion proteins for 1 hour at 4°C. The GST-Sepharose beads were centrifuged (1 min. x 5,000 rpm) and resuspended in 50 µl 1X Tris-Gly loading buffer. Samples were electrophoresed with HMW marker and T7 control protein as described above. b) **sequence alignment of subclone PI-4.** The sequence of PI-4 was compared to known sequences in the database by BLAST homology search (www.ncbi.nlm.nih.gov/). Strong homology to an unidentified protein on chromosome 21q22.1 was noted – sequence identity was 90% (195 / 215 nucleotides) with a 1% frequency of gaps (4 / 215). The significance of a given alignment with score S is represented by the E (expect) value, the expected number of chance alignments with a score of S or better. The E value decreases exponentially as the score S that is assigned to a match between two sequences increases. The most significant alignments (lowest E values) are at the top.

Figure 7

a)



b)

Sequences producing significant alignments:		Score (bits)	E value
dbj |AP000190.1|AP000190	Homo sapiens genomic DNA, chromosom....	268	3e-69
dbj |AP000046.1|AP000046	Homo sapiens genomic DNA, chromosom....	268	3e-69
dbj |AP000114.1|AP000114	Homo sapiens genomic DNA of 21q22.1.....	268	3e-69
emb|AL135749.2|HSN14	Homo sapiens *** SEQUENCING IN PROG.	260	8e-67
gb|AC007688.15|AC007688	Homo sapiens 12p12-27.2-31.7 BAC RP.....	256	1e-65
gb|AC002404.1|AC002404	Human Chromosome X PAC RFCI1-290C9..	252	2e-64
gb|AF196970.1|AF196970	Homo sapiens WAS protein (WAS) and S....	238	3e-60
emb|AL031283.26|HS467K16	Human DNA sequence from clone RP3-.....	236	1e-59
gb|AC018751.30|AC018751	Homo sapiens chromosome unknown clo.....	220	7e-55
gb|AC010170.3|AC010170	Homo sapiens 3p22-21.3 BAC RPCI11-48...	212	2e-52

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cells. *Cell.* **77**, 841-852.

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Identification of the *in vivo* phosphorylation sites of the Ras Suppressor Rsu-1

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Running Title: TPA Stimulates Serine Phosphorylation of Rsu-1

SUMMARY

The Ras Suppressor Rsu-1 is a 33 kDa protein containing potential phosphorylation sites for protein kinase C (PKC), protein kinase A (PKA) and casein kinase II. Previous experiments showed that Rsu-1 is phosphorylated *in vitro* by PKA and PKC, and also *in vivo* in response to growth factor and 12-O-tetradecanoylphorbol-13-acetate (TPA), a known activator of PKC. Phosphoamino acid analysis of Rsu-1 suggests that *in vivo* phosphorylation occurs on serine residues. In this study site-directed mutagenesis of individual Rsu-1 consensus PKC sites revealed that two serine residues (serine-4 and serine-163) are phosphorylated *in vivo* in response to TPA. This conclusion is supported by evidence that Rsu-1 phosphorylation can be specifically blocked by broad-spectrum serine kinase inhibitors and the PKC inhibitor bisindolylmaleimide I (BIM), but not by inhibitors of tyrosine kinase, MEK and casein kinase. Since transient expression of wild type Rsu-1 is associated with increased protein levels of p53 and $p21^{CIP}$, as well as an increase in activated ERK, stable transfectants in MCF7 of Rsu-1 mutants S4A and S163G were tested for effect on these proteins. HA-Rsu-1 phosphorylation mutants failed to show ERK activation and stabilization of p53 in contrast to phosphorylatable HA-Rsu-1. Since Rsu-1 can inhibit growth and enhance apoptosis in MCF7 cell lines, S4A and S163G mutants were tested for these effects. Wild type and non-phosphorylatable Rsu-1 transfectants S4A and S163G inhibited anchorage-dependent and –independent growth in comparison to control MCF7 cells; however, the Rsu-1 mutants S4A and S163G failed to enhance apoptosis in response to TNF-α and staurosporine compared to wild type Rsu-1. These data support the hypotheses

that PKC or a PKC-dependent serine kinase phosphorylates Rsu-1 *in vivo*, and that Rsu-1 phosphorylation contributes to its effect on apoptosis but not growth inhibition.

INTRODUCTION

Our laboratory has focused on the molecular and biological properties of the Rsu-1 gene. Rsu-1 was originally cloned as a cDNA that suppressed v-*ras* transformation but not v-*src*, *mos*, or *raf* - *myc* transformation in NIH-3T3 fibroblasts (Cutler, 1992). Rsu-1 is well conserved between human and *C.elegans* (58% genetic identity and 76% amino acid similarity), and its RNA is ubiquitously expressed in higher eukaryotes. The amino-terminal portion of Rsu-1 is composed of seven adjacent leucine-rich repeats (LRR). A related LRR region is present in three proteins known to interact with *ras*: yeast adenylyl cyclase, SUR-8 and most recently Ce-FLI-1 (Colicelli, 1990; Sieburth, 1998; Goshima, 1999). Leucine-Rich Repeats are not exclusive to binding Ras, however, and represent a general protein structure found in prokaryotic, plant, and mammalian proteins that likely mediate protein-protein interactions (reviewed in Kobe, 1995 and Kajava, 1998).

To further characterize the role of Rsu-1 in Ras-initiated signal transduction, the signaling pathway from Ras to its downstream effectors was investigated. Initial experiments demonstrated that Rsu-1 expression in NIH-3T3 cells and PC12 cells inhibited RasGAP activity, and in response to Epidermal Growth Factor (EGF) and 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), showed reduced Ras-dependent JNK activation but increased ERK activation (Masuelli, 1996). Further studies with the cell line PC12, which is a model for NGF- and Ras dependent differentiation (Bar-Sagi, 1985; Noda, 1985; Wood, 1992) demonstrated that Rsu-1 expression enhanced the differentiation process in these cells, characterized by neurite outgrowth and sustained ERK activity (Masuelli, 1999). Additionally, Rsu-1 expression in PC12

cells led to expression of the CDK inhibitor $p21^{CIP}$ and growth inhibition (Masuelli, 1999).

Rsu-1 has also been studied in cell cycle regulation and the EGF signaling pathway in MCF7 breast carcinoma cells (Vasaturo, 2000). It was shown that MCF7 cells stably transfected with Rsu-1 show decreased Rho-associated kinase (ROK), cyclin-dependent kinase 2 (CDK2), and cyclin D-associated activity, increased levels of $p21^{CIP}$, and a decrease in growth rate and anchorage-independent growth (Vasaturo, 2000). Similar to previous cell lines tested, there was increased ERK activation and decreased JNK activation correlated with Rsu-1 expression. Most recently, Rsu-1 MCF7 transfectants have demonstrated stabilization of p53 protein levels and an enhancement of p53-dependent apoptosis (Vasaturo, submitted), suggesting that growth inhibition via Rsu-1 may be p53-dependent. Together this data suggested that Rsu-1 acts in a Ras-dependent fashion to inhibit proliferation and promote the differentiation pathway while blocking the transformation pathway.

Analysis of the predicted amino acid sequence of Rsu-1 identified consensus sites for phosphorylation by PKC, PKA and casein kinase II (PROSITE, www.ncbi.nlm.nih.gov). The Rsu-1 sequence does not predict ATP binding or identify a kinase domain, and recombinant Rsu-1 does not contain these activities. Because the regulation of the activity of numerous proteins is accomplished via phosphorylation, we examined the level of Rsu-1 phosphorylation in response to stimulators of the Ras pathway. This approach was designed to determine if the Rsu-1 protein was phosphorylated in response to signals which resulted in an increase in the level of GTP-bound Ras and increased signal transduction through

Ras. Previous experiments have demonstrated by phosphoamino acid analysis that Rsu-1 is phosphorylated on serine in response to TPA. In this study we attempt to identify the specific amino acids of Rsu-1 which become phosphorylated *in vivo*, as well as the effect of phosphorylation on Rsu-1 activity.

EXPERIMENTAL PROCEDURES

Construction of HA-Rsu-1 Phosphorylation Mutants. Consensus Protein kinase C (PKC) phosphorylation sites (PROSITE) in the mouse Rsu-1 open reading frame (ORF) were altered by site-directed mutagenesis (figure 1). Rsu-1 plasmid DNA containing the epitope tag Hemagglutinin (HA) in the cloning vector pCRII (Invitrogen) was mutagenized using Quik Change Mutagenesis Kit (Stratagene) or a previously described PCR-based procedure (Jones, 1991). The primers used for site-directed mutagenesis are listed (see appendix). Mutant plasmids were transformed into X_L-Blue competent bacterial cells (Stratagene) and selected on agar plates containing ampicillin. Mutant plasmid DNA was isolated and digested with the restriction enzymes Not I and Spe I (New England Biolabs) to excise the HA-Rsu-1 ORF fragment. The insert DNA was separated by electrophoresis, recovered from agarose and purified (Prep-A-Gene kit, Bio Rad) for ligation (Rapid DNA Ligation, Roche Molecular Biochemicals) to the expression vector p521, described previously (Masuelli, 1996). p521 was digested with Not I and Spe I enzymes and purified by phenol / chloroform extraction and ethanol precipitation in preparation for ligation. These ligation reactions containing mutant plasmids were transformed into X_L-Blue cells and selected on agar plates with kanamycin. Not I and Spe I enzyme digestion products which showed an insert size of 1.3 kb by agarose gel electrophoresis, the expected size of the mouse HA-Rsu-1 ORF, were selected for further analysis. For transfection assays and other experiments, mutant DNA was isolated using High Purity Concert Maxiprep Kit (Life Technologies) to remove endotoxin. All site-

specific alterations in the mutant plasmids were confirmed by sequencing using the PCR chain-termination reactions (Big Dye, ABI Biosystems).

***In Vivo* Phosphorylation of Rsu-1.** For analysis of HA-Rsu-1 phosphorylation mutants, Cos-1 cells were plated in 6-well or 12-well plates (Costar) at 2.5 or 5.0 x 10^5 cells / well. Cells were transfected with 1.0 μg of vector control (p521), wild type HA-Rsu-1 (3v65) or phosphorylation-mutant DNA described above using Lipofectamine 2000 Reagent (Life Technologies). Cos-1 cells were maintained in Dulbecco Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum, 1% glutamine and 1% penicillin / streptomycin. Approximately 48 hours post transfection, Cos-1 cells were preincubated in phosphate – free DMEM with 25mM Hepes for 30 – 60 minutes. Cells were labeled with ^{32}P orthophosphate (0.5mCi / ml) in phosphate – free DMEM for 4 hours and then stimulated with 200 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA) per well for 20 or 30 minutes. Briefly, cells were washed with PBS and lysed in RIPA buffer plus inhibitors (sodium vanadate, AEBSF, aprotinin, β-glycerol phosphate, and leupeptin). Lysates were precleared with fixed Staph A and then immunoprecipitated with mouse anti-HA antibody (clone 12CA5, Boehringer Mannheim) and 50% protein A Sepharose solution for 1 hour at 4°C. Immunoprecipitates were washed four times with 1.0 ml of RIPA buffer and resuspended in 50 μl 1X gel buffer plus 10mM dithiothreitol (DTT). Protein samples were boiled for 5 minutes and electrophoresed on either 12% Tris-Gly or 10% Tricine gels. Protein was transferred to PVDF or nitrocellulose membranes and subjected to autoradiography (Kodak X-OMAT film). The presence of immunoprecipitated HA-Rsu-1 protein was confirmed by Western

blotting using rat anti-HA antibody (clone 3F10, Roche Molecular Biochemicals) and ECL detection (Amersham).

Inhibition of Rsu-1 Phosphorylation In Vivo. The effect of specific inhibitors on phosphorylation of HA-Rsu-1 DNA was determined: NIH-3T3 cells incubated in low serum (1%) for 16 hours were labeled for 3 hours following addition of ^{32}P orthophosphate to the media. The following inhibitors were then added to the medium for the final hour of labeling: bisindoylmaleimide (BIM), 10 nM, CalBiochem), PD 98059 (50 μM, CalBiochem), wortmannin (200 nM, CalBiochem), and tyrphostin 25 (50 υM, CalBiochem). TPA (200 ng / ml) was added to stimulate phosphorylation of HA-Rsu-1 and cells were lysed prior to immunoprecipitation similar to the procedure described above.

Construction of Rsu-1 Phosphorylation Mutant Transfectants. MCF7 cells were transfected with HA-Rsu-1 phosphorylation mutant DNA and selected as stable transfectants by selection in G418 in a procedure described previously (Vasaturo, 2000). MCF7 cells were maintained in essential modified eagle medium (EMEM) with 10% fetal bovine serum, 2 mM glutamine, non essential amino acids and 200 μg / ml G418 sulfate.

Western Blotting. Lysates were prepared from 100mm plates of MCF7 control and transfectants in RIPA buffer plus inhibitors as described previously (Vasaturo, 2000). For Western blotting of soluble proteins, lysates were centrifuged to remove nuclear debris, normalized for protein concentration and separated by SDS-PAGE. Protein was transferred to PVDF membranes and probed with antibody to the following proteins: phospho ERK (Promega), ERK 1/2 (Transduction Laboratories),

Rac (Transduction Laboratories), pan Ras (Oncogene Science), rat anti-HA (Roche), and cyclin D1 (Upstate Biotechnology). For Western blotting of nuclear proteins, an equal amount of 2X gel buffer plus 10 mM DTT was added to total lysates and boiled immediately. Protein was transferred to membrane and probed with monoclonal antibodies to the following proteins: p53 (clone D0-1, Santa Cruz Biotechnology), $p27^{KIP}$ (Transduction Laboratories), and $p21^{CIP}$ (Pharmingen).

Growth Assays. For soft agar cell growth, MCF7 control and transfectants were plated in top agar (0.3%) in EMEM in 60-mm gridded tissue culture plates (Nunc Brand Products) over a bottom layer of 2ml EMEM containing 0.6% agar. Plates were incubated for 10-14 days in a 5% CO_2 atmosphere at 37° C. Colonies larger than 60 μM were scored as positive. The number reported was the average of two plates, and the data was reported as the percentage of cells plated which formed colonies. For anchorage-dependent growth, MCF7 control and transfectants were plated at 5×10^5 cells per 60 mm plate in EMEM with 10% FBS. Cells were treated with or without TPA (1ng / ml) and counted after 24 or 48 hours; the number of cells reported at each time point was the average of four plates.

Apoptosis Assay. Cells were seeded into 60 mm plates 48 hours prior to treatment. A combination of TNFα (15 ng / ml) and cyclohexamide (10 μg / ml), or staurosporine (1 μM) was added to the cells in complete growth media. Detached cells were harvested and combined with the trypsinized cell population at specific time points (12 or 16 hours) after treatment. Cell death was assayed by uptake of trypan blue. Each time point represents the average of three plates.

RESULTS

The primary sequence of Rsu-1 predicted that the major post-translational modification of the protein is phosphorylation. Several consensus sites for phosphorylation by PKC, PKA and casein kinase II are distributed throughout the molecule (**Fig. 1a**). Our laboratory has previously found that Rsu-1 can be phosphorylated *in vitro* by PKA and PKC, and phosphoamino analysis suggests the *in vivo* phosphorylation of Rsu-1 occurs on serine residues. In this study we examined the hypotheses that activation of PKC phosphorylates Rsu-1 and that phosphorylation positively regulates the biological activity of Rsu-1.

Since previous studies suggested that Rsu-1 interacts with Ras or Ras effector molecules, known Ras activators were chosen to determine the conditions of Rsu-1 phosphorylation. Several compounds were tested for stimulation of Rsu-1 phosphorylation in NIH-3T3 cells, including acidic FGF, NGF, EGF, and the phorbol ester TPA. While these stimuli all phosphorylated Rsu-1 *in vivo*, TPA demonstrated the most efficient phosphorylation of Rsu-1 (data not shown). Accordingly, TPA was chosen to test stimulation of Rsu-1 phosphorylation in Cos-1 cells, which expressed transiently-transfected Rsu-1 at higher levels than NIH-3T3 cells. Results show that TPA efficiently stimulated Rsu-1 phosphorylation under conditions of serum starvation and normal (10%) serum concentration (**Fig. 1b**), suggesting that Rsu-1 phosphorylation is induced by TPA and is not constitutive in this system. Since TPA is a well known activator of PKC, and since PKC is implicated in Ras-mediated signal transduction, the possibility that PKC or a PKC-dependent kinase phosphorylates Rsu-1 was examined. Inhibition of Rsu-1

phosphorylation was tested in NIH-3T3 cells transiently transfected with HA-Rsu-1 with a series of compounds: the PKC inhibitor bisindolylmaleimide I (BIM), the tyrosine kinase inhibitor tyrphostin 25, wortmannin, an inhibitor of phosphatidylinositol-3-kinase (PI-3-K), and the MEK inhibitor PD98059. Results indicated that phosphorylation of Rsu-1 was blocked by BIM but not the other inhibitors (**Fig 1c**). The addition of casein kinase II inhibitor also failed to prevent Rsu-1 phosphorylation in response to TPA (data not shown). This suggested that Rsu-1 phosphorylation was mediated by PKC or a PKC-dependent serine kinase, and not by PI-3-K, MEK, CK II or a tyrosine kinase.

Next, the individual consensus PKC sites of Rsu-1 (**Fig. 1a**) were tested as *in vivo* sites of Rsu-1 phosphorylation. Serine or threonine residues in the consensus phosphorylation sites were altered by site-directed mutagenesis to non-phosphorylatable amino acids, either glycine or alanine. To utilize the SV40 origin of replication of the expression vector p521, the Rsu-1 phosphorylation mutants were tested for stable expression in Cos-1 and 293T cells, which both contain large T antigen and efficiently express protein from this plasmid. Cells were harvested 48 hours post-transfection and protein expression was determined by Western blotting using anti-HA antibody. Results showed that amino acid substitutions in the consensus sites did not alter the stability of the Rsu-1 protein (**Fig. 2a**). The HA-Rsu-1 phosphorylation mutants were then transiently transfected into Cos-1 cells and tested for response to TPA stimulation. 48 hours post-transfection, cells were labeled with ^{32}P-orthophosphate for 4 hours, stimulated with TPA, and harvested. The phosphorylation-mutant Rsu-1 proteins were immunoprecipitated using antibody

to the HA-epitope tag and observed for labeling by SDS-PAGE and autoradiography. As shown (**Fig. 2b**), the HA-Rsu-1 construct encoding the amino acid substitution S163G was not phosphorylated in response to TPA. Interestingly, the S4A mutant appeared to label less efficiently and displayed a band slightly higher than the radiolabeled band of the wild type Rsu-1. In contrast, proteins containing the other altered consensus sites (S23G, T135A, T241A, S264A and S268G) showed robust phosphorylation comparable to the wild type HA-Rsu-1 (3v65). The same filter was probed with anti-HA antibody and indicates comparable levels of HA-Rsu-1 protein for all mutants tested. These results demonstrate that HA-Rsu-1 mutant S163G is not phosphorylated, and mutant S4A is less efficiently phosphorylated, in response to TPA.

To test the biological effect of serine-4 and serine-163 on Rsu-1 function, the MCF7 breast cancer cell line was transfected with plasmids encoding Rsu-1 mutants S4A and S163G, and stable transfectants expressing these Rsu-1 mutant proteins were selected. The MCF7 cell line is a model for estrogen receptor (ER) positive breast cancer and has been previously used to test biological activity of Rsu-1 expression (Vasaturo, 2000). Transfectants of Rsu-1 mutants S4A and S163G expressing HA-reactive protein were identified by Western blotting (**Fig. 3a**). The same filter containing transfectant cell lysates was also immunoblotted with antibody specific to Rac and Ras (**Fig. 3b, 3c**); no significant difference of Rac and Ras protein levels was noted among HA-positive or negative MCF7 clones.

As previously mentioned, overexpression of Rsu-1 in a variety of cell lines resulted in increased activation of ERK, stabilization of p53 and elevated $p21^{CIP}$

levels. We tested Rsu-1 phosphorylation mutants S4A and S163G for the activation of ERK in comparison to wild type, phosphorylatable HA-Rsu-1 as well as the control vector. Results indicate that cells expressing both phosphorylation mutants S4A and S163A showed levels of activated ERK comparable to the vector control, in contrast to wild-type, phosphorylatable Rsu-1 clone 9B (**Fig 3d**). As a control, levels of total ERK protein were immunoblotted and results showed no significant difference of ERK protein among the wild type, control, and phosphophorylation mutants of Rsu-1 (**Fig 3e**).

Additionally, total lysates from cells expressing S4A and S163G Rsu-1 mutants were assayed for effect on p53 and $p21^{CIP}$ expression. As shown (**Fig. 4a**), levels of p53 protein in Rsu-1 mutants S4A and S163G are comparable to the MCF7 control, in contrast to elevated levels of p53 protein in wild type Rsu-1 (clone 9B). Interestingly, the S4A mutant, which expresses HA-Rsu-1 levels comparable to wild type Rsu-1 clone 9B, does not show a dramatic increase in levels of $p21^{CIP}$ (**Fig 4b**). The levels of cyclin D1 and p27 protein appeared comparable among the wild type, control, and phosphorylation mutants of Rsu-1 (**Fig 4c, 4d**), confirming previous results between control and wild type MCF7 transfectants (clone 9B) and indicating that equal amounts of protein were loaded in the gels.

Experiments have previously shown that Rsu-1 overexpression can reduce anchorge-independent growth of MCF7 and U251 cancer cell lines (Vasaturo, 2000; Tsuda, 1995). Additionally, Rsu-1 overexpression effectively reduces the growth rates of several cell lines (PC12, MCF7, U251). To examine if phosphorylation is required for the growth-inhibitory properties of Rsu-1, the S4A and S163G MCF7

transfectants were tested for colony formation in soft agar (anchorage-independent growth) and anchorage-dependent growth rate in the presence or absence of TPA. Interestingly, results from the soft agar assay indicated little difference in colony formation between cells expressing the non-phosphorylatable mutants of Rsu-1 (S4A and S163G) and cells expressing wild type Rsu-1 (clone 14B), compared to the positive control MCF7 (**Fig 5a**). Additionally, the growth rate of cells expressing non-phosphorylatable Rsu-1 mutants was similar to wild type Rsu-1 (**Fig 5b**). All cell lines also showed growth inhibition in response to TPA. These results suggested that phosphorylation is not required for Rsu-1-mediated growth inhibition.

Recently, experiments have shown that Rsu-1 enhances apoptosis in MCF7 cells in response to TNF-α and staurosporine (Vasaturo, submitted). To test whether phosphorylation is required for Rsu-1-mediated apoptosis in this cell line, the Rsu-1 phosphorylation mutants S4A and S163G were tested by trypan blue exclusion for effect on apoptosis. Results show that cells expressing S4A and S163G mutants failed to enhance apoptosis in response to both TNF-α and staurosporine, in contrast to wild type Rsu-1 (**Table 1, Fig 6**). This data suggests that phosphorylation of serine-4 and serine-163 of Rsu-1 may be required for the proapoptotic response of Rsu-1 in MCF7 cell lines.

DISCUSSION

Rsu-1 is a 33 kDa protein which suppresses v-*ras* transformation when ectopically expressed in fibroblasts. The amino acid sequence of Rsu-1 predicts phosphorylation sites for protein kinase C, protein kinase A and casein kinase II, suggesting the major post-translational modification of the protein is phosphorylation. There is no ATP binding site or kinase domain predicted by the amino acid sequence, and these activities have not been detected in the recombinant Rsu-1 protein. Our results demonstrate that Rsu-1 is efficiently phosphorylated by TPA in the presence or absence of serum. This phosphorylation is completely inhibited by BIM, which is a potent inhibitor of PKC. Furthermore, amino acid substitutions at two consensus PKC sites, serine-4 and serine-163, reduce or prevent Rsu-1 phosphorylation.

While the Rsu-1 mutant S163G showed complete inhibition of phosphorylation in response to TPA, the mutant S4A displayed a band of reduced phosphorylation that migrated slightly higher than the phosphorylated Rsu-1 band. This suggested that both residues might become phosphorylated in a stepwise fashion – first serine-163, and then serine-4. If only serine-163 is altered, then no Rsu-1 phosphorylation occurs. However, if only serine-4 is altered (and serine-163 is intact), Rsu-1 phosphorylation is less efficient but still possible. Interestingly, serine-163 is located in the fifth segment of the LRR region of Rsu-1, while serine-4 is located in the extreme end of the amino-terminus of Rsu-1. Accordingly, it is possible that serine-4 only becomes accessible to the appropriate kinase after serine-163 is

phosphorylated; future studies might address the dynamics of Rsu-1 phosphorylation and test this possibility.

MCF7 stable transfectants of Rsu-1 mutants S4A and S163G were assayed for biochemical and biological effects. As predicted, these Rsu-1 phosphorylation mutants failed to activate ERK, and did not result in stabilization of the p53 protein. While wild type Rsu-1 clone 9B normally displays a two-fold induction of $p21^{CIP}$ compared to the control MCF7, mutant S4A showed only slight elevation of $p21^{CIP}$. This may be explained by the observation that clone 9B expresses HA-Rsu-1 levels slightly higher than mutant S4A. Therefore, induction of $p21^{CIP}$ may be related to Rsu-1 in a quantitative manner, rather than the qualitative effect of substitution at serine-4.

Due to studies that established the growth inhibitory properties of Rsu-1, it was predicted that phosphorylation regulated this activity of the Rsu-1 protein. However, assays for both anchorage-dependent and anchorage-independent growth do not show a significant alteration in the profile of growth inhibition induced by wild type Rsu-1 among the mutants S4A and S163G. In response to TPA, Rsu-1 mutants S4A and S163G show a comparable inhibition of growth to wild type Rsu-1 clone 9B. This demonstrates that non-phosphorylatable Rsu-1 inhibits growth as well as phosphorylatable Rsu-1 in this cell line.

Recent experiments have demonstrated that Rsu-1 overexpression in MCF7 cell line enhances p53-dependent apoptosis in response to TNF-α and staurosporine. Stable expression of S4A and S163G Rsu-1 mutants fails to enhance apoptosis of MCF7 cells in response to TNF-α and staurosporine. The lack of apoptotic response

is especially pronounced in response to staurosporine, in which the degree of apoptosis induced by mutants S4A and S163G was comparable to the MCF7 control. Future studies will attempt to confirm the level of apoptotic response in mutants S4A and S163G by immunoblotting for cleavage products of PARP and Rb.

Previous studies have demonstrated that high-dose (500 nM), long-term (> 24 hr) TPA treatment of MCF7 cells induces apoptosis (deVente, 1995; Chen, 1995). This contrasts the effect of lower dosages (1 ng / ml) used in our studies, which promotes growth inhibition. This suggested that TPA activates the apoptotic response indirectly by induction of PKC isoforms that stimulate apoptotosis (Majumder, 2000). Very recently, a mechanism has been proposed that links TPA treatment and apoptosis, characterized by cytochrome c release, to the action of the PKC isoform PKC-δ (Majumder, 2000). This finding makes PKC-δ an attractive candidate as the upstream kinase of Rsu-1 phosphorylation. Additional evidence supports the role of PKC-δ as a proapoptotic kinase, including its identification as a kinase of nuclear lamins (Cross, 2000) and its induction of phospholipid scramblase activity during apoptosis (Frasch, 2000). The TPA-sensitive residues serine-4 and serine-163 of Rsu-1 are likely dependent on phosphorylation by PKC-δ or a similar kinase for its proapoptotic response.

The lack of stabilization of p53 protein levels in Rsu-1 mutants S4A and S163G also contributes to its decreased apoptotic activity. The p53 protein is central to the apoptotic response – by sensing DNA damage it can stimulate mitochondria to release cytochrome c via upregulation of the protein Bax (Hanahan, 2000). Recent studies in MCF7 cell line demonstrated that TNF-α-induced apoptosis is p53-

dependent (Cai, 1997; Ameyar, 1999). More recently it has been proposed that a portion of stabilized p53 protein localizes to mitochondrial membranes, where it promotes apoptosis as opposed to cell-cycle arrest (Marchenko, 2000). Interestingly, Rsu-1 has been shown to interact with Raf-1 *in vitro* (Masuelli, 1996); under certain circumstances, activation of Raf-1 and MAPK has been shown to promote apoptosis (Cross, 2000). The finding that MCF7 Rsu-1 transfectants S4A and S163G have reduced activation of ERK support a role for Raf-1 / ERK activation in the proapoptotic response in MCF7 cell lines. Strong activation of Raf-1 causes p53 dependent cell-cycle arrest, whereas normal Raf-1 function appears to promote proliferation (Downward, 1998). Perhaps Rsu-1 cooperates with Raf-1 to promote apoptosis in response to TPA and PKC isoforms such as PKC-δ.

This paper demonstrates that Rsu-1 is phosphorylated *in vivo* by TPA, and that Rsu-1 phosphorylation occurs on serine-4 and serine-163. Alteration of serine-4 and serine-163 does not significantly alter growth inhibition of Rsu-1 in MCF7. However, serine-4 and serine-163 appear to regulate the ability of Rsu-1 to enhance apoptosis in MCF7. This is supported by the observation that stable transfectants of Rsu-1 mutants S4A and S163G lack activation of ERK and stabilization of p53 protein. Rsu-1 phosphorylation is PKC dependent, either directly or indirectly, since the PKC inhibitor BIM effectively blocks Rsu-1 phosphorylation in response to TPA. The growth inhibitory property of Rsu-1 is likely phosphorylation-independent, whereas its role as a proapoptotic protein is inducible by a TPA-dependent process. Future studies might address the direct serine kinase of Rsu-1 and expand its role as a TPA- and PKC-dependent proapoptotic molecule

APPENDIX A

Primers used for site-directed mutagenesis of HA-Rsu-1 consensus phosphorylation sites:

S4A: 5' CAGATTACGCTGGTTCCAAGGCACTGAAGAAGCTGGTG

3' CACCAGCTTCTTCAGTGCCTTGGAACCAGCGTAATCTG

S23G: 5' GGAAGTGGACATGGGTGACAGGGGTATCTCC

3' GGAGATACCCCTGTCACCCATGTCCACTTCC

T135A: 5' GGAAACTTCTTCTACCTCACCGCCCTGGCACTCTATCTAAGC

3' GCTTAGATAGAGTGCACGCAGGGCGGTGAGGTAGAAGAA

GTTTCC.

S163G: 5' GTTGCAGATACTCGGCCTCAGGGATAATGACC

3' GGTCATTATCCCTGAGGCCGAGTATCTGCAAC

T241A: 5' GCTTACAAGTACCTCTACGGCAGACACATGCAAGCGAAC

3' GTGTCTGCCGTAGAGGTACTTGTAAGCTTCTGAACGAA

S264A / S268G:

5' AAACCAAAAAGATCGGCCGGAAACCCCTAGCA

3' CGGCCGATCTTTTTTGGTTTGTCGTTATTCTTCTTTGG

APPENDIX B

HA-Rsu-1 Phosphorylation Mutants – Site-Directed Mutagenesis Constructs

Controls

p521: empty expression vector

3v70: wild type Rsu-1 in pSVZeo

3v65: wild type Rsu-1 in p521

Single Mutants

3v84: T241A in cloning vector pCRII

3v85: T241A in expression vector p521

3v88: T135A in expression vector pSVZeo

3v95: T135A in expression vector p521

3v96: S4A in cloning vector pCRII

3v97: S4A in expression vector p521

3v98: S23G in cloning vector pCRII

3v99: S23G in expression vector p521

3v100: S163G in cloning vector pCRII

3v101: S163G in expression vector p521

3v114: S163A in cloning vector pCRII

3v115: S163A in expression vector p521

Double Mutants

3v68: S264A + S268G in cloning vector pCRII

3v69: S264A + S268G in expression vector p521

3v104: T241A + T135A in cloning vector pCRII

3v105: T241A + T135A in expression vector p521

3v106: S4A + S23G in cloning vector pCRII

3v107: S4A + S23G in expression vector p521

3v110: S4A + S163A in cloning vector pCRII

3v111: S4A + S163A in expression vector p521

3v112: S23G + S163G in cloning vector pCRII

3v113: S23G + S163G in expression vector p521

Triple Mutants

3v102: T135A + S264A + S268G in cloning vector pCRII

3v103: T135A + S264A + S268G in expression vector p521

3v108: S4A + S23G + S163G in cloning vector pCRII

3v109: S4A + S23G + S163G in expression vector p521

Figure 1. a) **Rsu-1 contains several consensus phosphorylation sites**. PROSITE analysis of the Rsu-1 protein sequence suggests the presence of eight potential phosphorylation sites for PKC, PKA or CKII. The amino acids predicted to be phosphorylated are either serine or threonine residues distributed throughout the Rsu-1 molecule. b) ***in vivo* Rsu-1 phosphorylation is stimulated by TPA**. Rsu-1 phosphorylation was tested by TPA stimulation in the presence or absence of serum. The vector control (p521) received TPA to control for background. Both the vector and wild type Rsu-1 were immunoprecipitated with mouse monoclonal antibody specific to the HA-epitope tag of Rsu-1. After electrophoresis, transfer and autoradiography, the same filter was probed with rat monoclonal antibody to HA to confirm the expression of HA-Rsu-1. c) ***in vivo* Rsu-1 phosphorylation is blocked by the PKC inhibitor BIM.** The ability of specific inhibitors to block Rsu-1 phosphorylation in response to TPA (200 ng / ml) was tested. BIM (10 nM), PD 98059 (50 μM), Wortmannin (200 nM) and tyrphostin 25 (50 μM) were added to NIH-3T3 cells transiently transfected with HA-Rsu-1. Radiolabeled HA-Rsu-1 was detected as described above by autoradiography after immunoprecipitation with monoclonal anti-HA antibody. The control reaction was immunoprecipitated with anti-IgG antibody.

Figure 1

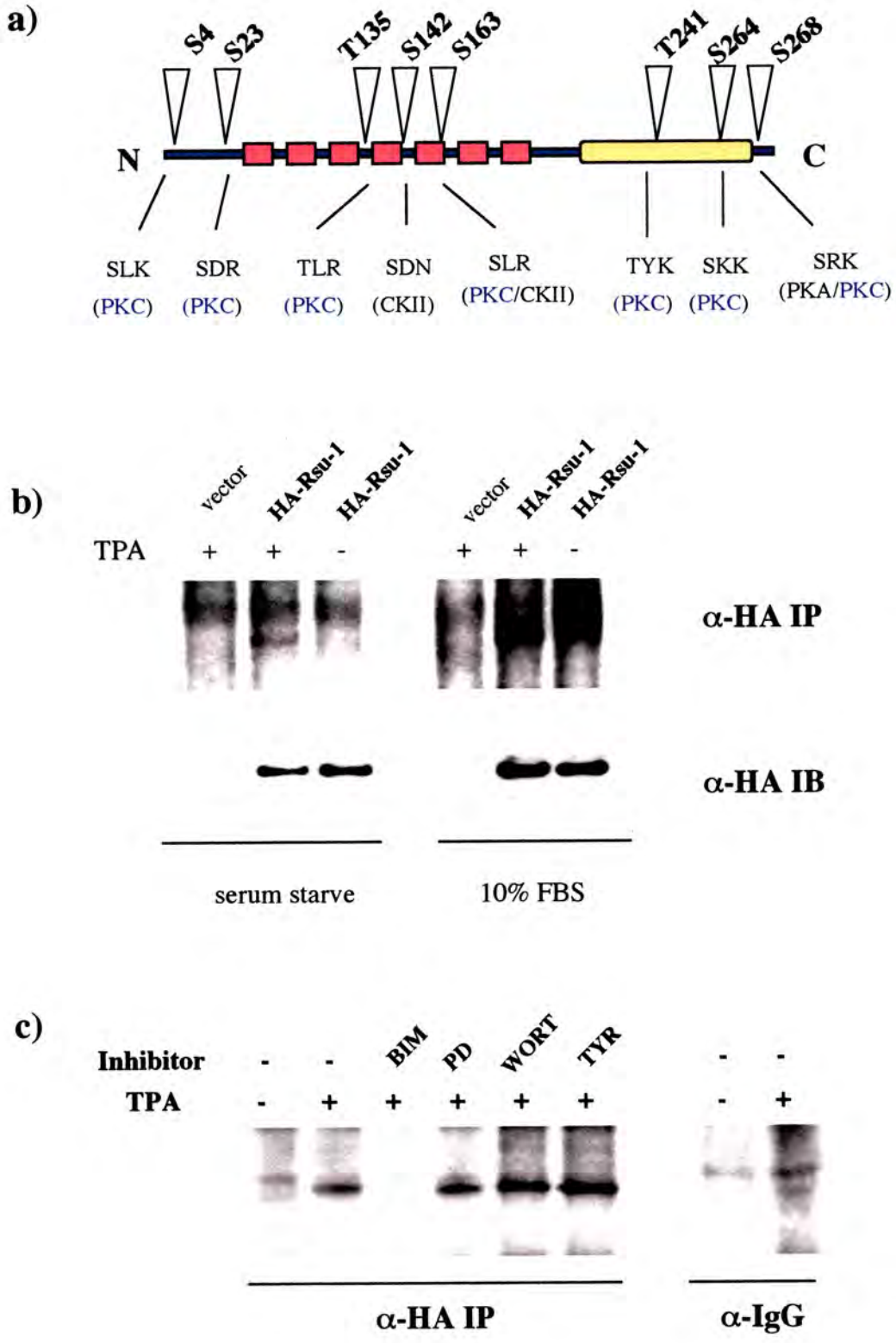

a)
S4
S23
T135
S142
S163
T241
S264
S268
N
C
SLK (PKC)
SDR (PKC)
TLR (PKC)
SDN (CKII)
SLR (PKC/CKII)
TYK (PKC)
SKK (PKC)
SRK (PKA/PKC)
b)
vector
HA-Rsu-1
HA-Rsu-1
vector
HA-Rsu-1
HA-Rsu-1
TPA
+ + - + + -
α-HA IP
α-HA IB
serum starve
10% FBS
c)
Inhibitor
TPA
- - BIM PD WORT TYR - -
- + + + + + - +
α-HA IP
α-IgG

Figure 2. a) **Stable expression of Rsu-1 constructs containing altered consensus phosphorylation sites.** HA-Rsu-1 constructs in which consensus PKC phosphorylation sites were altered by site-directed mutagenesis were tested for transient expression in Cos-1 cells and 293T cells. Five single-site constructs (T241A, T135A, S4A, S23G, and S163G) and one double-site mutant (S264A / S268G) were stably expressed in these cell lines. Two additional mutants, S163A and S4A / S163A, were also tested for transient expression in Cos-1 cells. The single CKII site (S142) was not tested in these studies. b) **TPA-induced phosphorylation of Rsu-1 constructs with altered consensus phosphorylation sites**. HA-Rsu-1 constructs encoding amino acid substitutions at the designated sites were transiently expressed in Cos-1 cells and tested for phosphorylation in response to TPA as described in Experimental Procedures. All HA-proteins were immunoprecipitated with mouse monoclonal anti-HA antibody and the expression of all constructs was confirmed by immunoblotting the same filters with rat anti-HA antibody.

Figure 2

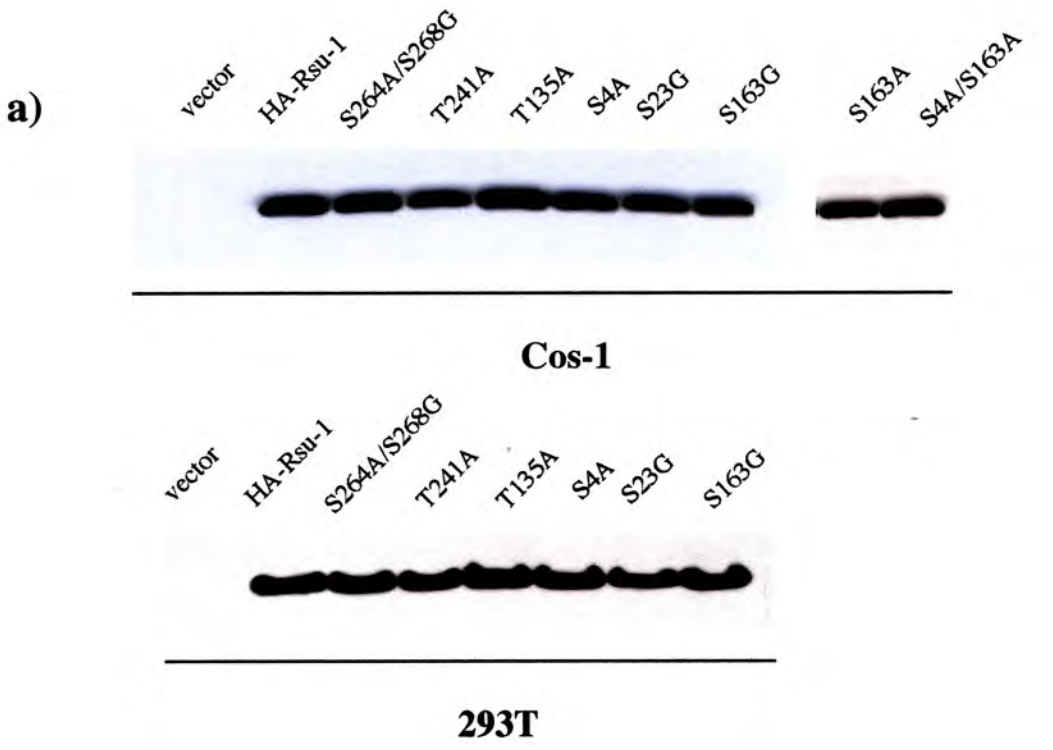

a)
vector
HA-Rsu-1
S264A/S268G
T241A
T135A
S4A
S23G
S163G
S163A
S4A/S163A
Cos-1
vector
HA-Rsu-1
S264A/S268G
T241A
T135A
S4A
S23G
S163G
293T
b)
vector
HA-Rsu-1
S264A/S268G
T241A
T135A
vector
HA-Rsu-1
T135A
S4A
S23G
S163G
S163G
α-HA IP
α-HA IP + α-HA IB

Figure 3. a) **Isolation of stable transfectants of Rsu-1 phosphorylation mutants in MCF7**. HA-Rsu-1 constructs encoding S4A and S163G substitutions were transfected in the MCF7 cell line and selected as stable transfectants. Transfectants that retained the HA-Rsu-1 protein after selection (S4A clone #2 and S163G clone #3) were chosen for further analysis. b, c) **Rac and Ras levels are not altered by HA-Rsu-1 expression**. In agreement with previous results, the levels of Rac and Ras are not altered among MCF7 control, wild type clone 9B (data not shown), and HA-Rsu-1 mutants S4A and S163G. d, e) **HA-Rsu-1 mutants S4A and S163G show reduced activation of ERK**. Rsu-1 mutants S4A and S163G were tested for ERK activation by Western blotting with antibody specific for activated (phosphorylated) ERK. The same filter was sequentially probed with antibody to total ERK to confirm comparable levels of protein among the samples.

Figure 3

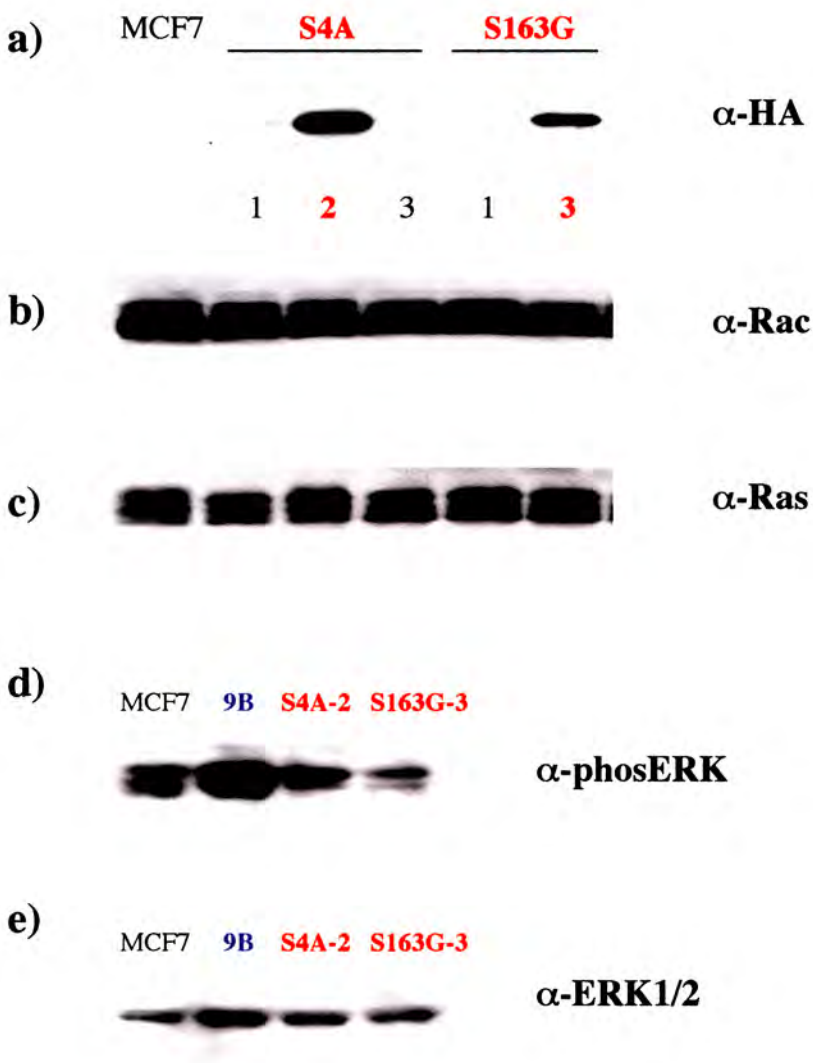

a)
MCF7
S4A
S163G
α-HA
1
2
3
1
3
b)
α-Rac
c)
α-Ras
d)
MCF7
9B
S4A-2
S163G-3
α-phosERK
e)
MCF7
9B
S4A-2
S163G-3
α-ERK1/2

Figure 4

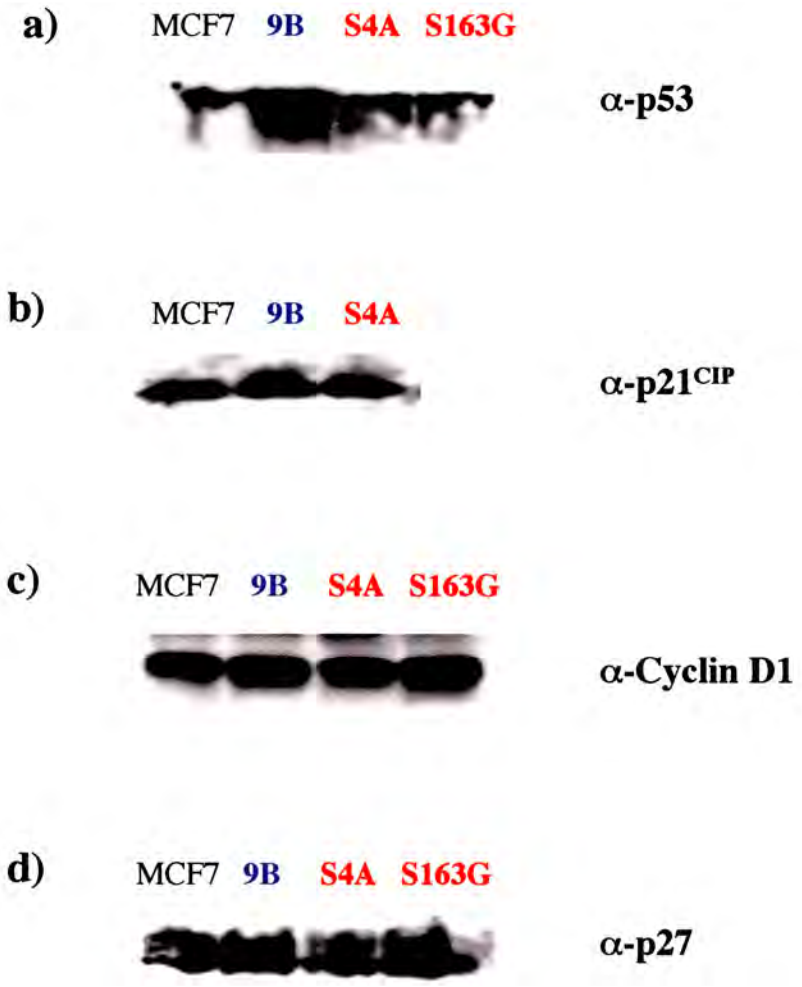
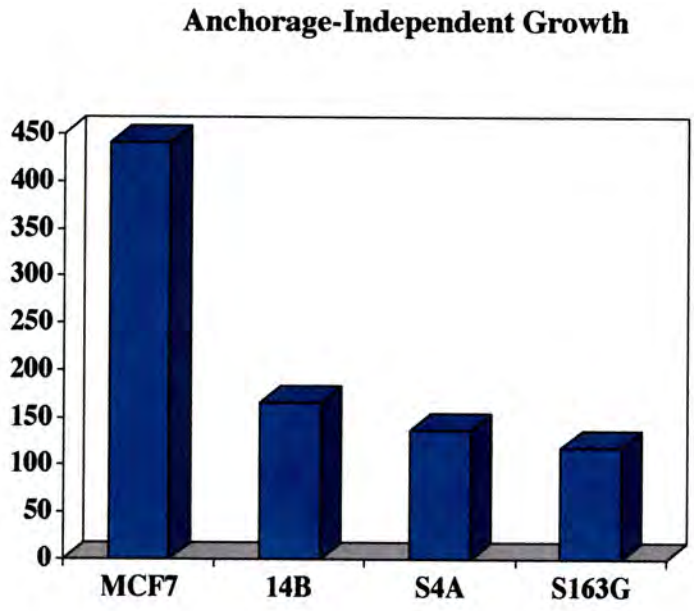


Figure 4. Rsu-1 Mutants Fail To Enhance Stabilization of p53. Total lysates of Rsu-1 mutants S4A and S163G in addition to wild type Rsu-1 clone 9B and the MCF7 control were analyzed for level of p53 a) and $p21^{CIP}$ b), as well as levels of cyclin D1 (soluble lysate) and $p27^{KIP}$ (total lysate) by Western blotting as described in Experimental Procedures.

Figure 5. Rsu-1 mutants S4A and S163G retain growth inhibitory properties in MCF7. a) Rsu-1 phosphorylation mutants were tested for anchorage-independent growth (colony formation in soft agar). The data represents the total number of colonies scored after 14 days of growth in agar. Colonies of seven (7) or more cells were scored as positive. A total of 100 grids was counted for each determination. For this experiment wild type clone 14B, which express HA-Rsu-1 levels comparable to clone 9B, was used. b) Rsu-1 mutants were tested for anchorage-dependent growth in the presence or absence of TPA. 48 hours after seeding cells, TPA (1 ng / ml) was added and the total number of cells was counted after an additional 48 hours. The data represents the total number of cells counted from four separate determinations for each sample. Growth rate of wild type clone 9B and Rsu-1 phosphorylation mutants S4A and S163G was significantly reduced in comparison to MCF7 control. Means of multiple groups were compared by one-way ANOVA followed by Tukey's multiple range test, $P \leq 0.05$.

Figure 5

a)


Anchorage-Independent Growth
450
400
350
300
250
200
150
100
50
0
MCF7
14B
S4A
S163G

b)

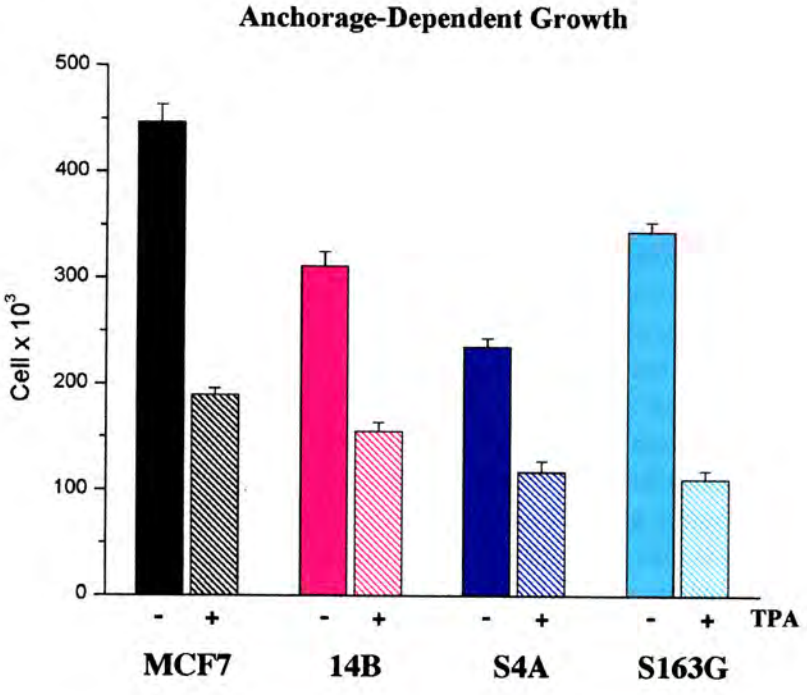

Anchorage-Dependent Growth
Cell x 10³
500
400
300
200
100
0
- + - + - + - + TPA
MCF7
14B
S4A
S163G

Figure 6

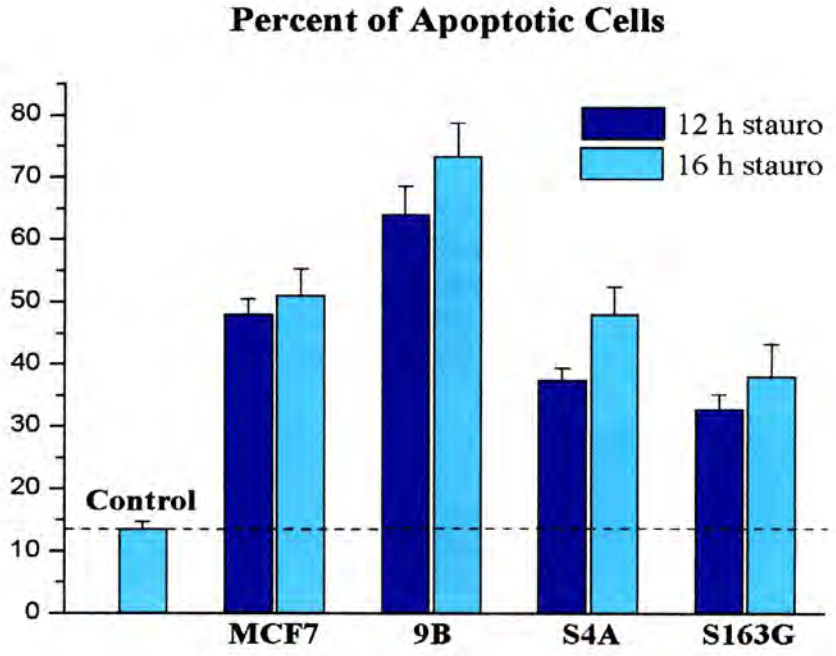


Figure 6. Rsu-1 phosphorylation mutants fail to enhance apoptosis in MCF7 cells. Rsu-1 mutants S4A and S163G were tested for enhancement of apoptosis in response to TNF-α / cyclohexamide and staurosporine. Cells were scored for apoptosis by trypan blue exclusion. Briefly, cells were harvested at 12 or 16 hours, centrifuged, resuspended in 1.0ml of media, and 50 µl of cells was mixed with 50 µl of trypan blue and counted. Data is represented as percent of apoptotic cells, from three separate determinations for both TNF-α and staurosporine treatment (Table 1). Apoptosis was demonstrated to be signifcantly reduced among mutants S4A, S163G and the MCF7 control in contrast to wild type clone 9B by statistical analysis (means of multiple groups were compared by one-way ANOVA followed by Tukey's multiple range test, $P \leq 0.05$).

Table 1

Cell line	Treatment [a]	% apoptotic cells
MCF7-control	(-)	< 0.1
	Stauro 12 hours	41
	Stauro 16 hours	47
	TNFα+CHX 12 hours	26
	TNFα+CHX 16 hours	34
MCF7-Rsu-1-9B	(-)	< 0.1
	Stauro 12 hours	58
	Stauro 16 hours	70
	TNFα+CHX 12 hours	32
	TNFα+CHX 16 hours	44
MCF7-Rsu-1-S4A	(-)	< 0.1
	Stauro 12 hours	32
	Stauro 16 hours	45
	TNFα+CHX 12 hours	28
	TNFα+CHX 16 hours	38
MCF7-Rsu-1- S163G	(-)	< 0.1
	Stauro 12 hours	36
	Stauro 16 hours	39
	TNFα+CHX 12 hours	25
	TNFα+CHX 16 hours	35

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DISCUSSION

This thesis presents the results of two specific aims: the identification of binding proteins to C-terminal Rsu-1, and the identification of *in vivo* Rsu-1 phosphorylation sites. This discussion attempts to integrate these results with previous data on Rsu-1 activity and to build a model of Rsu-1 regulation.

Results from chapter 2 demonstrate the *in vitro* binding of a 100 kD fusion protein to C-terminal Rsu-1; the 30kD portion of this protein is encoded by the cDNA insert. The DNA sequence encoding this protein shows strongest homology to an as yet unidentified gene sequence located on the long arm of chromosome 21. Further studies are required to 1) identify the full length sequence of the gene and 2) determine if there is an *in vivo* association with C-terminal Rsu-1. Interestingly, this unidentified sequence also suggests similarity to the Wiskott-Aldrich Syndrome Protein (WASP).

The WASP protein has been shown to bind Cdc42 (Miki, 1996; Aspenstrom, 1996; Kolluri, 1996) and promote actin polymerization via the Arp2/3 complex (Machesky, 1998; Machesky, 1999; Winter, 1999; Machesky, 1999). While speculative, it is reasonable to assume that Rsu-1 might bind a Rho-associated molecule such as WASP, since Rsu-1 has been shown to decrease ROK activity and JNK activation (Masuelli, 1996; Vasaturo, 2000). Also, a body of evidence suggests that Ras-dependent transformation requires JNK activation, which is mediated at least in part by the Rho-related proteins Cdc42 / Rac (Clark, 1996; Coso, 1995; Minden, 1995; Olson, 1995).

Previous studies in the laboratory have shown an *in vitro* association of Rsu-1 to Raf (Masuelli, 1996), and have also identified the protein KSR (Kinase Suppressor of Ras) as an HA-Rsu-1 co-immunoprecipitating protein from transiently transfected Cos cells (Cutler, unpublished results). KSR and Raf-1 are related proteins - both contain a serine / threonine kinase domain and a cysteine-rich zinc finger domain which binds phorbol esters and diglycerides, indicating homology to the PKC family of enzymes (Downward, 1995). However, KSR does not contain the serine / threonine – rich CR2 domain found in Raf family members and does not show similarity to the Ras-binding region of Raf (Downward, 1995). Interestingly, a recent report links KSR and Raf-1 as components of a multimolecular complex that enhances MAP kinase signaling (Stewart, 1999). Other proteins within the complex included MEK-1 and -2, Hsp90, Hsp70, Cdc37 and 14-3-3, as well as three unidentified proteins of 36kD, 34kD and 33kD. The molecular weight of Rsu-1 is 33kD, suggesting that this unidentified protein might represent Rsu-1. Ectopic expression of Rsu-1 in a variety of cell lines results in enhanced ERK activation (Masuelli, 1996; Masuelli, 1999; Vasaturo, 2000). While these data imply association of Rsu-1 with a Raf-1 and KSR – containing complex, further studies are required to test these possible relationships.

Interestingly, the leucine-rich repeat (LRR) protein SUR-8, identified in a genetic screen in *C. elegans* as a suppressor of Ras-dependent vulval differentiation (Sieburth, 1998), has been found to bind both Ras-GTP and Raf, and to enhance MAP kinase activation (Li, 2000). One model has been proposed where SUR-8, KSR, Raf-1, MEK and ERK form a complex linking receptor tyrosine kinases

(RTKs) and Ras activation to downstream MAP kinase signaling (Sternberg, 1998). Very recently the LRR region of RanGAP has been shown to mediate binding to the GTPase Ran and subsequent GTPase activation (Haberland, 1999). While LRR proteins are not limited to interaction with GTPases, it is possible that one subset of LRR proteins are specific for G-binding proteins such as Ras and Ran. Studies are currently underway to determine interactions specific to the LRR region of Rsu-1 using the yeast two-hybrid system – two non-GTPase proteins have been identified as potential binding proteins to Rsu-1 to date. Also, it should be noted that no high-affinity association or GTP-dependent binding to Ras has been detected either *in vitro* or *in vivo* using Ras and Rsu-1 constructs.

Results from chapter 2 demonstrate that PKC can phosphorylate Rsu-1 *in vitro*. Additionally, results from chapter 3 show that Rsu-1 is phosphorylated on serine-4 and serine-163 following stimulation of cells with the PKC-activator TPA, and that this phosphorylation is blocked by the PKC inhibitor BIM. This suggests that a phorbol ester – activated or PKC-activated enzyme catalyzes *in vivo* Rsu-1 phosphorylation. Since the best known phorbol ester - activatable enzymes represent the family of PKC isoforms (reviewed in Ron, 1999), all TPA - sensitive PKC isoforms are attractive candidates of Rsu-1 phosphorylation. This group includes all PKC isoforms except the atypical PKCs (ζ, ι, and λ) and the PKC homologue PRK / PKN.

To date over one hundred substrates have been shown to be phosphorylated by PKC isoforms, including proteins involved in the Ras – MAP kinase signaling

pathway (reviewed in Liu, 1996). Ras, Raf-1 and MEK have all been identified as targets of PKC phosphorylation (Jeng, 1987; Kolch, 1993; Kribben, 1993).
A consensus phosphorylation motif for PKC substrates was originally determined as RXXS/TXRX, where X represents any amino acid, and R represents arginine or a similar basic amino acid. Recently, a series of studies have refined the consensus phosphorylation sites among a large group of serine / threonine kinases (Songyang, 1998). Using an oriented peptide library screening technique, a "library" of identical-length peptides is mixed with the kinase and phosphorylated in a reaction. The phosphorylated peptides are then isolated and sequenced, which reveals a pattern of conserved amino acids required for phosphorylation by that particular kinase. This powerful technique allows literally millions of potential substrates to be screened for the optimal phosphorylation recognition sequence of each kinase (Songyang, 1996; Nishikawa, 1997; Wang, 1998).

Table 1 is an adaptation of results obtained by Songyang *et al.* (1998) comparing the determined substrate specificity of known serine / threonine kinases and the TPA – dependent phosphorylated sequences of Rsu-1. These serine / threonine kinases are classified as proline – directed, basophilic, or acidophilic. The PKC isoforms are basophilic, and show a strong preference for basic residues, particularly arginine, at positions –3 and –2 to the phosphorylated serine residue. PKCs also seem to prefer hydrophobic amino acids such as isoleucine or phenylalanine at the +1 position of phosphorylation. PKCμ is quite specific for peptides that contain leucine at the –5 position of phosphorylation.

Amino Acid Position

	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5
Proline-Directed													
ERK1				T	G	P	L	S	P	G	P	F	
CDK5				K	H	H	K	S	P	K	H	R	
Cyclin B / CDC2				H	H	H	K	S	P	R	R	R	
Cyclin A / CDK2				H	H	H	R	S	P	R	K	R	
Basophilic													
PKA				R	R	R	R	S	I	I	P	I	
PKCα	R	R	R	R	R	R	G	S	F	R	R	K	A
PKCβ-1,-2	R	K	L	K	R	K	G	S	F	K	K	F	A
PKCγ	R	R	R	R	R	K	G	S	F	K	R	K	A
PKCδ	A	A	R	K	R	K	G	S	F	F	Y	G	G
PKCε	Y	Y	X	K	R	K	M	S	F	F	E	F	D
PKCη	A	R	L	R	R	R	R	S	F	R	R	X	R
PKCζ	R	R	F	K	R	Q	G	S	F	F	Y	F	F
PKCμ	A	A	L	V	R	Q	M	S	V	A	F	F	F
CaM Kinase II				K	R	Q	Q	S	F	D	L	F	
Phosphorylase Kinase				F	R	M	M	S	F	F	L	F	
SLK1				R	R	E	G	S	L	R	R	F	
SRPK2				R	R	R	H	S	R	R	R	R	
NIMA				R	F	R	R	S	R	R	M	I	
Acidophilic													
CK1δ			E	F	D	T	G	S	I	I	I	F	F
CK1γ			Y	Y	D	A	A	S	I	I	I	F	F
CK2				E	D	E	E	S	E	D	E	E	
Rsu-1													
Amino acids 1 - 9					M	S	K	S	L	K	K	L	V
Amino acids 156 - 168	L	T	K	L	Q	I	L	S	L	R	D	N	D

Table 1. Phosphorylation Recognition Sequences Of Selected Serine Protein Kinases. A major development in the prediction of catalytic sites of potential serine/ threonine kinase substrates has been the use of oriented peptide library mapping. The kinases listed above were incubated in a phosphorylation reaction with a mixture of identical length peptides, after which the mixture of peptides were isolated and sequenced for identity. This reveals the consensus optimal substrate sequence for each kinase. The phosphorylated residue, serine, is represented by blue. Bold letters indicate amino acids which appear critical for kinase recognition. Red letters indicate the consensus similarity between the kinases SLR1, SRPK2, and NIMA with the known phosphorylated regions of Rsu-1 (Adapted from Songyang, 1998)

Comparison of the phosphorylated sequences of Rsu-1 to the consensus phosphorylation sequences shows that the PKC isoforms PKCα, -β, -γ, and –η share a patten of charged residues, either lysine or arginine, at the +2 position of serine. However, the consensus substrate specificity of SLK1 kinase matches the amino acid positions at +1 and +2 surrounding serine-163 of Rsu-1. The SLK1 kinase was originally identified in yeast and is implicated in nutrient sensing and growth control in yeast (Costigan, 1992; Costigan, 1994). SLK1 is activated by PKC and contains a catalytic domain in the carboxy terminus that exhibits amino acid sequence similarity to members of the PKC family. Also known as BCK1, the SLK1 enzyme is a homolog of eukaryotic MEK kinase and can activate a Rho-like pathway in yeast (Blumer, 1996; Von Pawel – Rammingen, 2000). These data suggest that SLK1 / BCK1 or a related kinase, presumably a member of the MEK kinase family, might be the serine kinase that phosphorylates Rsu-1.

Alternatively, KSR or Raf-1 might directly phosphorylate Rsu-1. Previous results demonstrate that Rsu-1 binds Raf-1 *in vitro* and KSR co-immunoprecipitates with Rsu-1 *in vivo*. Both KSR and Raf-1 contain phorbol ester – sensitive domains, and Raf-1 can be activated by TPA *in vitro* (Troppmair, 1994). Several substrates of the serine / threonine kinase Raf-1 have been identified, including MEK1/2 (Dent, 1992; Kyriakis, 1992), Cdc25 (Galaktionov, 1995), Bad (Wang, 1996), Rb (Wang, 1998) and most recently Tvl-1 (Lin, 1999). The representative phosphorylation site of Tvl-1 by Raf-1 is SALSLASMGG, which is similar to the MEK1 phosphorylation motif, (L/A)-S-(T/S)-G (Lin, 1999). Interestingly, serine-163 of Rsu-1 is flanked by leucine residues, suggesting similarity to the Tvl-1 sequence

(Table 1). While the protein structure of KSR predicts a kinase domain, no definitive substrate has been found - reports that KSR is a ceramide-activated kinase (Zhang, 1997) that phosphorylates Raf-1 (Yao, 1995) have been disputed by others (Muller, 1997; Michaud, 1997). Several experiments aimed at testing the ability of Raf-1 or KSR to phosphorylate Rsu-1 *in vitro* have yielded negative results. However, future studies might address the role of these or other TPA – dependent enzymes on Rsu-1 phosphorylation.

While it is likely that PKC does not directly phosphorylate Rsu-1, this phosphorylation is still PKC – dependent. This is suggested by the observation that the PKC inhibitor BIM can block Rsu-1 phosphorylation *in vivo*. BIM has been reported to show high selective inhibition of PKCα, -β, -γ, -δ, and –ε (Gekeler, 1996; Kiss, 1995). Thus, Rsu-1 phosphorylation may be indirectly activated by one of these TPA - responsive PKC isoforms. One PKC isoform in particular, PKCε, has been implicated in the MAP kinase pathway (reviewed in Toker, 1998). PKCε has been shown to bind Raf-1 and enhance MAP kinase activation (Cacace, 1996; Ueffing, 1997), and shows oncogenic potential in fibroblasts (Cacace, 1993; Mischak, 1993) and rat colonic epithelial cells (Perletti, 1996; Perletti, 1998). PKCε can enhance NGF – dependent MAP kinase activation and differentiation in PC12 cells (Ohmichi, 1993), similar to results seen with ectopic expression of Rsu-1 in this cell line. Interestingly, PKCε contains a unique actin – binding motif which suggests a role in actin – cytoskeletal regulation (Prekeris, 1996). Recent studies suggest that PKCε may transduce signals from the extracellular matrix via integrins to promote

cytoskeleton activation (Chun, 1996; Haller, 1998). Evidence also suggests that PKCε regulates the activity of another serine kinase, PKCζ (reviewed in Toker).

In light of data which suggests that serine phosphorylation of Rsu-1 may be required for enhanced apoptosis in MCF7 cells, the PKC isoform PKC-δ is another attractive candidate as an indirect activator of Rsu-1 phosphorylation. It is now appreciated that TPA stimulation of certain cells either enhance or inhibit apoptosis (Deacon, 1997). Data suggests that novel PKC isoforms such as PKC-ε and PKC-δ promote apoptosis, whereas the classical and atypical PKC isoforms promote cell survival (reviewed in Cross, 2000). PKC-δ has been categorized as a tumor suppressor gene since loss of PKC-δ is correlated with tumor growth (Lu, 1997) and overexpression of PKC-δ leads to growth arrest in G2/M (Griffiths, 1996; Watanabe, 1992). Several reports have now established PKC-δ as a positive regulator of apoptosis (Emoto, 1995; Basu, 1999; Bharti, 1998; Lord, 1995; Scheel-Toellner, 1999). Most recently, PKC-δ has been proposed as a kinase for nuclear lamina phosphorylation during apoptosis (Cross, 2000) and to translocate to mitochondria during release of cytochrome C in response to TPA – mediated apoptosis (Majumder, 2000).

In MCF7 breast cancer cells, phosphorylation appears to be required for the ability of Rsu-1 to enhance apoptosis in response to TNF-α and staurosporine, but not its ability to inhibit anchorage-dependent and anchorage-independent growth. The remainder of this discussion will focus on these two properties of Rsu-1, growth inhibition and apoptosis, and how this relates to its role as an inhibitor of Ras - dependent transformation. A current view of carcinogenesis is also incorporated into

the discussion to highlight the potential contribution of Rsu-1 as a tumor suppressor gene.

Previous studies have demonstrated that ectopic expression of Rsu-1 in a variety of cell lines results in growth inhibition, and most recently MCF7 breast carcinoma cells stably transfected with Rsu-1 were found to have slower rates of both anchorage – independent growth (soft agar colony formation) and anchorage–dependent growth than control MCF7 cells (Vasaturo, 2000). This growth inhibition in MCF7 cells is accompanied by increased levels of p53 and $p21^{CIP}$ and reduced activity of CDK2 and cyclin-D associated kinase activity. Results from chapter 3 demonstrate that expression of non-phosphorylatable mutants of Rsu-1 (S4A and S163G) in MCF7 cells result in growth inhibition comparable to the wild type Rsu-1 clone. In addition, anchorage-dependent growth rate is not significantly altered by low level TPA stimulation, suggesting that Rsu-1 mediated growth inhibition is not affected by phosphorylation but rather is constitutive and likely dependent on overall protein levels of Rsu-1. Rsu-1 mediated-growth inhibition has also been shown to be partially p53 dependent in L929 fibroblasts (Vasaturo, submitted). Because the expression of Rsu-1 mutants S4A and S163G results in growth inhibition without stabilization of p53, these proteins will be useful in dissecting the mechanism of Rsu-1 mediated – growth inhibition.

Recently a comprehensive model of how cancer cells develop has been proposed (Hanahan, 2000). It is suggested that tumorigenesis reflects the evolution of a single cell that has acquired, through a series of genetic alterations, the ability to become a malignant and invasive cancer. The trademarks of these cancers include

self-sufficient growth stimulation, insensitivity to growth inhibition, insensitivity to apoptosis, limitless replicative potential, angiogenic properties, and tissue invasion and metastasis (Hanahan, 2000). The characteristic of self-sufficient growth stimulation is exemplified by the Ras – Raf – MAP kinase pathway (Hunter, 1997). While direct mutation of *ras* is only observed in 25% of human cancers, it is suspected that most if not all tumors contain a deregulated Ras signaling pathway that mimics *ras* oncogene activation (Hunter, 1997; Kinzler, 1996). One critical role of the Ras – MAP kinase pathway is induction of cyclin D expression, which is required for G_1 cell cycle progression (Lavoie, 1996). As previously mentioned, $p21^{CIP}$ is a potent inhibitor of D - type cyclins. Data suggests that Rsu-1 might inhibit growth by enhancing stabilization of p53 and upregulate $p21^{CIP}$, and also by inhibiting cyclin – D associated kinase activity and CDK2 kinase activity (Vasaturo, 2000; Vasaturo, submitted). Interestingly, Rsu-1 phosphorylation mutants S4A and S163G retained growth inhibition comparable to wild type Rsu-1, but show reduced stabilization of p53 protein levels. This suggests that Rsu-1 can mediate growth inhibition independently of p53 in MCF7 cells. Future studies will address the mechanism by which Rsu-1 modulates p53 levels and affects p53-dependent and –independent growth inhibition.

In addition to self-sufficiency of growth signaling, cancers also exhibit the ability to evade apoptosis (Hanahan, 2000). In 1972 it was first observed that hormone-dependent tumor cells could die from hormone withdrawal by apoptosis (Kerr, 1972). Apoptosis is uniformly present in metazoans as a mechanism used in development and as a response to cellular injury (Hengartner, 2000). Apoptosis can

function as a tumor- suppressive mechanism by eliminating cells which have acquired genetic damage – conversely, when the apoptotic response is compromised, cells that sustain genetic damage can persist and likely evolve to a more cancerous state (Rich, 2000). Initial evidence that apoptosis could indeed be altered in cancer came from the observation that follicular lymphomas overexpress the anti-apoptotic protein Bcl-2 (Korsmeyer, 1992; Vaux, 1988).

Stimuli that activate apoptosis include DNA damage, activation of an oncogene, survival factor insufficiency, environmental stress and hypoxia (Evan, 1998). Most proapoptotic signals converge on the mitochondria, which release cytochrome C (Green, 1998). Cytochrome C activates two proteins, caspase 8 and –9, which ultimately elicit the apoptotic response (Thornberry, 1998). The p53 tumor suppressor protein is central to the apoptotic response. By sensing DNA damage, p53 can stimulate mitochondria to release cytochrome C via upregulation of the protein Bax (Hanahan, 2000).

The observation that wild type Rsu-1 can stabilize levels of the p53 protein and enhance apoptosis in MCF7 cells (Vasaturo, submitted), in addition to observations that the non-phosphorylatable mutants of Rsu-1 fail to enhance apoptosis in MCF7 cells, suggests that Rsu-1 phosphorylation is required for an enhanced apoptotic response in these cells. The fact that the non-phosphorylatable Rsu-1 mutants do not stabilize p53 supports the observation that the Rsu-1 dependent proapoptotic response appears to be p53 dependent. Rsu-1 can elicit either growth inhibition or apoptosis in the same cell type, depending on the stimuli. The molecular mechanisms underlying when and how a cell chooses growth inhibition versus

apoptosis are active areas of research, and many studies have focused on the p53 protein, which may be the pivotal switch controlling these very different cellular outcomes (Levine, 1997). The link between Rsu-1 and stabilized p53 protein levels raises the possibility that Rsu1 might contribute to this decision – making process by the cell. When Rsu-1 protein levels are reduced or absent, such as in certain gliomas and humor tumor cell lines, perhaps p53-dependent apoptosis is significantly comprimised.

The connection of Ras to p53 and apoptosis comes from studies of Ras expression in primary cells and on PI-3 kinase (Kauffmann-Zeh, 1997; Downward, 1998). PI-3 kinase and its downstream effector, Akt/PKB, transmit antiapoptotic survival signals when activated by Ras, and this pathway is negatively regulated by PTEN, a phospholipid phosphatase of Akt (Cantley, 1999). PTEN is a tumor suppressor protein that is deleted in a variety of cancers, including gliomas and carcinomas of the prostate, endometrium, kidney and lung (Li, 1997; Steck, 1997). Thus, activated Ras is not only capable of stimulating mitogenesis via the Raf – MAP kinase pathway, but it is also capable of evading apoptosis by activation of the PI-3 kinase pathway. Clearly the Ras-dependent pathways of mitogenesis, anti-apoptosis and cytoskeletal activation, when deregulated, cooperate to foster the selection of tumor cells (McCormick, 1999). Interestingly, when only one of these signals dominate and other pathways are competent, cells are much less likely to become transformed. For example, when activated Ras is expressed in primary cells, it triggers a profound p53-dependent growth arrest (Lloyd, 1997; Serrano, 1997), frequently accompanied by apoptosis (Kauffmann-Zeh, 1997) or senescence

(Serrano, 1997), both of which actions are mediated by the Ras kinase, Raf (Lloyd, 1997; Kauffmann-Zeh, 1997). Only when the p53 pathway is inactivated, by mutation or loss of p53 or the INK4a locus, can activated Ras efficiently transform cells (Lin, 1998; Ries, 2000).

This requirement of multiple pathways to transform a cell may explain why cancer is relatively rare. The generation of potentially neoplastic clones in the human body occurs daily, yet cancers arise on average less than once in every three lifetimes out of a total of some 10^7 possible cellular targets (Evan, 1998). While the major pathways that lead to tumorigenesis seem to have been identified, the intricate balance and cross-talk of these pathway effector molecules to eventuate cancer remains to be elucidated. This thesis has presented data supporting the role of Rsu-1 phosphorylation as a requirement for enhanced apoptosis but not growth inhibition in MCF7 breast cancer cells. A current model of Rsu-1 regulation is presented that highlights the interaction of Rsu-1 with several other key effector molecules involved in Ras-dependent transformation, growth inhibition and apoptosis (figure 4). Continued studies with Rsu-1 will hopefully provide further insight into the biology of growth inhibition and apoptosis, and also its contribution as a suppressor of the Ras-transformed phenotype and tumorigenesis.

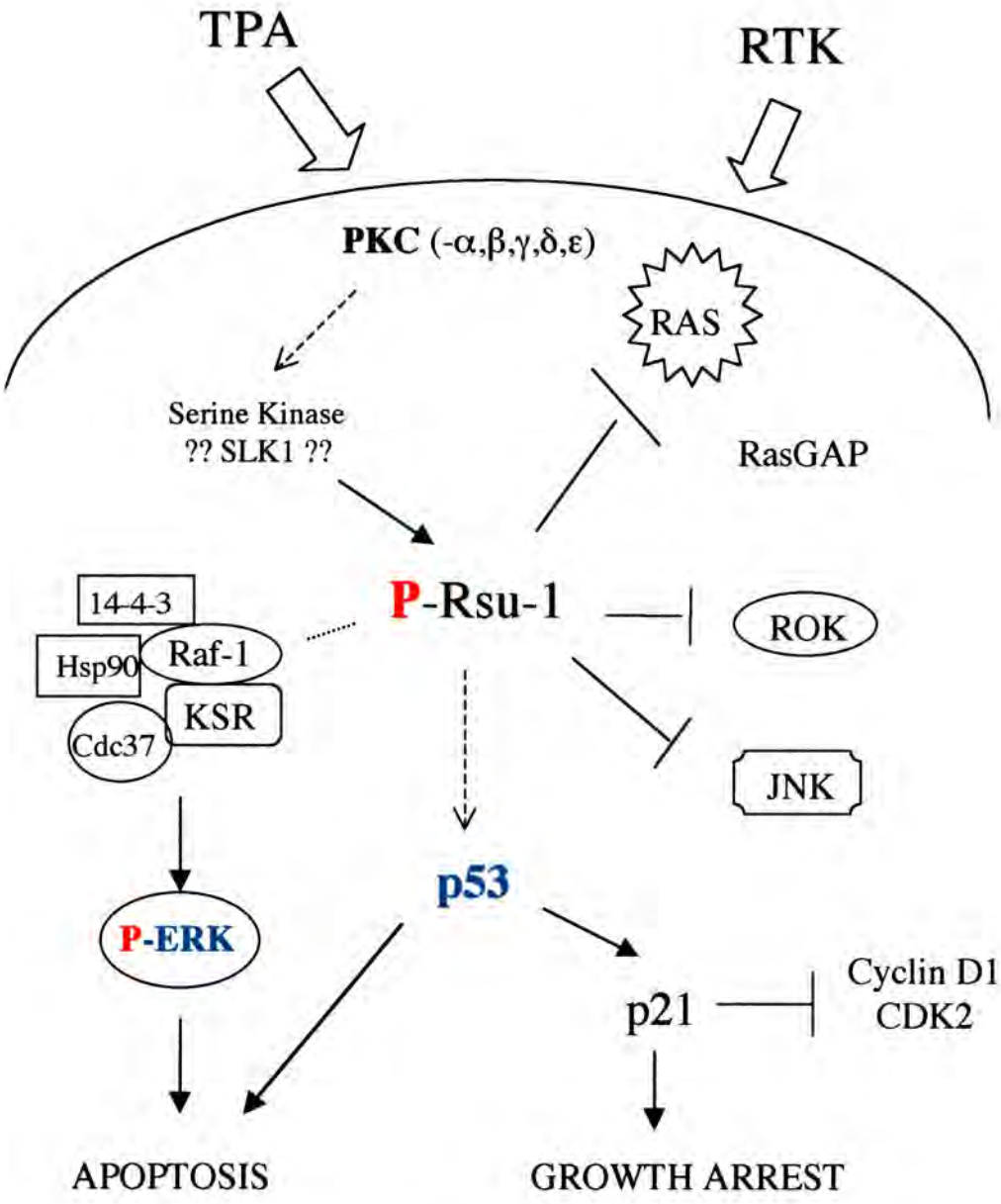

Figure 4. Model of Rsu-1 Regulation. Rsu-1 was originally discovered to suppress v-Ras transformation and was subsequently shown to enhance ERK and reduce JNK activation. The primary function of Rsu-1 appears to be growth inhibition, and recent studies suggest that apoptosis is an inducible function of Rsu-1. Phosphorylation of serine-4 and serine-163 are required for ERK and p53-dependent enhancement of apoptosis in MCF7 cell line. Growth inhibition by Rsu-1 does not appear to require phosphorylation, and from studies in MCF7 may be p53-independent.

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