Regulation of Cell Signaling by the Small G Protein Rap1

by

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

LIST OF FIGURES

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ABSTRACT

Cellular responses to environmental cues depend on the coordinated regulation of multiple signaling cascades that mediate cellular functions such as proliferation, differentiation, and gene transcription. Many of these signaling cascades are regulated by the cell surface receptor dependent activation of small G proteins. The small G proteins Ras and Rap1 regulate the extracellular-regulated mitogen-activated protein kinase cascade (ERK) in a cell-type specific manner. In certain cell types, Rap1 is able to activate ERKs and induce proliferation or differentiation. However, in other cell types, Rap1 can function as a Ras antagonist. This is because the major isoform of Raf, Raf-1, is ubiquitously expressed. B-Raf, an isoform of Raf-1, is expressed in a subset of cells. In cells that express B-Raf, Rap1 is able to activate ERKs via activation of B-Raf. In cells that don't express B-Raf, Rap1 is an ERK antagonist, inhibiting Raf-1 kinase activation. The major goal of this thesis has been to understand the biochemical mechanism of the cell-type specific actions of Rap1 on the ERK cascade. We find that in T cells, where B-Raf is not expressed, Rap1 functions as a Ras antagonist and can inhibit ERK activation. This function of Rap1 may be to regulate the level of ERK activation to promote an appropriate immune response. We find that augmentation of ERK stimulation and transcriptional regulation of the c-fos promoter by co-stimulation of T cell antigen receptor and the CD28 receptor is due to the activation of a Rap1-specific GAP activity. This activity is dependent on the recruitment of the non-receptor tyrosine kinase Lck to the cytoplasmic domain of the CD28 receptor. Therefore, the magnitude and duration of ERK signaling is via the control of RapGEF and RapGAP activities,

which mediate the activation of Rap1. Our findings suggest that the mechanism of Rap1 antagonism of ERKs is to sequester Raf-1 to a membrane compartment that is deficient in at least one of two key phosphorylation events that are required for Raf-1 kinase activation. These phosphorylation events occur on tyrosine residue 341 (Y341) and serine residue 338 (S338) of Raf-1. We find that Y341 phosphorylation is required to allow Raf-1 to enter a membrane microdomain that has the properties of a lipid raft which contains a S338 kinase. Interestingly, due to sequence differences between Raf-1 and B-Raf, a Rap1-B-Raf interaction results in the activation of ERKs. Ras, however, is functionally different from Rap1 due to post-translational modifications that occur in the C-terminus of the protein and localizes to a membrane domain that is able to support both tyrosine 341 and 338 phosphorylation. Surprisingly, both Ras and Rap1 localize to lipid rafts suggesting that rafts comprise multiple functionally distinct membrane microdomains in the cell. We propose that Rap1 signaling specificity for regulating the ERK cascade in a cell is determined by its subcellular localization and by the differential expression of Raf-1 and B-Raf.

CHAPTER ONE

INTRODUCTION

The mitogen-activated protein kinase pathway

Cellular responses to extracellular stimuli are mediated by the activation of intracellular signaling cascades. Different growth factors and receptors can activate the same signaling cascade but the biological outcome can be cell-type specific. Cell-type specificity is determined by several different mechanisms including localization, effector usage, and the dynamic regulation of components of the signaling cascade. The coordinated regulation of signaling pathways can be mediated in part by G proteins, which can regulate the localization and activity of many components of cellular signaling pathways. Cross talk among these proteins allows for cell type-specific responses through the regulation of the mitogen-activated protein kinase pathway. In this thesis, I explore some biochemical mechanisms that are involved in the cell-type specific regulation of the extracellular-regulated mitogen-activated protein kinase cascade by the small G proteins Ras and Rap1.

The mitogen-activated protein kinase (MAPK) family consists of three different kinase subfamilies, the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), and the p38 family of kinases (Martin-Blanco, 2000; Schaeffer and Weber, 1999). Together they form a network of signal transduction cascades that mediate cellular responses to a wide range of stimuli, including growth factors, hormones, cytokines, chemical and osmotic stress, and irradiation (Schaeffe and Weber, 1999; Davis, 2000). MAPKs are activated by dual phosphorylation by MAPK kinases (MKKs or MEKs) on a tripeptide Thr-Xaa-Tyr motif resulting in a conformational

change that enables the kinase to become activated and interact with substrates (Boulton and Cobb, 1991; Canagarajah et al., 1997). The MEKs are activated by phosphorylation on two serine residues by MAPK kinase kinases(MKKKs), each of which is associated with activating a distinct MEK (Hagemann and Blank, 2001; Hagemann and Rapp, 1999). Different signaling cascades mediate the activation of these MKKKs. MAPK cascades frequently function as multi-protein complexes in which the different components are assembled on a scaffold protein and by specific protein-protein interactions, thereby increasing the speed and specificity of the cascade (Karandikar and Cobb, 1999; Morrison and R. E. Cutler, 1997). MAPKs phosphorylate their substrates on serine or threonine residues which preceed a proline, but their specificity *in vivo* is further enhanced by the presence of distinct docking sites that facilitate interaction with substrates (Cobb and Goldsmith, 1995; Pearson et al., 2001). To date, twelve different MAPK family members have been identified in mammalian cells, and homologues are found in all eukaryotic cells (Widmann et al., 1999).

One of the best-studied MAPK cascades in mammalian cells is the classical ERK cascade. This cascade, comprised of extracellular signal regulated kinase 1 and extracellular signal regulated kinase 2 (ERK1 and ERK2), is activated by mitogens and growth factors and plays an important role in the control of cell growth and differentiation (Cobb et al., 1994). The ERKs are activated by the Raf family of MKKKs, which then phosphorylate and activate the MKKs MEK1 and MEK2 (Morrison and R. E. Cutler, 1997). The mechanism for Raf family kinase activation is poorly understood, but requires membrane recruitment, phosphorylation, and formation of a

complex with several adapter proteins (Morrison and R. E. Cutler, 1997). Once activated, ERKs can phosphorylate a number of different cytoplasmic targets or translocate to the nucleus and phosphorylate transcription factors such as Elk to initiate a particular biological response (Schaeffer and Weber, 1999). The initiation of the ERK cascade by growth factors is dependent on the regulation of the Ras family of small GTPases. Among the members of the Ras family, three Ras proteins (H-Ras, K-Ras, and N-Ras) and two Rap1 proteins (Rap1A and Rap1B) play a central role in regulating the activation of Raf kinases in response to different stimuli. Cell-type specificity of ERK signaling is dependent on the activation of these two small G proteins in several different systems (figure 1.1). The distinct mechanisms of control of Raf activation by Ras and Rap are the major theme of this thesis.

Ras and Rap1 signaling

The Ras superfamily of small GTPase activating proteins are mediators of multiple intracellular signaling cascades that regulate cellular proliferation, differentiation, survival, and gene transcription. Currently, this family has 80 members, consisting of four large subfamilies (Reuther and Der, 2000). These proteins act as molecular switches cycling between the inactive GDP-bound state and the active GTP-bound state. In their GTP-bound form, these proteins undergo a conformational change enabling them to interact with their effectors. Environmental cues are transmitted through the activation of cell surface receptors, which regulate the activation and inhibition of G proteins through the activation of guanine nucleotide exchange factors

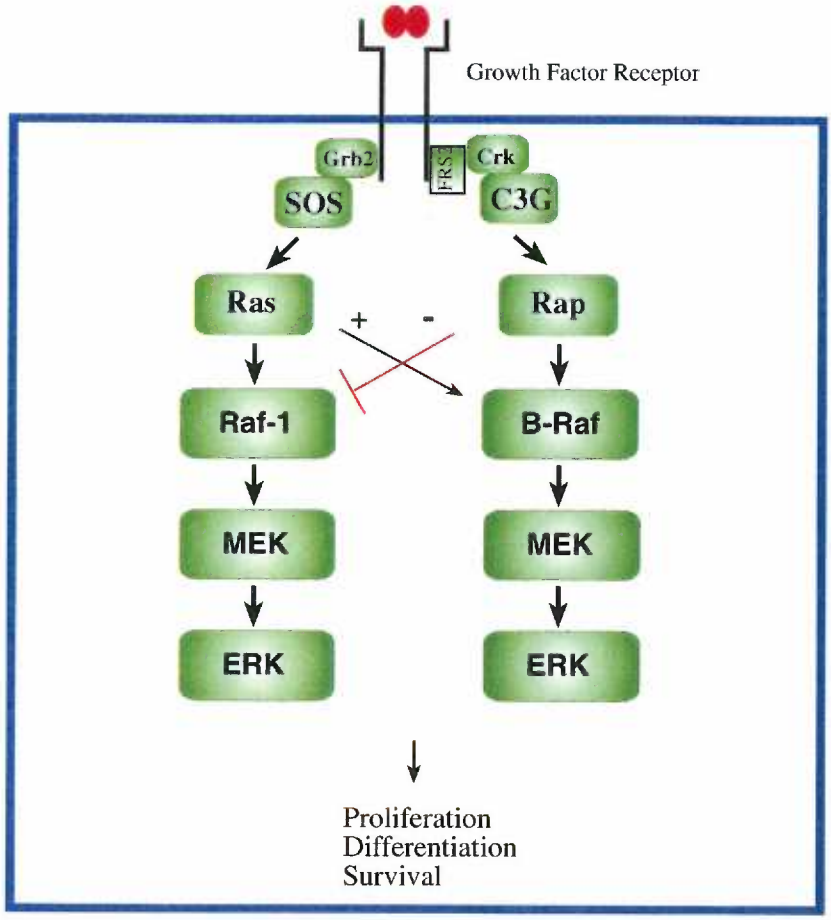

Figure 1.1 Ras and Rap1 activation initiates the MAPK/ERK cascade. Growth factor activation of receptor tyrosine kinases recruit adapter protein complexes that include guanine nucleotide exchange factors (GEFs) such as SOS and C3G to the membrane. SOS and C3G activate the small G proteins Ras and Rap1 that in turn initiate the MAPK/ERK cascade. Ras and Rap1 selectively control the dynamics and amplitude of the ERK cascade by the recruitment of Raf-1 and B-Raf to the membrane. Raf-1 and B-Raf phosphorylate and activate MEK, which in turn, phosphorylates and activates ERK 1,2. ERK is then able to phosphorylate multiple intracellular targets leading to cell proliferation, gene transcription, and survival.

(GEFs) and GTPase activating proteins(GAPs). The diversity of biological responses that result from activation of Ras family members is due to cross talk and cooperation with other small G proteins (Bar-Sagi and Hall, 2000). For example, Ras can activate Rac through the activation of one of its effectors, PI3K (Walsh and Bar-Sagi, 2001). Ras and Rac cooperation leads to an enhancement of Raf-1 kinase activation and ERK signaling (Li et al., 2001a; Sun et al., 2000). Another small G protein that cross talks with Ras, is Rap1(Bos et al., 1997). Rap1 can either antagonize or synergize with Ras to regulate the dynamics of the MAPK cascade in a cell-type specific manner.

Rap1 was originally cloned by low stringency hybridization as homologues to the Drosophila gene DRas3 (Pizon et al., 1988). The Rap family of small G proteins has four members consisting of Rap1A, Rap1B, Rap2A, and Rap2B (Bos, 1998). Rap1A and Rap1B are most closely related to Ras with 70% overall homology and both proteins have an identical effector loop domain. Rap and Ras proteins have been shown to bind *in vitro* to many of the same effectors, including Raf-1, RalGDS, PI3K, and AF6 (Bos et al., 2001). Both Ras and Rap are ubiquitously expressed, structurally related and many of the activating and inhibiting mutations that occur in Ras function are the same in Rap1 (Bos, 1998). A crystal structure of Rap with the Ras-binding domain of Raf-1 or PI3K have been reported (Nassar et al., 1995; Pacold et al., 2000). This has led to the suggestion that Rap and Ras have overlapping or antagonistic roles in regulating intracellular signaling (Bos et al., 1997). Consistent with the hypothesis that Rap1 antagonizes Ras signaling, Rap1 was independently identified in a screen to identify proteins that can suppress the morphological phenotype in Ki-Ras-transformed fibroblasts (Kitayama et al., 1989). The ability of Rap1 to suppress Ras-dependent cellular transformation has

also been observed in other cell types (Buss et al., 1991; Cox et al., 1992; Lin et al., 2000). Overexpression of active Rap1 inhibits both Ras-dependent germinal vesicle breakdown in Xenopus oocytes (Campa et al., 1991) and Ras-dependent activation of ERKs in Rat-1 fibroblasts (Cook et al., 1993; Schmitt and Stork, 2001). Rap1 can also antagonize Ras-dependent gene transcription (Boussiotis et al., 1997; Sakoda et al., 1992). The mechanism of Rap1 antagonism of Ras signaling is however unknown.

Although Rap1 can antagonize Ras signaling in fibroblasts, overexpression of a constitutively active Rap1, RapV12, has been shown to induce proliferation in certain cell types (Altschuler and Ribeiro-Neto, 1998; Yoshida et al., 1992), suggesting that Rap1 is involved in signaling pathways that are shared with Ras. This independent signaling role of Rap1 may depend on the stimulus and effector usage. For example, cAMP has been shown to activate Rap1 (Altschuler et al., 1995; Vossler et al., 1997), and can either be a positive or negative regulator of proliferation depending on the cell type (Altschuler and Ribeiro-Neto, 1998; Cook et al., 1993; Yao et al., 1995). The cell-type specific actions of cAMP have been shown by our laboratory to be due to the differential expression of the Raf kinase isoforms Raf-1 and B-Raf (Grewal et al., 2000a; Schmitt and Stork, 2001; Vossler et al., 1997). B-Raf is activated by Rap1 *in vitro* (Ohtsuka et al., 1996), and co-immunoprecipitates with activate Rap1 upon hormonal or growth factor stimulation (Grewal et al., 2000b; Schmitt and Stork, 2001; Vossler et al., 1997; York et al., 2000; York et al., 1998). Our laboratory has shown that the Rap1-B-raf pathway positively regulates MAPK signaling in both primary neurons and in PC12 cells stimulated with either cAMP or NGF (Grewal et al., 2000b; York et al., 1998).

Other laboratories have shown that expression of B-raf in non-neuronal cells can convert cAMP into a survival factor (Dugan et al., 1999). B-raf expression may thus be considered as a switch, converting Rap1 into a positive regulator of MAPK signaling.

A crucial role of Rap1 in regulating the biological response to an extracellular stimulus may be to regulate the dynamics of the MAPK casacade through the controlled regulation of Raf-1 and B-Raf. Previous studies in our laboratory have shown that NGF induced neuronal differentiation is through the coordinated activation of Ras and Rap1 (York et al., 1998). Rap and Ras activation dynamics also seem to play a role in regulating muscle and mekakaryocyte differentiation (Garcia et al., 2001; Pizon and Baldacci, 2000; Pizon et al., 1996). This raises the possibility that the dynamic regulation of Erk signaling by Rap1 may be dependent on the mechanism of Rap1 activation by growth factors, the temporal role of Erk signaling mediated by Raf-1 and B-Raf, or the expression of effectors and their activation by Ras and Rap1.

This thesis focuses on the mechanism of Rap1 antagonism of Ras-dependent signals that activate ERKs. The first half will describe one such system involving T cell signaling. The second half will examine the mechanism of this inhibition. In the third chapter of this thesis, we show that Ras and Rap1 can cooperate to activate signaling through a mechanism that regulates the activation of a shared effector, Raf-1.

Rap1 and T cell signaling

An appropriate immune response depends on the careful regulation of lymphocyte activation. Lymphocytes require at least two independent receptor activation events to initiate a multitude of intracellular signaling cascades in order to become fully activated (Bernard et al., 2002). For T cells, activation of the T cell antigen receptor initiates many signaling cascades that synergize with signaling events resulting from the activation of co-stimulatory receptors, such as CD28 (Lenschow et al., 1996; Weiss and Littman, 1994). Signaling pathways initiated through both the T cell antigen receptor and a distinct pathway from the CD28 receptor effect the activation of Ras and Rap1 (Reedquist and Bos, 1998). Rap1 has been proposed to antagonize Ras signaling in Jurkat T cells, inhibiting IL-2 transcription (Boussiotis et al., 1997). In chapter two of this thesis, we have used Jurkat T cells as a model system to examine the mechanism of Rap1 antagonism of Ras.

T cell receptor activation

T cell activation begins by T cell receptor (TCR) recognition of antigen presented on the surface of an antigen presenting cell (APC) as a peptide fragment bound to the major histocompatability complex molecule (MHC) (Garcia et al., 1999). The TCR is organized into an antigen binding heterodimer ($\alpha\beta$ and $\gamma\delta$) in a complex with four membrane bound signaling polypeptide chains (CD3ε, CD3γ, CD3δ, and CD3ζ) which associate with either the CD4 and CD8 receptor to form a multicomponent structure (Hennecke and Wiley, 2001). The cytoplasmic domains of the signaling chains of the TCR contain immunoreceptor tyrosine-bound activation motifs (ITAMS) which become phosphorylated upon optimal TCR engagement by antigen (Johnson et al., 1995). ITAM

phosphorylation is mediated primarily by the Src family tyrosine kinases Lck and Fyn which are associated with the intracellular tail of the CD4 receptor (Straus and Weiss, 1992) (Germain and Stefanova, 1999). The phosphorylated ITAMS then interact with the SH2 domain of ZAP-70, a Syk family protein tyrosine kinase, which can then become subsequently phosphorylated by Lck or Fyn (Hashimoto et al., 1996; Weiss, 1993). Phosphorylation of ZAP-70 results in new binding sites for SH2 containing proteins, such as the adapters LAT , Vav, and Cbl (Salojin et al., 2000). These adapters in turn recruit additional proteins, such as Grb2 and SOS and Crk/C3G (Wange, 2000) that can regulate the activation of the small G proteins Ras and Rap1. This multiprotein complex assembled by the TCR leads to the activation of multiple intracellular signaling cascades, including activation of ERKs (Figure 1.2).

Costimulation by the CD28 receptor is required for full activation of T cells

In order to prevent the activation of an improper immune response, mechanisms have evolved to ensure that antigen is presented by an APC in the proper context by the MHC and by the activation of other receptors on the surface of the T cell by a process known as co-stimulation (Bernard et al., 2002). TCR stimulation alone fails to result in cytokine production and proliferation and the T cell can become unresponsive to subsequent stimulation or undergo apoptosis (Appleman et al., 2001). The best-characterized co-stimulatory receptor is CD28. CD28 is a 44-kDa homodimeric glycoprotein that is expressed on the surface of all mature T cells. CD28 interacts with its ligands (B7-1 and B7-2) that are expressed on the surface of the APC, becomes phosphorylated by tyrosine kinases, and initiates a number of intracellular signaling pathways some of which are dependent on the co-activation of the TCR (June et al.,

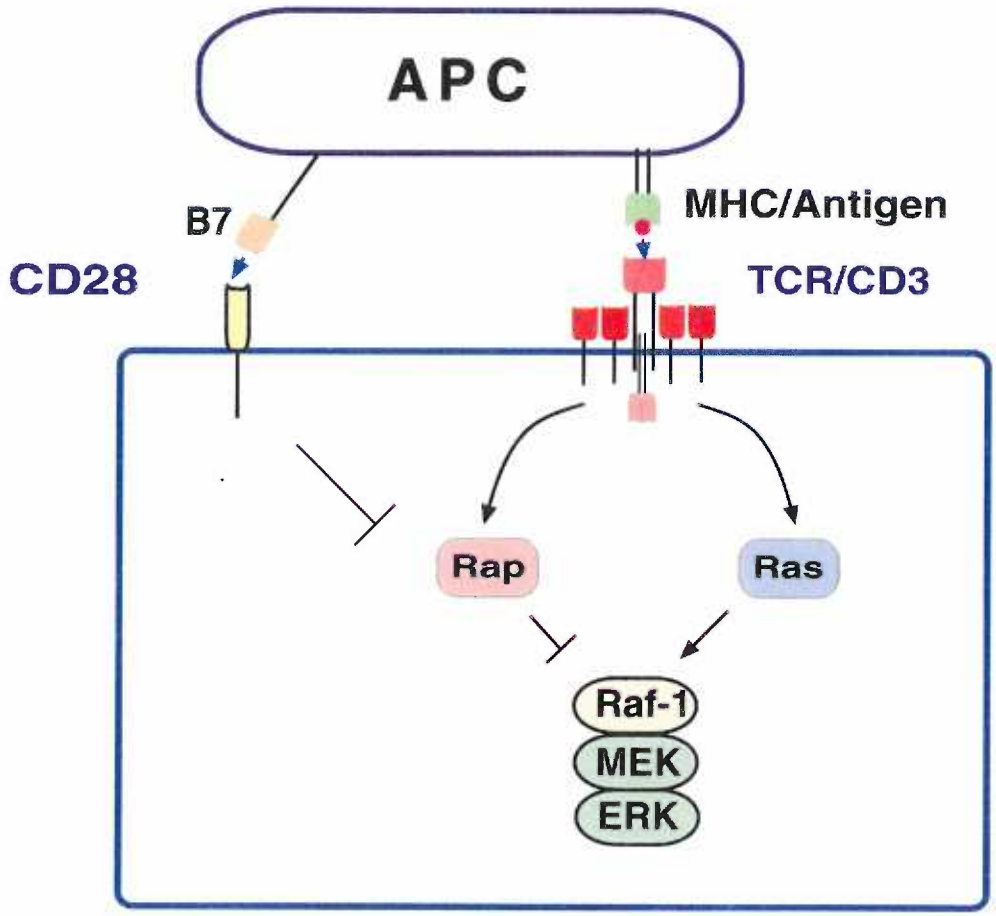

Figure 1.2 Co-stimulation by the CD28 receptor augments TCR activation of the MAPK/ERK cascade. An antigen presenting cell (APC) presents antigen to the T Cell Receptor complex (TCR) as a peptide fragment bound to the major histocompatability complex (MHC). This initiates a number of intracellular signaling events, including the activation of Ras and Rap1. TCR stimulation and engagement of the CD28 receptor by its ligand B7, or by cross-linking with an anti-CD28 antibody, can block TCR-dependent Rap1 activation and augments ERK activation. Thus, T cells are a convenient system to study the role of endogenous Rap1 in the regulation of the ERK cascade.

1994)(Lenschow et al., 1996). The activation of both the TCR and CD28 synergize to augment multiple intracellular signaling pathways, including ERKs (Bernard et al., 2002; Lenschow et al., 1996). This augmentation results in full T cell activation, IL-2 production, and cellular proliferation (Yang and Linsley, 1992).

Despite a powerful influence on T cell activation, the mechanism of signaling by CD28 remains uncertain. The intracellular domain of CD28 contains four tyrosine residues that have been shown to be absolutely required for co-stimulation (Truitt et al., 1996). These tyrosine residues reside in two motifs that have been shown to be important for costimulation (Holdorf et al., 1999; Ward et al., 1992). The first is a dual tyrosine (YXXMY) motif that can recruit the p85 subunit of PI3K, and other SH2 containing proteins (Truitt et al., 1994). The second is a proline-rich motif that is present in the last 16 amino acids of the intracellular domain and can recruit the SH3 domain of Lck thereby augmenting its kinase activity (Holdorf et al., 1999; Holdorf et al., 2002). Although there is evidence to support a role for both domains in the regulation of T cell activation, the domain participating in ERK regulation is unknown. In the second chapter of this thesis we determine a mechanism for the regulation of TCR/CD28 co-stimulation of ERKs.

Ras and Rap1 signaling participate in T cell regulation

A number of studies have shown Ras proteins to be essential components of immune cell signaling, where they regulate proliferation, maturation and activation of many cell types (Genot and Cantrell, 2000). T cell receptor stimulation causes a rapid

activation of Ras and Rap1 (Downward et al., 1990; Reedquist and Bos, 1998). TCR dependent activation of the MAPK cascade through Ras has been shown to play an important role during the development of the mature thymus in mice by regulating a process known as positive and negative selection (Alberola-Ila et al., 1996) (Bommhardt et al., 2000). Transgenic mice expressing a dominant negative form of H-Ras (RasN17) in the thymus fail to undergo normal T cell selection of immature double positive to mature single positive cells (Swan et al., 1995). A critical parameter determining the fate of thymocytes is the manner in which immature T cells are stimulated. In T cell anergy, in which the TCR is activated in the absence of other costimulatory signals, MAPK is not activated, and interestingly Rap1 is constitutively activated (Boussiotis et al., 1997). Activation of CD28 receptor can rescue the anergic phenotype in T cells, possibly by activating signaling pathways that rescue MAPK activation (Appleman et al., 2001). Cross-linking the CD28 receptor has been shown to inhibit TCR dependent activation of Rap1, suggesting that Rap1 activation may antagonize ERK dependent pathways in anergy (Boussiotis et al., 1997; Reedquist and Bos, 1998). Therefore, T cells provide a useful model system to evaluate the biological role of Rap1 in antagonizing ERK signaling.

TCR-dependent activation of both Ras and Rap1 may serve a physiological role to regulate T cell activation. In the absence of appropriate costimulatory receptor activation, Ras and Rap1 coactivation would limit the T cell response thereby ensuring fidelity of the immune response. A system to test this model is in the Jurkat T cell line. Upon T cell receptor activation both Rap1 and Ras become activated (Reedquist and Bos,

1998). Other studies have shown that the coactivation of the CD28 receptor along with TCR activation leads to increased ERK activation by the TCR in these cells (Nunes et al., 1996). These cells represent a convenient *in vivo* model system to test the role of Rap1 activation on the regulation of ERKs, since we can control the regulation of Ras and Rap1 activation through the TCR and CD28 receptors (Reedquist and Bos, 1998). This is made possible by the recent development of methodologies that make it possible to study the activation of endogenous Ras and Rap1 (Franke et al., 1997; Zhang et al., 1993), and are described in further detail in appendix 1 in this thesis. The hypothesis that Rap1 can antagonize ERK activation by Ras-dependent Raf-1 activation is tested in chapter two of this thesis.

Regulation of Rap1 activation

The regulation of several signaling pathways is dependent on the activation of two classes of regulatory proteins Rap1 guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) by regulating the proportion of GTP and GDP bound forms of Ras and Rap1. The GTP-bound form of Ras and Rap1 are then able to interact with their respective effectors to initiate signaling pathways. Mechanisms that regulate these proteins can therefore influence the dynamics of the ERK cascade through the activation of Raf-1 kinases, which are effectors of Ras and Rap1. The control of Ras signaling pathways may then be considered to be dependent on the formation of active Rap1 through the balance of the activities of RapGEFs and RapGAPs (Figure 1.3).

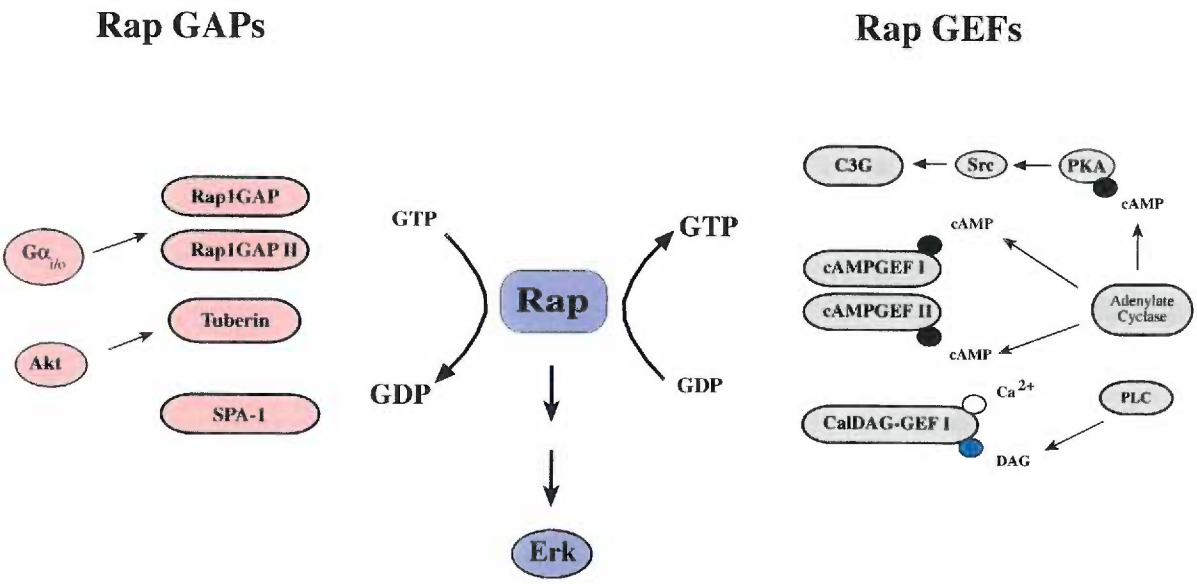

Figure 1.3 Regulation of Rap1 activation. Rap1 signaling is initiated by the activation of Rap1 guanine nucleotide exchange factors (Rap GEFs). Rap GEFs bind Rap1 when activated by growth factor receptors or intracellular second messangers and promote the exchange of GDP for GTP. Rap1-GTP can then recruit effectors such as B-Raf to initiate the MAPK/ERK cascade. Rap1 GAPs augment the intrinsic GTPase activity of Rap1 by directly binding to Rap1 accelerating the release of GTP. Rap GAPs reduce Rap1-GTP levels in the cell and may be important regulators of ERK signaling.

Rap GEFs initiate Rap signaling

The control of Ras signaling events by Rap1 is dependent on the formation of active Rap1. A number of different growth factors, hormones, and second messenger pathways have been reported to activate Rap1 (Bos et al., 2001). The formation of active, GTP-bound Rap1 is regulated by several different families of guanine nucleotide exchange factors (GEF) that are regulated transcriptionally, post-translationally, by phosphorylation, or directly by second messengers. C3G, which regulates receptor tyrosine kinase-induced Rap1 activation, contains a proline-rich domain that binds to the SH3 domain of Crk-L, an adapter protein (Gotoh et al., 1995). The SH2 domain of Crk-L is recruited to activated tyrosine kinase receptors or to phosphorylated adapter proteins such as Cbl , Gab1, or CAS (Reedquist et al., 1996; Sakkab et al., 2000; Xing et al., 2000; York et al., 1998). The recruitment of Crk-C3G to the membrane or to these adapters may be required to correctly localize C3G in order to activate Rap1. It has also been shown that the adapter protein FRS2 can differentially regulate the kinetics of Rap1 activation in PC12 cells (Kao et al., 2001). C3G GEF activity can be further modified by direct phosphorylation on Tyrosine residue 504 by Src family kinases leading to increased GEF activity (Ichiba et al., 1997). C3G can also be activated by cAMP via PKA-dependent activation of Src, leading to the phosphorylation of Cbl in fibroblasts (Schmitt and Stork, 2002). While C3G is ubiquitously expressed, other Rap GEFs are expressed in a tissue specific manner. The CalDAG-GEFs are activated by calcium and diacylglycerol and are expressed in the basal ganglia of the brain and the hematopoietic system (Ebinu et al., 1998; Kawasaki et al., 1998a). Another family of Rap GEFs that are differentially expressed are the Epac proteins (exchange protein activated by cAMP).

These Rap GEFs are activated directly by cAMP and are expressed predominantly in the brain (de Rooij et al., 1998; Kawasaki et al., 1998b). The PDZ-GEFs, have been detected at the synapse of neurons, and are able to activate both Rap1 and Rap2 (de Rooij et al., 1999) (Ohtsuka et al., 1999). The tissue-specific expression and post-translational regulation of these proteins may dictate whether Rap1 can antagonize Ras signaling in different cell types.

Rap is inactivated by RapGAPs

Compared with Ras, Rap has a very low intrinsic GTPase activity *in vitro* (Frech et al., 1990). However, the *in vivo* duration of Rap1 dependent signaling events does not correlate with the kinetics of Rap1 GTP hydrolysis, suggesting that control of Rap1 mediated cellular responses is also dependent on proteins that accelerate the intrinsic GTPase activity of Rap1, RapGAPs. Currently, there are six different mammalian RapGAPs that have been identified based on sequence homology in the GAP domain, Rap1GAP, SPA-1, tuberin, E6TP1, SPAR, and GAP^{IP4BP} (Bos et al., 2001). However, each RapGAP has a different tissue distribution, subcellular localization, and specificity towards members of the Ras family of small GTPases. The first identified, Rap1GAP, is specific for Rap1A and Rap1B, having no detectable GAP activity for Rap2A or Rap2B, or Ras isoforms (Rubinfeld et al., 1991). Rap1GAP can be either cytosolic or membrane localized depending on proteolytic cleavage and is predominantly expressed in neuronal tissue, although it may be ubiquitously expressed during development (Kurachi et al., 1997; Polakis et al., 1991; Rubinfeld et al., 1991; Tsukamoto et al., 1999). SPA-1 is expressed predominantly in lymphoid tissues and has GAP activity towards both Rap1

and Rap2 both *in vitro* and *in vivo* (Kurachi et al., 1997). SPA-1 seems to play an important role in cellular adhesion (Tsukamoto et al., 1999), although it is unclear if this is due to GAP activity towards Rap1 or Rap2. Tuberin, so named for one of two genes (TSC-1, TSC-2) mutated in tuberous sclerosis, has been shown to have GAP activity for Rap1 *in vitro*, and colocalizes with Rap1 (Wienecke et al., 1995; Wienecke et al., 1997). Tuberous sclerosis is a disease having aberrant growth in tissues, suggesting the possibility that defective Rap regulation may have a pathological role (Gutmann et al., 1997; Maheshwar et al., 1997). The Drosophila tuberin gene homologue restricts cell growth and proliferation (Ito and Rubin, 1999; Tapon et al., 2001). However, tuberin also has GAP activity towards Rab5, and it is unclear if Rap1 plays a role in this disease (Xiao et al., 1997). The Drosophila homologue of Rap1GAP has been identified and overexpression *of Rapgap1* leads to a rough eye phenotype (Chen et al., 1997). It is unclear if these functions of RapGAPs are due to misregulation of the ERK cascade by inhibiting Rap1, or due to RapGAP regulation of some other G protein. SPAR, a PSD-95-associated RapGAP that is specific for both Rap1 and Rap2 regulates dendritic spine morphology, but has not been shown to influence ERK signaling in neurons (Pak and Sheng, 1999). The final member of the family, E6TP1, is a viral protein and is related to RapGAPs by sequence homology, however, it is unknown if this protein has GAP activity towards Rap1 (Gao et al., 1999). The diversity of RapGAPs and their divergent biological roles suggests that the control of Rap1 signaling by distinct groups of RapGAPs provide an additional level of biological control for Rap1 signaling.

As with RapGEFs, there is evidence that RapGAPs are also regulated. The expression of Rap1GAP can be induced in lymphoid cell lines by TPA stimulation, which concomitantly turns off the expression of SPA-1 (Kurachi et al., 1997). Rap1GAP is phosphorylated on serine, threonine, and tyrosine residues (Rubinfeld et al., 1992). PKA and cdc2 are both capable of phosphorylating Rap1GAP *in vitro*, however these phosphorylation events do not influence *in vitro* GAP activity but may instead play some other regulatory role possibly by regulating protein-protein interactions, localization, or protein stability (Polakis et al., 1992; Rubinfeld et al., 1992). Rap1GAP and a splice variant Rap1GAPII are able to interact with heterotrimeric G proteins $G\alpha_i$, $G\alpha_o$, and $G\alpha_z$, both *in vitro* and *in vivo* (Jordan et al., 1999; Meng et al., 1999; Mochizuki et al., 1999). This interaction is dependent on the amino terminus of Rap1GAP and can recruit Rap1GAP to the membrane upon hormonal stimulation (Mochizuki et al., 1999). For example, epinephrine can decrease endogenous RapGAP activity in platlets via the α_{2A}-adrenergic receptor which can be either $G\alpha_i$ or $G\alpha_z$ coupled (Marti and Lapetina, 1992). The RapGAP-heterotrimeric G protein interaction also seems to play a role in Erk regulation in both Jurkat cells and in PC12 cells (Jordan et al., 1999; Mochizuki et al., 1999), whether this is through the re-localization of RapGAP, or by direct phosphorylation is unclear.

The control of Rap1 regulation through the balance of Rap1 GEF activation and Rap1GAP regulation represents a way to fine tune Rap1-mediated signaling pathways. In chapter 2 of this thesis, we investigate the role of Rap1GAP activation by the CD28

receptor in regulating Rap1 activation by the TCR and how this regulation is important for T cell receptor dependent activation of ERKs.

Regulation of Raf kinase activation by Ras and Rap1

Ras recruitment of Raf-1 is the initiating step for the activation of the classical ERK signaling cascade (Morrison and R. E. Cutler, 1997). Numerous studies have demonstrated that Ras can co-immunoprecipitate with Raf-1 upon growth factor receptor activation (Zhang et al., 1993). Similarly, there is evidence that Raf-1 and Rap1 are constitutively associated in anergic T cells (Boussiotis et al., 1997). The association of Rap1 with Raf-1 can be regulated by insulin in Chineese hamster ovary cells expressing the human insulin receptor (Okada et al., 1998). We propose that Rap1 may limit TCR dependent ERK activation by competing with Ras for Raf-1.

Ras and Rap1 bind Raf-1

Three Raf isoforms are expressed in vertebrates, C-Raf-1, A-Raf and B-Raf. All three proteins share three conserved regions (CR1, CR2, and CR3), but differ significantly in other parts of the protein which may contribute to signaling specificity (Daum, 1994; Morrison and R. E. Cutler, 1997). The amino-terminus (CR1 and CR2) encodes a regulatory function and the C-terminus (CR3) contains the kinase domain. The CR1 domain of Raf-1 contains a Ras-binding domain (RBD), which has low affinity for GDP-bound Ras, but high affinity for GTP-bound Ras (Daub et al., 1998). Ligands

which activate Ras result in the formation of Ras-Raf-1 complexes (Hallberg et al., 1994; Marais et al., 1998). Mutations in the Raf-1 RBD or Ras which disrupt the Ras-Raf-1 interaction, or agents which inhibit Ras function block Raf-1 activation (Dent et al., 1996; Dent et al., 1995b; Fabian, 1993; Marais et al., 1995; Tamada et al., 1997). In addition to binding to the RBD, the switch 2 domain of Ras makes additional contacts with the CR1 domain in a region known as the cysteine-rich domain (CRD) which are required for full activation of Raf-1 by Ras *in vivo* (Drugan et al., 1996; Mineo et al., 1997; Roy et al., 1997). The amino-terminus of Raf-1 and B-raf share an identical Ras binding domain, but differ in size and sequence, particularly in the CRD domain. It is this difference between CRD domains which has recently been proposed to account for the ability of Rap1 to activate B-raf, but not Raf-1 (Okada et al., 1999). However, the interaction between Raf-1 and Ras is not sufficient for activation, because activated Ras cannot activate Raf-1 *in vitro* unless Ras is membrane bound and unidentified factors are present (Dent et al., 1995a; Stokoe and McCormick, 1997; Tamada et al., 1997; Zhang et al., 1993). Similar findings have been reported for the Rap1-B-raf interaction (Kuroda et al., 1996; Ohtsuka et al., 1996). Therefore, Ras-dependent Raf activation is dependent not only on the physical association with Ras, but also requires additional modifications to become fully activated. This will be addressed directly in the second half of this thesis.

Raf-1 activation is regulated by phosphorylation

Raf-1 is active when constitutively localized to the plasma membrane by adding a Ras farnesylation motif to the carboxy terminus (Leevers et al., 1994; Stokoe et al., 1994). This suggests that the main role of Ras may be to recruit Raf-1 to the membrane

where subsequent post-translational modifications and protein-protein interactions can occur. However, the nature of these membrane events that activate Raf-1 are poorly understood. Multiple phosphorylation events on serine, threonine and tyrosine residues have been demonstrated to regulate Raf-1 kinase activity *in vivo*, both positively and negatively (Figure 1.4). Incubation of immunoprecipitated Raf-1 with phosphatases has been shown to inhinbit Raf kinase activity, and pharmacological agents which influence these activating phosphorylation events can inhibit kinase activation in response to growth factors or constitutively active Ras (Dent et al., 1995a; Jelinek et al., 1996; Zhao et al., 1996). This suggests a key role for tyrosine phosphorylation in Ras- and growth factor-dependent Raf-1 activation. Src family tyrosine kinases have been shown to activate Raf-1, and can further augment Ras activation of Raf-1 (Fabian, 1993; Mason et al., 1999). However, evidence for Src, Fyn, or Yes tyrosine kinase phosphorylation requirement is not supported by genetic studies showing no deficits in Erk activation by PDGF in Src family kinase deficient mice (Klinghoffer et al., 1999). Other Raf-1 phosphorylation events have been shown to be regulated by multiple different kinase cascades, including PI3K/Akt, PKA, Pak, and PKC. The PI3K/Akt and PKC phosphorylation events, on Ser259 and Ser621 respectively, have been shown to be required for regulating the interaction with 14-3-3 proteins, which are necessary for Raf-1 to be both activated and for Raf-1 to couple to Erks (Rommel et al., 1999; Tzivion et al., 1998). It is also possible that these phosphorylation events may also be required for the interaction with scaffolding proteins such as Ksr, MP-1, and Sur-8 (Li et al., 2000; Schaeffer et al., 1998; Therrien et al., 1996). As has been demonstrated in yeast, these

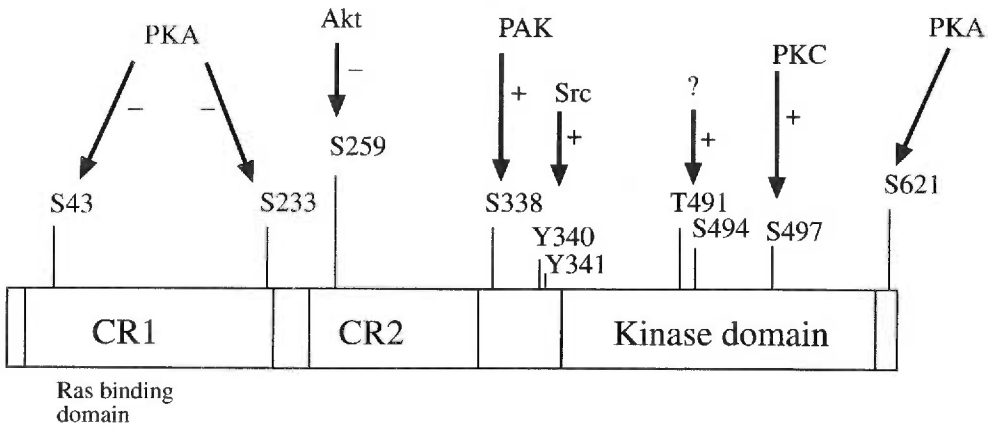

Figure 1.4 Multiple positive and negative phosphorylation events regulate Raf-1 kinase activation. Phosphorylation of serine residues 338 and 494, threonine residue 491, and tyrosine residue 341 is required for Raf-1 kinase activation. Raf-1 activation is inhibited upon phosphorylation of serine residue 259 by Akt, or by direct phosphorylation of residues serine 43, serine 233, and serine 621 by PKA. Phosphorylation of Raf-1 at S259 and S621 regulates 14-3-3 association and S259 phosphorylation has also been reported to regulate Raf-1 membrane association. The role of S338 Y340, and Y341 phosphorylation is unknown.

scaffolding protein interactions may mediate specificity in signaling to a particular extracellular stimulus thereby eliciting a specific biological response (Peyssonnaux and Eychene, 2001). The difference between the Ras-Raf-1 and Rap-Raf1 interaction may influence these key phosphorylation events.

Two key phosphorylation events that are absolutely required for growth factor and Ras-dependent activation of Raf-1 occur on tyrosine residue 341 and serine residue 338 (Fabian, 1993; Mason et al., 1999) (Diaz et al., 1997). The role for these kinase phosphorylation events is unknown, but it has been suggested to enable a conformational change in Raf-1 to initiate kinase activation by relieving autoinhibition by the amino terminus analogous to Src family kinase activation (Mason et al., 1999). Support for this model is provided by the observation that deletion of the first 300 amino acids of Raf-1 (ΔRaf) results in a constitutively active and oncogenic kinase (McCormick, 1992). However, ΔRaf still requires phosphorylation at Ser338 and Tyr 341 to be active (Sun et al., 2000). Neither Ras binding, nor 14-3-3 interactions are affected if these sites are mutated, suggesting some as yet undefined role for these phosphorylation events (Chong et al., 2001). The availability of phosphospecific antibodies for monitoring the phosphorylation of these two sites makes them good candidates for evaluating the role of Rap1 in regulating Raf-1 kinase activation.

Structural differences between B-Raf and Raf-1

A key difference between B-raf and Raf-1 is the presence of the tyrosine site at position 341 (Figure 1.5). B-raf substitutes two aspartate residues at this position and this

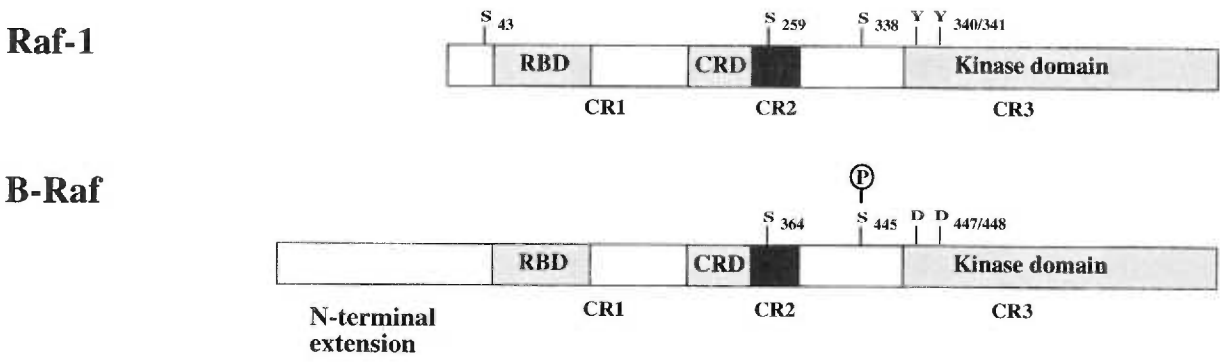

Figure 1.5 Raf-1 and B-Raf are structurally similar. Members of the Raf kinase family have high homology in the conserved regions (CR1-CR3), but have several sequence differences that contribute to kinase activity and regulation. Both Raf-1 and B-Raf have an identical Ras-binding domain (RBD), but differ in the cysteine-rich domain (CRD). The CRD of Raf-1 has been proposed to make additional contacts with the switch 2 domain of Ras enabling Ras, but not Rap1, to activate the kinase. Sequence differences in key regulatory phosphorylation sites, also may contribute to the signaling specificity of Raf-1 and B-Raf. Tyrosines 340 and 341 (Y340/Y341) are replaced with aspartic acid residues (D447/D448) in B-Raf. The negative charges mimic phosphorylation and it has been proposed to contribute to the high basal activity of B-Raf and constitutive phosphorylation of serine 445 (S338 in Raf-1). However, B-Raf still requires Ras and Rap1 activation to activate ERKs.

sequence difference has been proposed to explain the high basal kinase activity of B-raf *in vitro* (Mason et al., 1999). Alternatively, these differences in sequence may account for subtle differences in the regulation of B-raf and Raf-1 activation by Ras and Rap1. We hypothesized that the ability of Rap1 to activate B-raf but not Raf-1 may be due to Rap1 not being able to support tyrosine phosphorylation. These differences may be because of the subcellular localization of these kinases and if activated Ras and Rap colocalize to the membrane domains containing these kinases. This model is explored in chapter three of this thesis.

Ras and Rap localize to different subcellular compartments

Ras localization

The signals required for Ras proteins to localize to the plasma membrane consist of two components. The first component comprises farnesylation of the cysteine of the C-terminal CAAX motif, which occurs during protein synthesis in the ER (Hancock et al., 1989; Hancock et al., 1990; Magee and Marshall, 1999). The second component has been shown to be either S-acylation (palmitoylation) of cysteine residues in N- and H-Ras proteins or, in the case of K-Ras, a polybasic stretch of amino acids (Hancock et al., 1991). Both signals occur in the C-teminal 20 amino acid hypervariable domain (Figure 1.6). These different mechanisms of membrane attachment also influence how H-Ras and K-Ras traffic to and from the plasma membrane (Choy et al., 1999). The CAAX modifications alone target Ras to the endoplasmic reticulum (ER), while palmitolyation directs proteins to traffic through the Golgi prior to continuing on to the plasma membrane (Choy et al., 1999). This suggests that the polybasic sequence may either

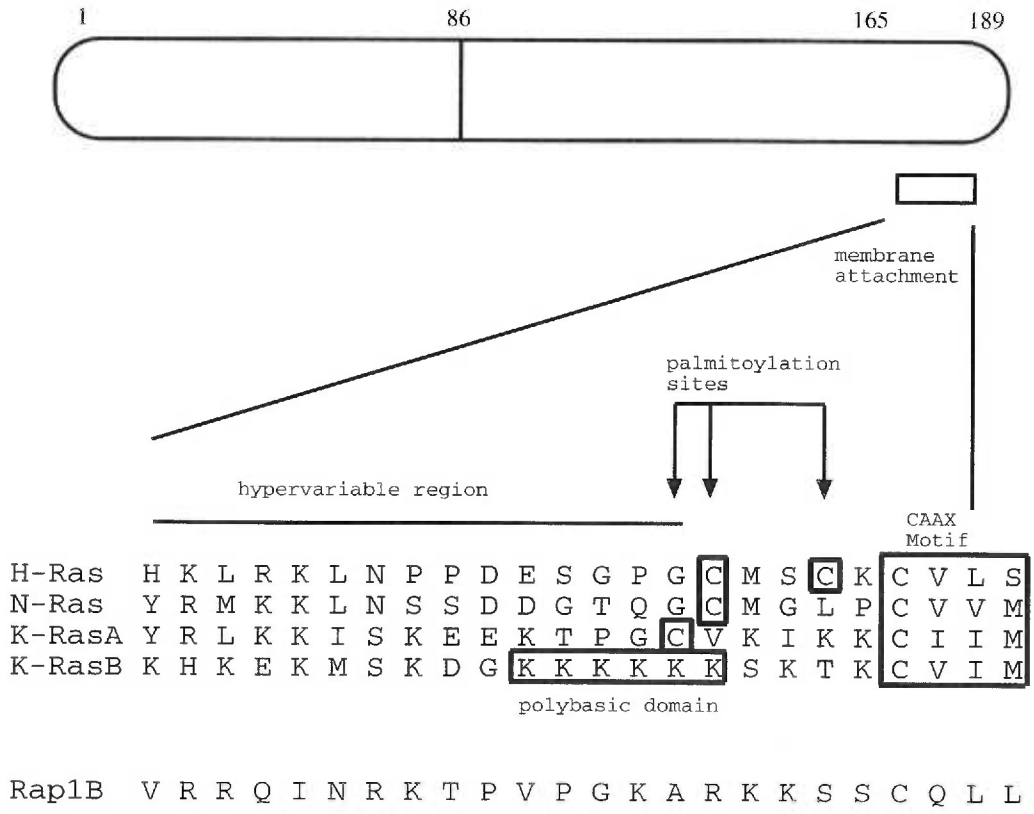

Figure 1.6 Ras and Rap1 localization is dependent on the post-translational lipid modifications in the CAAX motif and hypervariable region at the C-terminus of the protein. H-Ras localizes to the plasma membrane due to farnesylation of the CAAX motif and palmitolyation on two cysteine residues in the hypervariable domain. In contrast, Rap1 is carboxymethylated and geranylgeranylated on the cysteine residue of the CAAX motif and contains a polybasic hypervariable domain analogous to Ki-Ras. Rap1 is primarily found in endosomes and in the Golgi in many cell types as a result of these post-translational modifications. The CAAX and hypervariable domains of Ras family members are both necessary and sufficient for subcellular localization. The differences in the localization of these small G proteins may contribute to their signaling specificity.

exclude K-Ras from the Golgi or that other sequences in the hypervariable domain of H- and N-Ras influence trafficking. Studies using GFP as a tag show that the CAAX motif and hypervariable domain are both necessary and sufficient for proper tagetting of Ras proteins (Cadwallander et al., 1994; Choy et al. 1996). The two different membrane attachment mechanisms and trafficking of Ras proteins may influence H-Ras and K-Ras signaling properties.

The differences in the hypervariable domain among the Ras proteins with the two alternative membrane binding signals has been suggested to account for the functional differences among the Ras family (Prior and Hancock, 2001). For example, H-Ras is a stronger stimulator of phosphatidylinositide 3-kinase than K-Ras *in vivo*, and this has been attributed to palmitolyation of H-Ras (Jaumot et al., 2001; Yan et al., 1998). Swapping the CAAX motifs is sufficient to convert effector usage by these G proteins and can direct signaling events such as cell survival in fibroblasts (Walsh and Bar-Sagi, 2001). Although all Ras family members are able to bind similar effectors *in vitro*, they signal differently *in vivo*. It is possible that the early differences in trafficking of these proteins, or other sequences in or adjacent to the hypervariable domain may influence effector preference. Mice deficient in H-Ras, N-Ras, or K-Ras suggest that Ras family members have both overlapping and independent signaling roles (Bar-Sagi, 2001). This suggests that Ras function in regulating cellular signaling is dependent on subcellular localization.

Rap localization

While Ras primarily localizes to the inner leaflet of the plasma membrane, the localization of Rap1 appears to be more variable and depends on the cell type. Rap1 has been shown to localize to the Golgi (Béranger et al., 1991) or to early and late endocytic vesicles and lysosomes (Pizon et al., 1994), in neutrophils in the plasma membrane upon stimulation (Maridonneau-Parini et al., 1992; (Quinn et al., 1992), and in platelets Rap1 is found on the plasma membrane and α-granules (Berger et al., 1994). The divergence of Rap1 localization as compared to Ras may be due to differences in the post-translational modifications that occur in the Rap1 hypervariable domain and CAAX motif. Rap1 is geranylated instead of farnesylated and contains a polybasic stretch of amino acids resembling K-Ras (Winegar et al., 1991). It is unclear if the localization of Rap1A is the same as Rap1B, which differ in their C-termini. Rap1A is predominately expressed in hematopoetic cells, while Rap1B is expressed in neurons (Bos and Zwartkruis, 1999). Localization of Rap1 may also be regulated by PKA-dependent phosphorylation of serine residue 179 at the C-terminal end of the protein (Siess et al., 1990). Phosphorylation of S179 in neutrophils influences the membrane attachment of Rap1A (Quilliam et al., 1991; Quinn et al., 1992). Phosphorylation of this site has also been shown to influence the affinity of Rap1 for the CRD of Raf-1 (Hu et al., 1999), and substituting an aspartate residue at this site can influence Rap1 signaling in thyroid cells (Ribeiro-Neto et al., 2002). This suggests the possibility that activation of Rap1 and the subsequent response are determined by the localization of Rap1. This may explain why Ras and Rap1 induce different responses, even though their effector domains are identical.

The different localization of Rap1 has given rise to the hypothesis that Rap1 antagonizes Ras signaling pathways by sequestering Ras effectors. This view is supported from early studies using chimeric Ras-Rap1 constructs and the ability of these constructs to induce or revert the Ras transformed phenotype in fibroblasts (Cox et al., 1992; Zhang et al., 1991). Mutations in residues adjacent to the effector loop of Rap are sufficient to convert Rap into a transforming protein, suggesting that despite localization differences Rap1 and Ras are able to interact with the same effectors *in vivo* (Zhang et al., 1991). However, differences between how Rap and Ras interact with the same effectors does not account for the biological effects of Rap1 because Rap-Ras chimeras which have the same amino terminus but lack the Ras hypervariable domain and CAAX motif are not able to induce transformation (Cox et al., 1992; Zhang et al., 1991). Since transformation is dependent on the Erk pathway, these findings suggest that Rap1 reverts the transformed phenotype because Rap1 sequesters Raf1 away from Ras (Kitayama et al., 1989). This localization effect may explain why despite both Ras and Rap1 bind with high affinity to RalGDS *in vitro*, only Ras is able to activate the Ral pathway *in vivo* (Matsubara et al., 1999). This suggests that there are differences between Ras and Rap1 localization that regulate effector activation or coupling to downstream signaling cascades (Figure 1.7).

Role of lipid rafts in Ras signaling

It is possible that the differences in the localization of Ras and Rap1 directs the recruitment of their effectors to different membrane domains that will regulate the

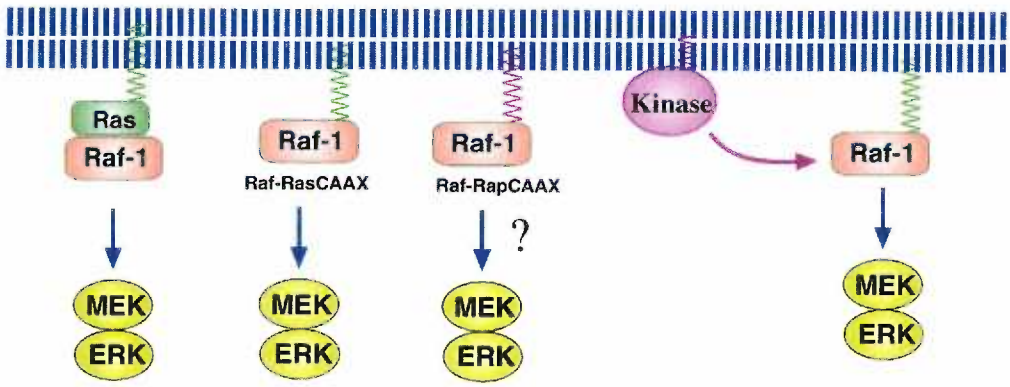

Figure 1.7 Raf-RasCAAX chimeras are constitutively active. Raf-1 kinase activation is dependent on Ras recruitment to the plasma membrane. Raf-Ras chimeras having the hypervariable and CAAX motif of Ras constitutively activate MEK and ERKs. This suggests that the primary role of Ras is to recruit Raf-1 to membrane associated kinases that phosphorylate Raf-1 on key regulatory residues. Rap1 does not activate Raf-1. This may be due to differences in the membrane or subcellular localization of Ras and Rap1.

activation or coupling of effectors to different signaling cascades. Many studies have reported association of Ras proteins with plasma membrane domains enriched in cholesterol and sphingolipids, also known as lipid rafts (Prior and Hancock, 2001; Waugh et al., 2001) or the related structures called caveolae (Anderson, 1998). The extent of association of different G proteins with these domains varies between studies, often depending on the method that has been used in their isolation (Prior et al., 2001; White and Anderson, 2001). A possible explanation for this variability is that the farnesyl group provides a weaker affinity to rafts than does a saturated long-chain acyl group and that this interaction is partially disrupted by detergent treatment (Brown and London, 1998). Recently, Hancock and coworkers have shown that disruption of rafts has different effects on the ability of activated, H- and K-Ras proteins to activate Raf-1 (Prior et al., 2001; Roy et al., 1999). Expression of a dominant negative caveolin, the principal structural protein of caveolae, or extraction of cholesterol from rafts with methyl-β-cyclodextrin, blocked H-Ras but not K-Ras activation of Raf-1 (Roy et al., 1999). Expression of the dominant negative caveolin displaces H-ras from caveolae but has little effect on K-Ras membrane association (Prior et al., 2001). In contrast, other studies suggest that both H-Ras and K-Ras both associate with lipid rafts, and this association is required for the activation of Raf-1 kinase activity (Kranenburg et al., 2001; Liu et al., 1996). It is unclear if there are signaling differences between caveolae and lipid rafts, or if the different methods used in these studies is looking at different types of lipid rafts, which may have distinct signaling properties.

Raft domains also seem to serve as scaffolds for kinases that are required for, or modify the intensity of different signaling components of intracellular signaling cascades. Several groups have documented the importance of rafts in TCR- and BCR-mediated signal transduction (Cheng et al., 2001; Dykstra et al., 2001; Viola et al., 1999). Upon TCR engagement, various signaling molecules, including LAT, Vav, and Lck become enriched in rafts (Samelson et al., 1999). The mechanism responsible for localization of these signal-transducing molecules to rafts is largely unknown. As is the case for H-Ras, palmitoylation seems to be essential for LAT and Lck targeting to lipid rafts (Samelson et al., 1999; Webb et al., 2000). Although the fact that molecules involved in TCR signal transduction migrate to rafts following T cell stimulation strongly suggests a functional role of rafts in TCR signaling, it is possible that the molecules migrate to rafts for purposes other than signal transduction. Many growth factor receptors such as PDGF and EGF receptor also localize to lipid rafts and EGF and PDGF-dependent Erk activation seems to require the recruitment of Raf1 to lipid rafts where many of the signaling components of the cascade reside (Liu et al., 1996; Mineo et al., 1996; Rizzo et al., 2001). Agents that disrupt lipid raft integrity, such as methyl-β-cyclodextrin, block Raf-1 kinase activation by growth factor stimulation (Furuchi and Anderson, 1998). For efficient signal transduction it seems reasonable that a high concentration of signaling molecules should be maintained at the receptor. Hence, it is also reasonable to consider that receptor and signaling molecules exist in a condensed functional compartment. Ras may recruit Raf-1 to lipid rafts where other kinases may reside that are required for Raf-1 kinase activation. The different intracellular location of Rap1 suggests that Rap1 may

recruit Raf-1 to a non-lipid raft containing membrane domain, or distinct membrane microdomain that is incapable of supporting Raf-1 kinase activation.

Two different models emerge for Rap1-dependent Ras antagonism. The first is a direct binding model where activation of Rap1 leads a non-productive interaction with a Ras effector, possibly Raf-1 because it is unable to interact with the CRD to support further activation events. The second model, which is not mutually exclusive, is that Rap1 blocks Ras signaling by sequestering effectors to an intracellular compartment that is unable to support effector activation due to absence of activating kinases, or the inability to couple to downstream components of the signaling cascade. In chapter three of this thesis, we test these models by constructing Raf-Rap and Raf-Ras chimeras and examine their activation in response to growth factor or Ras dependent signals.

This thesis will test the hypothesis that Rap1 antagonizes Ras dependent ERK signaling using Jurkat T cells as a model system. These studies will demonstrate that Rap1 limits signals to ERKs that are generated by activation of the TCR via Ras. Furthermore, we propose a model whereby co-stimulation is required to overcome the inhibition of ERK activation by Rap1. The final section will examine possible biochemical models to explain the distinct actions of Ras and Rap1 on the ERK cascade. We will test the hypothesis that cellular localization dictates the ability of Ras and Rap1 to regulate Raf-1 kinase activation. We will show data that the inability of Rap1 to activate Raf-1 is due, in part, to differences in the membrane compartments where these small G proteins localize Raf kinase isoforms.

THESIS AIMS

The major focus of this thesis to test the following:

(1) **Does Rap1 antagonize Ras signaling by inhibiting ERK activation?**

Rap1 can antagonize Ras-dependent signaling events in a number of different systems, including T cell lymphocytes. It is unclear if this is by inhibiting Ras-dependent ERK activation, or by some other mechanism. Using Jurkat T cells as a model system, we will test the hypothesis that Rap1 inhibits ERK activation. These cells provide a useful *in vivo* model system since T cell receptor (TCR) cross-linking can activate both Ras and Rap1. In T cells, co-stimulation of TCR and the CD28 receptor is required for activation, proliferation, and cytokine production. Co-stimulation through the CD28 receptor also results in the augmentation of TCR-dependent activation of ERKs. As CD28 inhibits Rap1, we will be able to inhibit the activation of endogenous Rap1 and test the effects on ERK activation.

(2) **How does CD28 regulate Rap1?**

In this part of the thesis, we will determine the mechanism of CD28 mediated Rap1 inhibition. We will test the hypothesis that CD28 signaling regulates either the activation of Rap1 exchange factors or Rap GTPase activating proteins. These studies will be performed in Jurkat T cells.

(3) Does Rap1 inhibit Raf-1 kinase activation?

In this part of the thesis we will examine the mechanism for the cell-type specific inhibition of ERKs by Rap1. Both Ras and Rap1 bind to Raf-1 *in vitro*, but localize to distinct intracellular compartments and may sequester Raf-1 from Ras. However, Rap1 is able to activate B-Raf. In this part of the thesis we will test the hypothesis that Rap1 and Ras regulate the activities of Raf-1 and B-Raf by either a localization mechanism or by direct binding.

CHAPTER TWO

CD28 and the tyrosine kinase Lck stimulate MAP kinase activity in T cells via inhibition of the small G protein Rap1.

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ABSTRACT

Proliferation of T cells via activation of the T cell receptor (TCR) requires concurrent engagement of accessory co-stimulatory molecules to achieve full activation. The best-studied co-stimulatory molecule, CD28, achieves these effects, in part, by augmenting signals from the TCR to the MAP kinase cascade. We show here that TCR-mediated stimulation of the MAP kinase ERK is limited by activation of the Ras-antagonist Rap1. CD28 increases ERK signaling by blocking Rap1 action. CD28 inhibits Rap1 activation because it selectively stimulates an extrinsic Rap1 GTPase activity. The ability of CD28 to stimulate Rap1 GTPase activity was dependent on the tyrosine kinase Lck. Our results suggest that CD28 mediated Rap1 GAP activation can help explain the augmentation of ERKs during CD28 co-stimulation.

INTRODUCTION

Maximal activation of T lymphocytes following antigen presentation is thought to require at least two signals. One signal is generated by engagement of the T cell receptor (TCR). A co-stimulatory molecule mediates the second signal. The best-studied co-stimulatory molecule is CD28, which is engaged by APCs during antigen presentation. Co-stimulation enhances production of interleukin-2 (IL-2) and T cell proliferation. The importance of co-stimulation is demonstrated by the fact that TCR engagement in the absence of co-stimulation leads to a state of T cell unresponsiveness termed anergy.

IL-2 production is governed by transcriptional activation of the IL-2 promoter as well as post-transcriptional effects reviewed by (Jain et al., 1995). The IL-2 promoter contains multiple control elements that respond to a variety of transcription factors including AP-1, NF-AT, and NF-κB (Tuosto and Acuto, 1998). One signaling pathway that is required for IL-2 expression is the Ras/Raf-1/ ERK (extra-cellular signal regulated kinase) or the MAP kinase cascade (Izquierdo et al., 1994; Whitehurst and Geppert, 1996). Interfering mutants of Ras (Baldari et al., 1993; Rayter et al., 1992), Raf-1 (Izquierdo et al., 1994), and the MAP kinase kinase MEK (Faris et al., 1996) can block IL-2 transcription following CD28 co-stimulation. The activation of ERK is thought to result in the activation of AP-1 (Frost et al., 1994; Westwick et al., 1994), presumably via the transcriptional activation of c-fos through the transcription factor Elk-1 (Marais et al., 1993). Indeed, a role for CD28 in c-fos expression has been demonstrated (Holdorf et al., 1999).

In primary T cells, CD3 stimulation by itself can activate ERK (Perez et al., 1997). However, ERK activation is enhanced by CD28 co-engagement (Nunes et al., 1994). Activation of the Ras/ERK pathway is strongly inhibited under experimental conditions of T cell unresponsiveness, or anergy, induced following stimulation via the TCR in the absence of CD28 co-stimulation (Boussiotis et al., 1997; Fields et al., 1996; Li et al., 1996b). One candidate effector of this blockade of Ras signaling is Rap1, a small G protein that was initially cloned as an antagonist of Ras-dependent transformation in fibroblasts (Kitayama et al., 1989). Rap1 is constitutively activated in anergic T cells, and activation of Rap1 inhibits both ERK activation and IL-2 expression (Boussiotis et al., 1997). Interestingly, TCR cross-linking activates Rap1 (Boussiotis et al., 1997; Reedquist and Bos, 1998) while co-engagement of CD28 blocks this activation (Reedquist and Bos, 1998). In this study, we examine the mechanism by which CD28 activates the ERK signaling cascade in T cells via its inhibition of Rap1.

MATERIALS AND METHODS

Cell Culture, Transfections, and Stimulations. The human T cell leukemia cell line Jurkat and JCaM1.6 T cell isolates (stably expressing Lck wild type and LckW97A) were maintained in RPMI medium with 10% FCS at 37°C, 5% CO_2. Jurkat cells expressing mCD28 receptor (Holdorf et al., 1999) were maintained in RPMI medium, 10% FCS, 50μg/ml G418. For transient transfections, $5x10^7$ cells were resuspended in 400μl cytomix (120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4/KH_2PO_4, pH 7.6, 25mM Hepes, pH 7.6, 2mM EGTA, pH 7.6, 5mM $MgCl_2$, 5mM glutathione) with the appropriate cDNAs and electroporated (250V, 950μF). All cDNAs were transfected at a concentration of $5μg/5x10^7$ cells, except dn.Lck (10μg), Rap1GAP1 (10μg), and fos-luciferase (20μg), and the total DNA transfected held constant with the addition of pCDNA3.1 (vector). The transfection efficiency of transfected plasmids was monitored using 5μg of cDNA encoding green fluorescent protein (EGFP, Clonetech). In all experiments, transfection efficiency was greater than 60%. After a 24 hr recovery in RPMI with 10% FCS, cells were incubated for 30 min on ice with or without anti-TCR/CD3 mAb (C305 mAb 1/40 hybridoma supernatant; gift from Dr. A. Weiss, University of California, S.F.) and/or anti-hCD28 mAb (CD28.2, 5μg/ml; Pharmingen) and/or anti-mCD28 mAb (PV-1.5μg/ml, gift from Dr. C. June, Naval Medical Research Institute, Bethesda, MD). Cells were stimulated by addition of 10 μg goat anti-mouse secondary antibody (Southern Biotech) at 37°C for the indicated times. When indicated, PD98059 (50μM), or UO126 (10μM) was added to cells for 30 min of pretreatment and remained in the incubation medium for the duration of the experiment.

T Cell Isolation and Antibody Stimulation. T cells were purified from C57BL6 splenocytes using a murine T cell enrichment column (R&D Systems) according to the manufacturers instructions. Ten million T cells were then incubated with 5 μg anti-CD3 antibody (145-2C11, Pharmingen) with or without co-stimulation using 10μg anti-CD28 antibody (37.51, Pharmingen) on ice for 30 minutes, washed, then incubated with 20 μg of goat anti-hamster immunoglobulin (Fisher) for 5 min at 37°C, lysed for RalGDS assay, as described below.

DNA Constructs and Mutants. Wild-type bovine Rap1b or wild-type human Ha-Ras (Vossler et al., 1997) were tagged at the amino terminus with 2x FLAG epitope (Kodak) by PCR and introduced into the BamHI and XbaI sites of pcDNA3.1 vector (Invitrogen). Rap1GAP1, (RG9T, gift from Dr. P. Polakis) was N-terminally tagged with the 2xFLAG epitope by PCR and introduced into the BamHI and XbaI sites of pcDNA3.1 pcDNA3.FLAG-RapE63/V12 (RapE63/V12) was generated from pcDNA3.FLAG-RapWT using the Quick-change PCR mutagenesis kit (Stratagene). Constitutively active and dominate negative versions of Lck and Fyn were constructed using PCR-directed mutagenesis.

***In vivo* Rap and Ras activation assays**. Activated Rap1 was isolated from cell lysates using a protocol adapted from Franke et. al. (Franke et al., 1997). Jurkat cells ($5x10^7$/ml) were stimulated with anti-TCR/CD3 and/or anti-CD28 mAb as previously described for the indicated times at 37°C. Cells were lysed in 400μl ice-cold Rap lysis buffer (10% glycerol, 1% NP-40, 50mM Tris-HCl, pH 8.0, 200mM NaCl, 5mM $MgCl_2$, 1mM

PMSF, 1μM leupeptin, 10μg/ml soybean trypsin inhibitor, 10mM NaF, 0.5mM aprotinin, and 1mM Na_3VO_4). Lysates were clarified by centrifugation and supernatants containing 0.5mg of total protein were incubated with 60μg of GST-RalGDS-RBD fusion protein (gift of Dr. J.L. Bos, Utrecht University, The Netherlands) coupled to glutathione agarose beads for 1 hr at 4°C. Beads were pelleted and rinsed three times with lysis buffer, and protein was eluted from the beads with Laemmli buffer. Activated Ras was isolated from stimulated cell lysates using agarose coupled GST-Raf1-RBD provided in the Ras Activation Assay Kit (Upstate Biotechnology, Inc., Lake Placid, N.Y.) following manufacturer's recommended protocol. Proteins were separated by electrophoresis in a 12% gel followed by transfer to a polyvinyldine diflouride membrane. Membranes were blocked in 5% milk and probed with either anti-Rap1 polyclonal antibody (anti-Krev-1, Santa Cruz Biotechnology Inc., Santa Cruz, CA.) or anti-Ras mAb (Upstate Biotechnology, Inc., Lake Placid, N.Y.) followed by an HRP-conjugated anti-mouse monoclonal secondary antibody (Amersham). Proteins were detected by enhanced chemiluminescence. Activation of ERK1/ERK2 was detected from 30μg of total cellular stimulated lysates by immunoblotting with a phospho-specific MAPK mAb (New England Biolabs, Beverly, MA.). When indicated, the densitometric analysis of the bands was performed using NIH Image.

GAP assay. Cos7 cells were transfected with FLAG-Rap1b, FLAG-Ras, or FLAG-RapE63, or with pcDNA3.1 vector alone using Lipofectamine (GIBCO, BRL). Cells were allowed to recover for 48 hr and then lysed in Rap lysis buffer and FLAG-epitope tagged proteins were immunoprecipitated with 10μg of M2-FLAG antibody (Sigma) coupled to protein A-Sepharose for 2 hr at 4°C. Expression of FLAG-Rap and FLAG-Ras was

confirmed by western blotting. Immune complexes retained on protein A-agarose beads and washed twice in lysis buffer, and once with Rap loading buffer (20mM Tris,pH 7.5, 100mM NaCl, 1mM $MgCl_2$, 1mM DTT, 5μg/ml BSA, 5% glycerol, 0.1% NP-40, 1μg/ml leupeptin, 0.5μg/ml aprotinin) or with Ras loading buffer (20mM Tris, pH 7.5, 5mM EDTA, 10mM NaCl, and 5μg/ml BSA, 1μg/ml leupeptin, 0.5μg/ml aprotinin). FLAG-Rap complexes were loaded with 0.1μM (γ-^{32}P)GTP (3000Ci/mmol) at 30°C for 20 min FLAG-Ras complexes were loaded with 0.1μM (γ-^{32}P)GTP at 30°C for 10 min The $MgCl_2$ concentration was adjusted to 10 mM to stabilize the FLAG-Rap-(γ-^{32}P)GTP or FLAG-Ras-(γ-^{32}P)GTP complex. Unincorporated GTP was removed by rinsing the complexes 4 times in ice-cold loading buffer containing 10mM $MgCl_2$. Jurkat cells were stimulated as described above and lysed in Rap lysis buffer. For each assay condition, 10μg of total cellular protein was added to 100μl exchange buffer (25mM Tris, pH 7.5, 5mM $MgCl_2$, 100mM NaCl, 1mM GTP, 1μg/ml BSA, 1μg/ml leupeptin, 0.5mg/ml aprotinin) along with (γ-^{32}P)GTP-loaded FLAG-Rap or FLAG-Ras complexes and incubated at 30°C for the indicated times. The reaction was stopped with 1 ml of stop buffer (20mM Tris, pH 7.5, 100mM NaCl, 10mM $MgCl_2$). The complexes were washed 4 times with 1 ml ice-cold stop buffer to remove released (^{32}P). Total radioactivity remaining associated with the immune complexes was measured by scintillation counting.

GEF assay. FLAG-Rap and FLAG-Ras immune complexes were prepared as described and resuspended in Rap exchange buffer (20mM Tris, pH 7.5, 1mM $MgCl_2$, 20mM EDTA, 100mM NaCl, 10mM β-mercaptoethanol, 5% glycerol, and 1mg/ml BSA) or Ras exchange buffer (20mM Tris, pH 7.5, 1mM $MgCl_2$, 10mM NaCl, 5μg/ml BSA, 1μg/ml

leupeptin, 0.5mg/ml aprotinin). FLAG-Rap immune complexes were loaded with 5μCi of (^{3}H)GDP (34Ci/mmol) at 30°C or 20 min FLAG-Ras immune complexes were loaded with 5μCi of (^{3}H)GDP (34Ci/mmol) at 37°C for 10 min FLAG-Rap-(^{3}H)GDP or FLAG-Ras-(^{3}H)GDP immune complexes were stabilized by adjusting the $MgCl_2$ concentration to 25mM. Unincorporated (^{3}H)GDP was removed by washing the beads 4 times in loading buffer containing 25mM $MgCl_2$. Jurkat cells were stimulated as described above and lysed in Rap lysis buffer. For each assay condition, 50μg of total cellular protein was added to 250 μl of exchange buffer containing 100μM GDP, 1.5mM GTP, and 50,000-100,000 cpm of FLAG-Rap-(^{3}H)GDP or FLAG-Ras-(^{3}H)GDP complexes and incubated at 30°C for the indicated times. The reaction was stopped by adding 1 ml of ice-cold stop buffer (20mM Tris, pH 7.5, 100mM NaCl, 25mM $MgCl_2$) and immediately applied to nitrocellulose filters under vacuum. The filters were rinsed 4 times with 3 mls of Stop buffer and retained radioactivity was measured by scintillation counting.

Calcium Flux Analyses. Calcium measurements were performed as previously described (Huby et al., 1998). Briefly, Jurkat cells were co-transfected with dn.Lck and cDNA encoding GFP, dn.Fyn and GFP or pcDNA3.1 (vector) and GFP as described above. After recovery, cells were resuspended to $2x10^6$/ml in complete medium supplemented with 2 mM of the Ca^{2+} indicator, Indo-1 and 0.02% pluronic F127 (Molecular Probes, Eugene, OR) and incubated at 37°C for 30 min. Cells were then washed in ice-cold buffer (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM KCl, 10 mM glycine, 15 mM HEPES, pH 7.4) and resuspended to $2x10^6$ cells/ml and then kept on ice until use. Ca^{2+} fluctuations, before and after the addition of anti-CD3 mAb (Pharmingen, San Diego, CA) at 10 μg/ml, were

monitored using a FACS Vantage flow cytometer by gating on the GFP positive cells. Cells were excited at 355 nm, and emission was measured at 480 nm, representing free Indo-1 and 405 nm representing Ca^{2+}-associated Indo-1, to give a ratio (405 nm/480 nm).

Luciferase assays. Luciferase assays were performed as previously described (Vossler et al., 1997).

RESULTS

CD28 co-stimulation augments ERK activation through the TCR/CD3. In Jurkat cells (Figure 2.1A, left panel) and in primary T cells harvested from mouse splenocytes (Figure 2.1A, right panel), activation of the TCR/CD3 by antibody crosslinking produces a modest activation of ERKs as measured by phospho-specific antibodies recognizing pT202pY204 of human ERK1/2 (pERK). However, this is strongly augmented upon activation of CD28 by crosslinking antibodies (Figure 2.1A). Similar results are seen examining ERK activity by *in vitro* kinase assay (Figure 2.1B). The data demonstrate the profound synergy between CD3 and CD28 on ERK activation in primary T cells, as well as in Jurkat T cells. This enhancement was reflected in the level of Raf-1 associating with Ras, which was highest following co-stimulation with anti-CD3 and anti-CD28 (Figure 2.1C). One potential downstream target of ERKs is the expression of c-fos. Activation of the c-fos promoter coupled to luciferase (Visvader et al., 1988) can also be enhanced by CD28 co-stimulation. This action of CD28 on the fos promoter was blocked by the MEK inhibitor PD98059 (Dudley et al., 1995) and UO126 (Duncia et al., 1998) (Figure 2.1D), suggesting that the enhancement of fos expression by CD3/CD28 reflected CD3/CD28's ability to augment ERK activity.

Rap1 limits ERK activation. It has been previously demonstrated that CD3 engagement stimulates activation of Rap1 (Reedquist and Bos, 1998). Rap1 is a small G protein that antagonizes Ras signaling to ERKs in a cell type-specific manner (Vossler et al., 1997), through it's antagonism of the MAP KKK, Raf-1. In cells that express the Raf isoform B-Raf, like PC12 cells, Rap1 has the opposite effect; it activates ERKs (Vossler et

al., 1997). In Jurkat T cells (Figure 2.2A), and primary T cells (data not shown), Rap1 is expressed but B-Raf is not. Therefore, we predict that Rap1 activation may antagonize ERK signaling in Jurkat cells, as has been shown in primary T cells (Boussiotis et al., 1997; Reedquist and Bos, 1998). Indeed, expression of the constitutively active mutant of Rap1 (Rap1E63) blunts CD3/CD28 activation of ERKs (Figure 2.2B). That CD3/CD28's activation of ERKs requires Ras was demonstrated by the ability of the interfering mutant RasN17 to block ERK activation through CD3/CD28 (Figure 2.2B). To examine the role of endogenous Rap1 to limit ERK activation following TCR/CD3 stimulation, Rap1 activity was inhibited by transfection of Rap1GAP1, a Rap1-specific GTPase activating protein (Jordan et al., 1999; Polakis et al., 1991). The inhibition of Rap1 by Rap1GAP1 augmented CD3's activation of ERKs (Figure 2.2C), suggesting that endogenous Rap1 serves to limit CD3 activation of ERKs. It has been proposed that the antagonism of signals to ERKs by Rap1 may be due to the sequestration of Raf-1 by activated Rap1 (Okada et al., 1999; Sakoda et al., 1992). We show that CD3 stimulation of Jurkat cells promotes the association of Raf-1 and Rap1 (Figure 2.2D). Interestingly, CD3 also promotes the association of Raf-1 with Ras (Figure 2.2D), consistent with the ability of CD3 to activate both Rap1 and Ras (Reedquist and Bos, 1998). This association of Ras and Raf-1 appears to be augmented following the inhibition of endogenous Rap1 by the Rap1 inhibitor Rap1GAP1 (Figure 2.2D), presumably because Rap1 sequesters Raf-1 only in the GTP-loaded state (Okada et al., 1999). This suggests that activation of Rap1 by CD3 limits ERK signaling by limiting Ras-dependent recruitment of Raf-1.

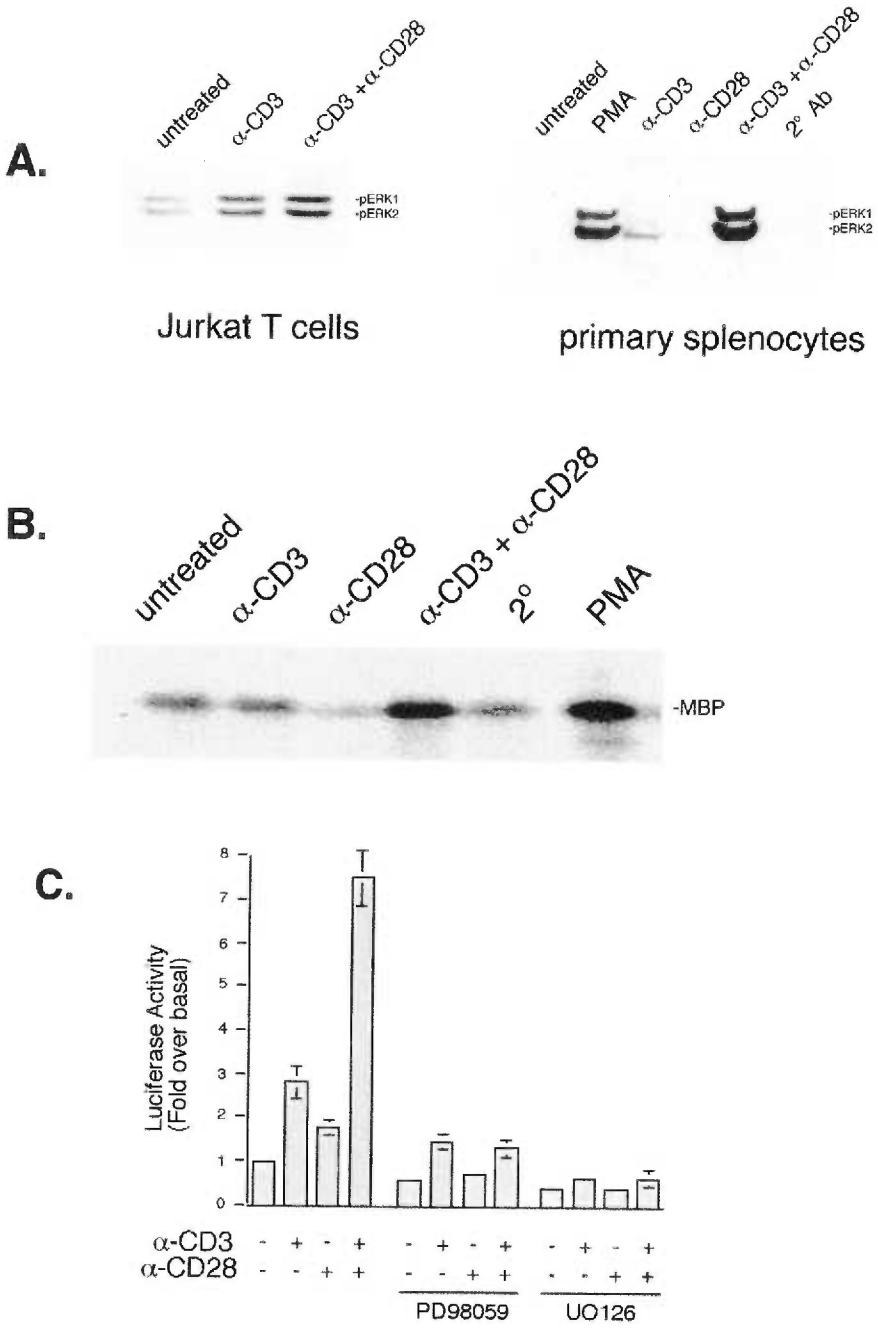

FIGURE 2.1 Co-stimulation with antibodies to CD28 Augments signals to ERKs

FIGURE 2.1. Co-stimulation with antibodies to CD28 augments signals to ERKs. (A) Jurkat T cells (left panel) were stimulated with α-TCR/CD3 +/- α-CD28 or left untreated, as indicated. Primary splenocytes (right panel) were stimulated with α-CD3 and/or α-CD28 as indicated, and PMA was used as a positive control. Incubation of cells with secondary antibody alone (2° Ab) served as a negative control. In both panels, phospho-ERK (pERK1/2) was measured using phospho-specific pERK antibodies. The positions of pERK1 and pERK2 are indicated. (B) Jurkat cells were incubated with anti-TCR/CD3 antibody, anti-CD28 antibody or PMA (50 ng/ml) for 30 min on ice, or left untreated as indicated, then stimulated at 37°C for 5 min following addition of 10 μg/ml crosslinking antibody. Secondary antibody alone (2° Ab) was used as a negative control. Cells were lysed and ERK2 was immunoprecipitated. Activation of immunoprecipitated ERK2 was measured by an *in vitro* kinase assay. Samples were subject to SDS-PAGE and analyzed with a phosphoimager. A representative gel with the position of the substrate MBP is presented. (C) Ras activation of Raf-1 is augmented by co-stimulation with α–CD28. Jurkat cells were transfected with Flag-Ras, and treated with α-CD3, α-CD28 or left untreated as indicated. The associated endogenous Raf-1 was measured following Flag immunoprecipitation and anti-Raf-1 western blot. For Flag-Ras, cells were also transfected with vector or Rap1GAP1 as indicated. A representative gel with the position of Raf-1 is shown (n=3). (D) Wild type Jurkat cells were transfected with a fragment of the c-fos promoter coupled to luciferase (c-fos-luciferase) (Visvader et al., 1988) as indicated and incubated with anti-human CD3 (α-CD3) and anti-human CD28 (α-CD28) for 6 hr in the absence and presence of the MEK inhibitors PD98059 or UO126, as indicated. For all luciferase assays, lysates were prepared andassayed for luciferase activity. The data is presented as fold activation above basal luciferase activity (lane 1), with standard error (s.e.) (n=3).

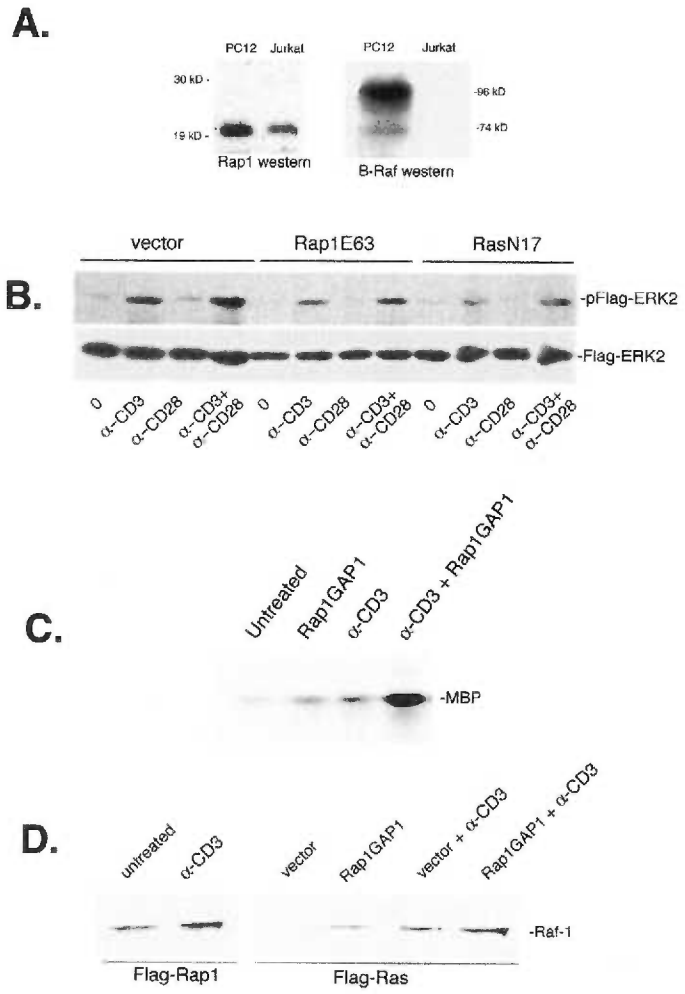

FIGURE 2.2 Rap1 limits signals from the TCR to ERKs

FIGURE 2.2. Rap 1 limits signals from TCR to ERKs. (A) Jurkat T cells express Rap 1, but not B-Raf. Jurkat cells express Rap1 but not B-Raf. Western blots for B-Raf and Rap1 expression in both PC12 cells and Jurkat cells. Left panel. Low levels of Rap1 are detected in Jurkat cells compared to PC12 cells. Right panel. B-Raf expression is very high in PC12 cells, cells where Rap1 activates ERK (Vossler et al., 1997), but absent in lymphocytes where Rap1 inhibits ERK (Boussiotis et al., 1997). (B) Constitutively active Rap1 blocks ERK activation by CD28 co-stimulation in Jurkat cells. Jurkat cells were transfected with vector, Rap1E63 or RasN17 as indicated. Cells were treated with α-CD3 and/or α-CD28 as indicated. Phosphorylation of ERK1/2 monitored by pERK western blot. The positions of pERK1/2 are shown. Lower panel: control showing equivalent protein loading. (C) Jurkat cells were transfected with 10 μg each of cDNAs encoding vector or Rap1GAP1 as indicated. All cells received 10μg of Flag-ERK2 cDNA. Subsequently, cells were incubated with α-TCR/CD3 for 30 min on ice or left untreated. Cells were then activated by incubation at 37°C for 10 min. Cells were lysed and Flag-ERK2 was immunoprecipitated. Activation of immunoprecipitated ERK2 was measured by *in vitro* kinase assay. Samples were subject to SDS-PAGE and analyzed with a Phosphoimager. A representative gel with the position of the substrate MBP is presented. (D) Ras activation of Raf-1 is limited by endogenous Rap1. Jurkat cells were transfected with either Flag-Rap1 or Flag-Ras, and treated with α-CD3 or left untreated as indicated. The associated endogenous Raf-1 was measured following Flag immunoprecipitation and anti-Raf-1 western blot. For Flag-Ras, cells were also transfected with vector or Rap1GAP1 as indicated. A representative gel with the position of Raf-1 is shown (n=3).

CD28 inhibits Rap1 activation. It was previously demonstrated that CD3's activation of Rap1 can be inhibited by co-engagement of CD28 in PHA blasts and in some T cell lines (Reedquist and Bos, 1998). We confirmed this finding using primary splenic T cells (Figure 2.3A) and in the Jurkat T cell line (Figure 2.3B). This was likely a direct effect on Rap1 rather than on an upstream activator of Rap1, as CD28 also blocked the activation of Rap1 triggered by overexpressing C3G, a Rap1-specific guanine nucleotide exchange factor (GEF) (York et al., 1998) (Figure 2.3C).

Mapping sequences of CD28 required to inhibit Rap1 activation. To determine which residues of CD28 were required to inhibit Rap1 activation, we utilized Jurkat cells stably expressing either full-length mouse CD28 (mCD28-WT) or a truncated form of mouse CD28 (mCD28-CΔ16), lacking the C terminal 16 residues (Holdorf et al., 1999). These cells express equivalent numbers of mouse CD28 molecules, as measured by FACS (data not shown). Similar to the results with the endogenous human CD28 in Jurkat cells, engagement of mouse CD28 in Jurkat cells was able to inhibit Rap1 activation by anti-CD3 antibody (Figure 2.4A). Importantly, engagement of mCD28-CΔ16 using anti-mouse CD28 was unable to inhibit Rap1 activation by anti-CD3 (Figure 2.4A). This demonstrates that the carboxy-terminal 16 residues of CD28 were required to inhibit Rap1 activation by CD3.

The carboxy-terminal 16 residues of CD28 are required for CD28's augmentation of ERKs but not Ras. We examined the action of CD3/CD28 on Ras activation using a Raf-1 fragment containing the Ras-binding domain (RBD) linked to Gst (Gst-Raf-1-RBD). Anti-CD3 was able to stimulate Ras activity; this was only slightly enhanced by the addition of antibodies to CD28 (Figure 2.4B, upper panel), similar to that seen in mCD28-CΔ16 cells (Figure 2.4B, lower panel). In contrast, ERK activation under the same conditions was strongly augmented by CD28 (Figure 2.4C, upper panel). However, CD28 co-stimulation of mCD28-CΔ16 cells was not able to enhance ERK activation (Figure 2.4C, lower panel), despite being able to enhance CD3 action in mCD28-WT cells. Taken together, these results suggest that the activation of Ras seen in mCD28-CΔ16 cells was not sufficient to activate ERKs. This result may explain previous results demonstrating that CD28 co-stimulation of mCD28-CΔ16 cells did not stimulate transcription of the c-fos promoter, while stimulation of the CD28 co-receptor of mCD28-WT cells did (Holdorf et al., 1999).

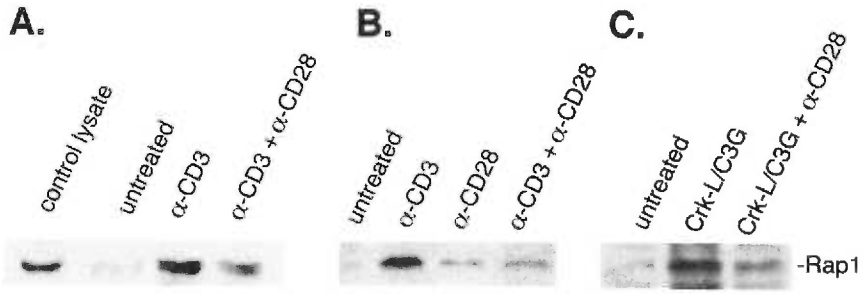

Figure 2.3 Inhibition of Rap1 by CD28 costimulation in primary splenic T cells and in human Jurkat cells. (A) Activation of Rap1 in primary splenic T cells. Primary splenic T cells were harvested and incubated with anti-mCD3 antibody (α-CD3) and/or anti-mCD28 antibody (α-CD28) for 5 min. or left untreated as indicated. (B) Activation of Rap1 in human Jurkat T cells. Jurkat cells were incubated with anti-human CD3 antibody (α-CD3) and/or anti-human CD28 antibody (α-CD28) for 5 min. or left untreated. (C) Inhibition of Rap1 by CD28 following transfection of human Jurkat T cells. Wild-type Jurkat cells were transfected with CrkL/C3G or the vector alone and incubated with α-CD28 as indicated. In all experiments, T cell lysates were prepared and assayed for Rap1 activation using GST-RalGDS and Western blotting was performed using Rap1 antiserum. The position of Rap1 in control lysates and following isolation of glutathione-bound proteins is shown. Representative Western blots are shown (n=3).

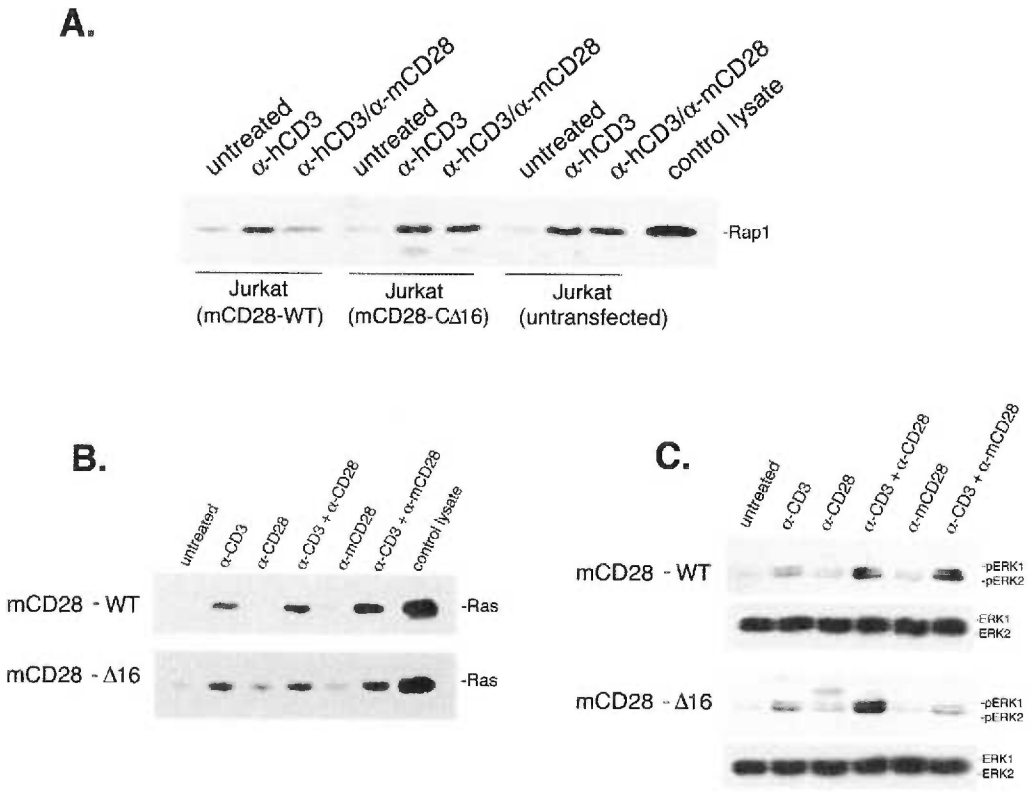

Figure 2.4 CD28's inhibition of Rap1 and enhancement of ERKs maps to the carboxy-terminal 16 residues of CD28. Jurkat cells and Jurkat cells stably expressing wild-type mCD28 [Jurkat (mCD28-WT)] or mCD28 with its 16 carboxy-terminal amino acids deleted [Jurkat (mCD28-CΔ16)] were incubated with anti-human CD3 antibody (α-hCD3) and/or anti-mouse CD28 antibody (α-mCD28) for 5 min. or treated with secondary antibody alone (untreated). In all experiments, T-cell lysates were prepared and assayed for Rap1 activation using GST-RalGDS and Western blotting was performed using polyclonal Rap1 antiserum. The position of Rap1 in control lysates (A and D) and following isolation of glutathione-bound proteins is shown. Representative Western blots are shown (n=3). (B) Ras activation does not require the carboxy terminus of CD28. Jurkat mCD28-WT cells and mCD28-CΔ16 cells were incubated with anit-human CD3 antibody (α-CD3), anti-human CD28 antibody (α-CD28), and/or anti-mouse CD28 antibody (α-mCD28) for 5 min. or left untreated as indicated. Lysates were prepared and assayed for Ras activation using GST-Raf-1-RBD, and Western blotting was performed using Ras antiserum. The position of Ras in control lysates and following isolation of glutathione-bound proteins is shown. (C) The 16 carboxy-terminal amino acid residues of CD28 that are required for inhibiting Rap1 are required for stimulating ERK activity. Jurkat mCD28-WT cells and mCD28-CΔ16 cells were incubated with anti-human CD3 antibody (α-CD3), anti-human CD28 antibody (α-CD28), and/or anti-mouse CD28 antibody (α-mCD28) for 5 min. or left untreated as indicated. Lysates were prepared and assayed for ERK activation using phospho-specific ERK (pERK) antibodies. Representative Western blots with the positions of pERK1 and pERK2 indicated are shown (n=3).

The tyrosine kinase Lck mediates CD28's inhibition of Rap1. Recently, it was shown that the carboxyl-terminal 16 amino acids of CD28 are important for binding and activation of Lck (Holdorf et al., 1999). To directly test whether activation of Lck is involved in inhibiting Rap1 activation, we expressed a constitutively active form of Lck (LckF505, or ca.Lck) and analyzed its effects on Rap1 (Figure 2.5A). Expression of LckF505 was able to block the activation of Rap1 by CD3. This was a specific effect, as expression of a constitutively active form of Fyn, a related src-family kinase (FynF531, or ca.Fyn), was unable to inhibit CD3's activation of Rap1 (data not shown). Indeed, overexpression of FynF531 stimulated Rap1 activation by itself suggesting that the activation of Rap1 by anti-CD3 might be mediated via the activation of Fyn, as has been suggested (Boussiotis et al., 1997). Importantly, LckF505 was also able to block the activation of Rap1 by FynF531 (Figure 2.5B). These results suggest that activation of Fyn is sufficient to activate Rap1 while activation of Lck is able to block this activation.

We also tested whether an interfering mutant of Lck (LckR273 or dn.Lck) could block CD28's actions on Rap1. In this mutant, the essential lysine at residue 273 has been replaced with an arginine (Carrera et al., 1993), to create a kinase-dead protein that has been shown to block Lck function selectively *in vivo* (Anderson et al., 1993; Hashimoto et al., 1996; Hernandez-Hoyos et al., 2000; Levin et al., 1993). Here we show that its expression, but not expression of the analagous mutant of the related tyrosine kinase fyn (dn.Fyn) (Cooke et al., 1991), results in a complete block of TCR-induced calcium flux in Jurkat T cells (Figure 2.6A), suggesting that this mutant is selective for Lck-dependent actions. Expression of dn.Lck in Jurkat cells blocked the ability of CD28 to inhibit Rap1 activation (Figure 2.6B)

demonstrating that Lck mediates CD28's regulation of Rap1. Expression of dn.Lck in Jurkat cells also blocked the ability of CD28 to augment ERK activation (Figure 2.6C). A similar results was found using JCaM1.6 cells that lack a functional Lck (Straus and Weiss, 1992). In JCaM1.6 cells, stimulation through the TCR did not regulate Rap1 (Fig. 6D,E). However, stable expresion of Lck restored Rap1 regulation by CD28. Interestingly, Lck expression also restored TCR stimulation of Rap1. This may be partially explained by the increased levels of Rap1 protein in JCaM1.6/Lck cells, compared to the parental JCaM1.6 cells (Figure 2.6D).

The ability of CD28 to inhibit Rap1 requires the expresion of an intact Lck SH3 domain. In addition to the kinase domain (SH1 domain), Lck contains an SH2 and SH3 doamin. Binding of Lck to the proline-rich domain (PRD) of CD28 is thought to require an intact SH3 domain (Holdorf et al., 1999), as is Lck-dependent activation of ERKs (Denny et al., 1999). The importance of the SH3 domain in ERK signaling is suggested by studies examining the expression of an Lck SH3-mutant (W978ALck) Lck. For example, Straus and colleagues have shown that wild type Lck, but not W978ALck, can restore ERK activation in Lck-defective JCaM1.6 cells (Denny et al., 1999). Using JCaM1.6 cells expressing either wild type Lck (JCaM1.6/LckWT) or LckW97A (JCaM1.6/LckW97A), we examined whether the SH3 domain was required in CD28's inihibition of Rap1. Activation of CD28 inhibits Rap1 activation in the JCaM1.6/LckWT cells, but not in the related JCaM1.6/LckW97A cells (Figure 2.6D,E). These data, coupled with the data using dn.Lck, show that CD28 inhibition of Rap1 requires an intact Lck protein.

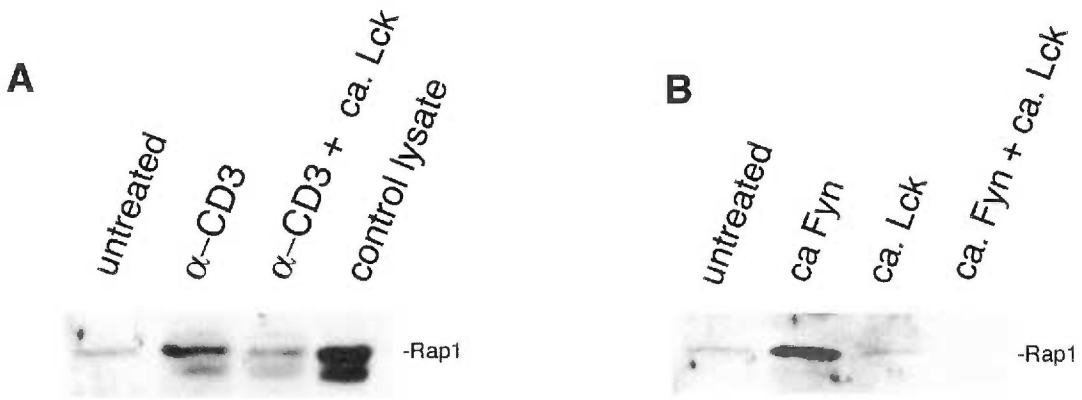

Figure 2.5 Lck is sufficient to inhibit TCR stimulation of Rap1. (A) Jurkat cells were transfected with ca.Lck or vector alone and incubated with anti-human CD3 antibody (α-CD3) for 2 min. or left untreated as indicated. (B) Jurkat cells were transfected with Lck505 (ca.Lck) and/or FynF531 (ca.Fyn) or the vector alone. In both experiments, T-cell lysates were prepared and assayed for Rap1 activation using GST-RalGDS and Western blotting was performed using Rap1 antiserum. The position of Rap1 in control lysates and following isolation of glutathione-bound proteins is shown. Representative Western blots are shown (n=3).

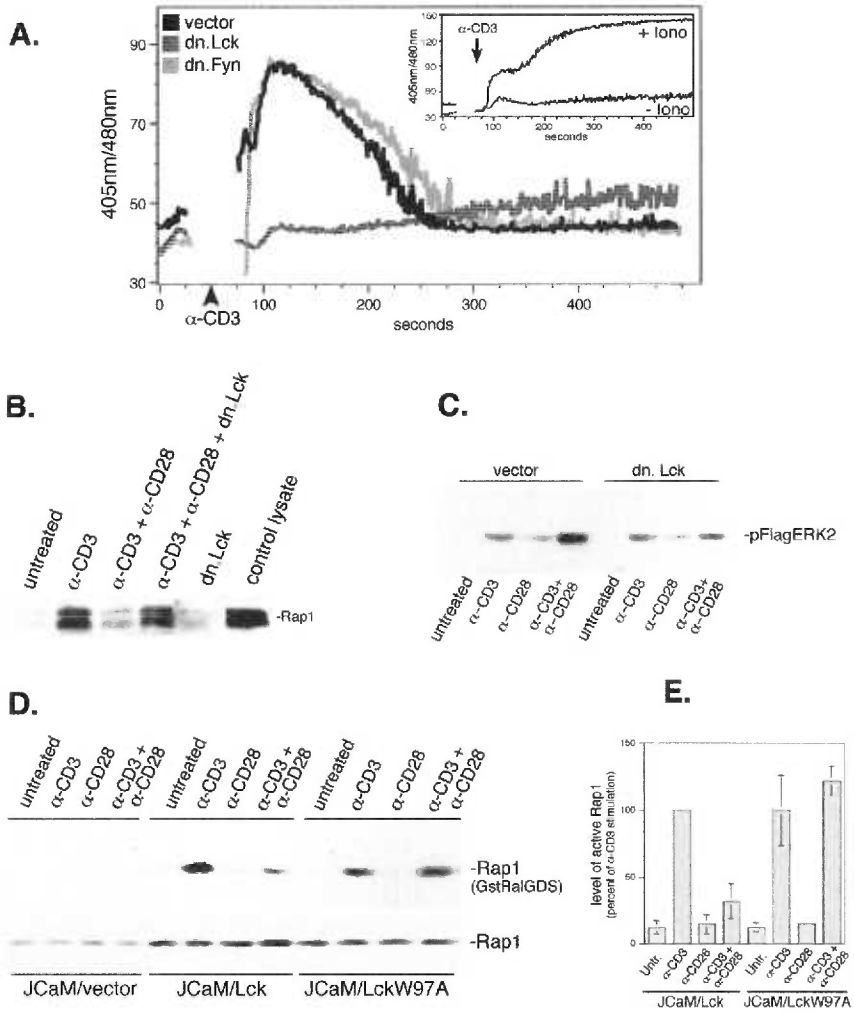

Figure 2.6 Lck is required for CD28's inhibition of Rap1 and augmentation of ERK

FIGURE 2.6 Lck is required for CD28's inhibition of Rap1 and and augmentation of ERK (A) dn.Lck inhibits mobilization of intracellular calcium. Jurkat T cells were transfected with vector (black line), dn.Lck (dark gray line),or dn.Fyn (light gray line) and preloaded with Indo-1. Cells were then stimulated with anti-CD3 mAb as indicated. Changes in the mobilization of intracellular free calcium, presented as a ratio of 405 nm/480 nm representing Ca^{2+}-associated Indo-1 (405 nm) and free Indo-1 (480 nm), are shown as a function of time. Expression of dn.Fyn had no effect on Ca^{2+} flux. However, expression of dn.Lck blocked Ca^{2+} in these cells. Inset graph: Successful loading of Jurkat cells, expressing dn.Lck, with Indo-1 was confirmed by treating cells with anti-CD3 mAb and ionomycin (+indo) which shows a Ca^{2+} flux, or treating with anti-CD3 alone (-indo) which shows a block in Ca^{2+} flux. A representative experiment is shown (n=3). (B) Jurkat cells were transfected with LckR273 (dn.Lck) or vector alone and incubated with α–hCD3 (α-CD3) and/or α-hCD28 (α-CD28) for 2 min. or left untreated as indicated. T cell lysates were prepared and assayed for Rap1 activation using Gst-RalGDS and western blots performed using Rap1 antisera. The position of Rap1 in control lysates and following isolation of Glutathione-bound proteins is shown. Representative western blots are shown (n=3). (C) Jurkat cells were transfected with dn.lck or vector along with Flag-ERK2 and treated with antibodies to CD3 and/or CD28 as indicated. The phosphorylation of Flag-ERK2 was monitored by pERK western. The position of pFlag-ERK2 is shown. A representative western blot is shown (n=3). (D) JCaM1.6/LckWT (JCaM/Lck) or JCaM1.6/LckW97A (JCaM/LckW97A) cells were incubated with anti-human CD3 (α-CD3) and/or anti-human CD28 (αCD28) for 5 min. or left untreated as indicated. Cell lysates were prepared and assayed for Rap1 activation using Gst-RalGDS and western blots performed using Rap1 antisera (upper panel). A representative western blot with the position of Rap1 is shown follwoing Gst-RalGDS pull-down (n=3). Lower panel depicts a western blot showing the relative expression of Rap1 in these cell lines. (E) The data in D is presented as the average of three independent experiments, with s.e.

CD28 inhibits Rap1 by stimulating extrinsic Rap1-specific GTPase activity. The activity of small G proteins like Rap1 is regulated by both positive and negative factors. They are positively regulated by GTP exchange factors (GEFs) which catalyze the exchange of GDP for GTP. They are negatively regulated by GTPase activating proteins (GAPs) which selectively enhance the intrinsic GTPase activity of specific G proteins. CD28's inhibitory effects on Rap1 could therefore be mediated by inhibition of Rap1 GEF activity or by enhancement of Rap1 GAP activity.

We first examined the ability of CD28 to modulate Rap1 GEF activity. GEF activity was measured in an *in vitro* assay, using recombinant Rap1 loaded with (^{3}H)-GDP, as previously described (Li et al., 1996a; Li et al., 1992). Anti-CD3 stimulated GEF activity, as measured by the exchange of (^{3}H)-GDP for cold GTP. Anti-CD28 had no effect on Rap1 GEF activity and, more importantly, did not inhibit GEF activity stimulated by anti-CD3 (Figure 2.7, left panel). Therefore, inhibition of Rap GEF did not contribute to CD28's inhibition of Rap1. CD28 had a modest effect on Ras exchange (right panel).

To examine the possibility that a Rap1 GAP was stimulated by CD28, we used an *in vitro* GAP assay. For these experiments, we used recombinant wild type Rap1 loaded with GTP-(γ-^{32}P) and measured the release of (^{32}P) catalyzed by exogenous GAP activities. To validate this assay, we used a cDNA encoding the Rap1GAP1 protein, a human Rap1 GTPase activating protein that displays Rap1-specific GAP activity when expressed in mammalian cells (Rubinfeld et al., 1991). In addition, we assayed the release of GTP-(α-

^{32}P), to confirm that CD28 was not stimulating exchange under the GAP assay conditions. The specificity of Rap1GAP1 for Rap1 was shown by its inability to hydrolyze GTP bound to Ras (Figure 2.8A). Using this assay, we measured Rap1 GAP activity from Jurkat cell lysates before and after treatment with antibodies to CD3, CD28 or both (Polakis et al., 1991). CD28 stimulation by itself was able to enhance a Rap1 specific GAP activity (Figure 2.8B, left panel). Anti-CD28 treatment stimulated the release of (^{32}P) from GTP-(γ-^{32}P)-loaded recombinant wild type Rap1, but it had no effect on GTP-(γ-^{32}P)-loaded RapV12/E63, a mutated form of Rap1 that cannot be regulated by Rap1GAP (Boussiotis et al., 1997) (Figure 2.8A, 8B; right panel). CD28 did not have any effect on Rap1 loaded with GTP-(α-^{32}P) under the conditions of this GAP assay (Figure 2.8C), consistent with the absence of CD28's regulation of Rap1 exchange activity (Figure 2.7). Consistent with this, the Rap1 GAP activity from anti-CD28 treated extracts was heat-sensitive (data not shown). These results suggest that this activity in anti-CD28 treated cell extracts represented a bona fide Rap1 GAP activity. Because the activity expressed from the transfected cDNA encoding Rap1GAP1 may be distinct from the Rap1GAP activity identified in stimulated Jurkat T cell lysates, we will refer to the cDNA as Rap1GAP1 and the GAP activity from the lysates as Rap1 GAP activity.

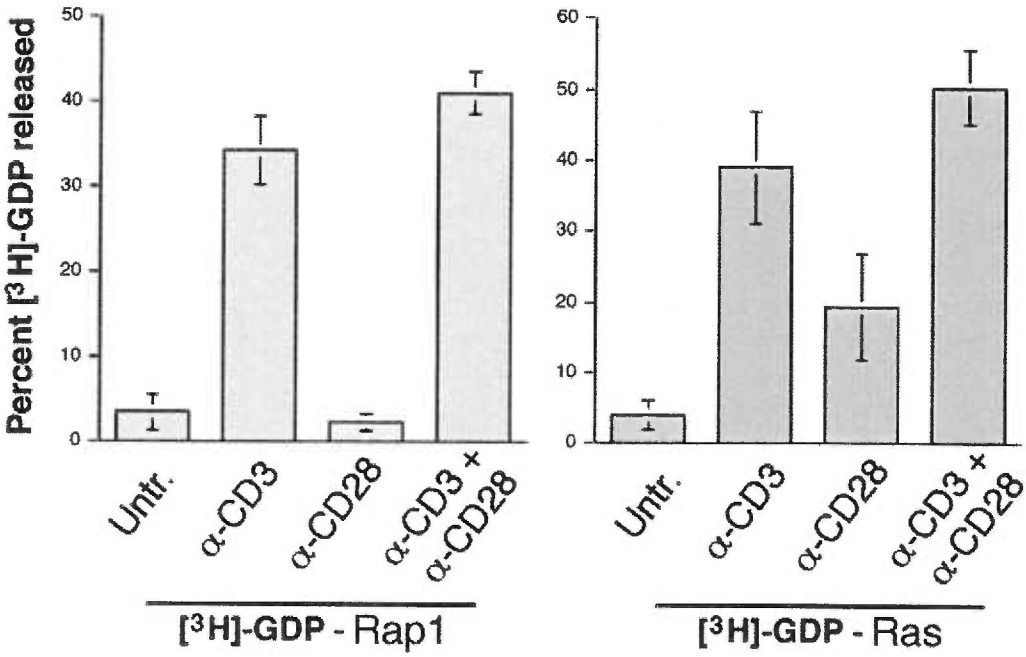

Figure 2.7 Lack of regulation of Rap1 GEF activity by CD28. Jurkat cells were incubated with anti-human CD3 antibody (α-CD3) and/or anti-human CD28 antibody (α-CD28) for 5 min. or left untreated (Untr.) as indicated. Lysates were prepared and incubated for 10 min. with recombinant [3H]GDP-loaded Rap1 protein (left panel) or Ras protein (right panel) bound to agarose beads. The percentages of [3H]GDP released from the beads are indicated. Standard errors are shown (n=3).

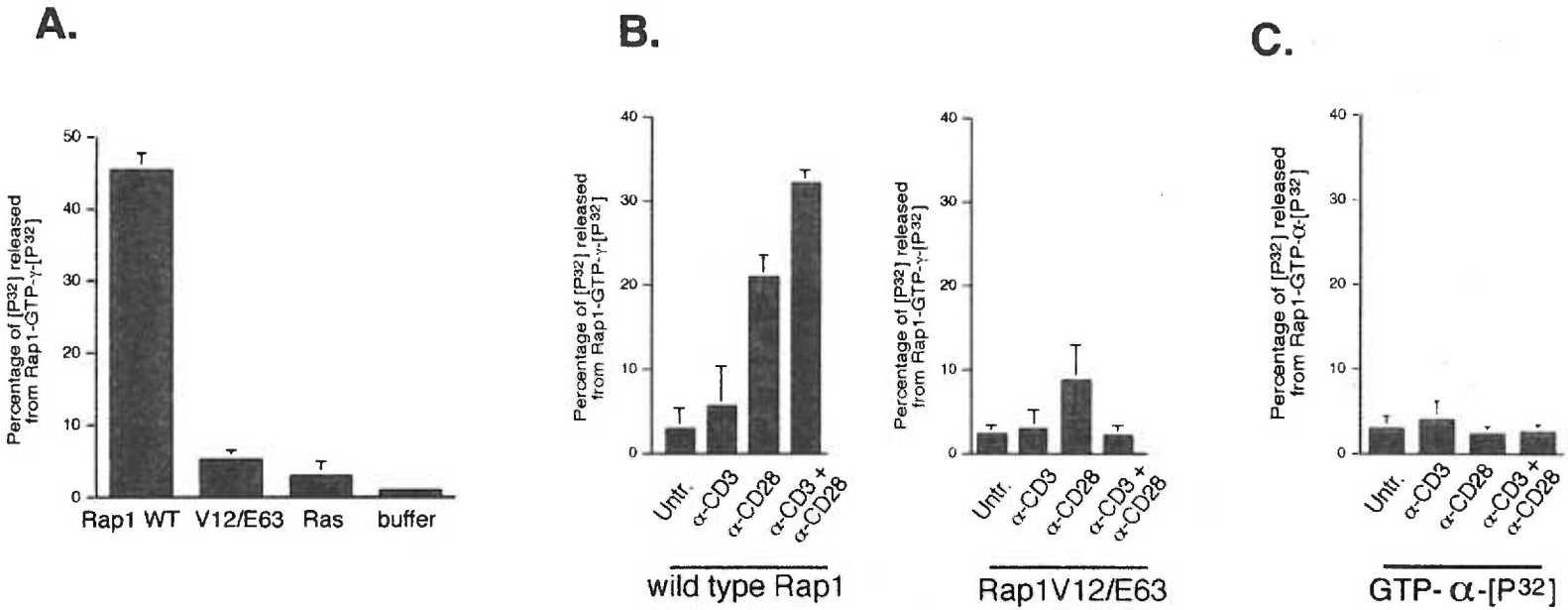

Figure 2.8 Enhancement of Rap1 GTPase activity by CD28. (A) Specificity of Rap1GAP1 for Ras and selected Rap1 mutants in vitro. Human Rap1GAP1 was expressed in Cos7 cells and incubated with recombinant Rap1 mutant proteins loaded with [γ-32P]GTP. The percentages of hydrolysis of [γ-32P]GTP from wild-type Rap1(Rap1 WT), RapV12/E63 (V12/E63), Ras, and buffer alone are indicated by the percentage of γ-32P released from Rap or Ras loaded in vitro. (B) CD28 stimulation of RapGAP activity and introduction of E63 into RapV12, which blocks GTPase activity. Jurkat cells were incubated with anti-human CD3 antibody (α-CD3) and/or anti-human CD28 antibody (α-CD28) for 5 min. or left untreated (Untr.) as indicated. Lysates were prepared and incubated for 20 min. with recombinant FLAG-tagged Rap1 (left graph) or RapV12/E63 (right graph) loaded in vitro with [γ-32P]GTP, as indicated. The percentages of release of [γ-32P]GTP are shown with standard errors (n=3). (C) Cells were treated as described for panel B and were assayed with [γ-32P]GTP. Release of α-32P was monitored as described in Materials and Methods, and the percentages of release of [γ-32P]GTPase is shown with standard errors (n=6).

We confirmed that the last sixteen residues of CD28 were required to stimulate Rap1 GTPase activity (Figure 2.9A). Truncation of the last 16 residues of CD28 blocked its ability to stimulate Rap1 GAP activity. Furthermore, expression of LckF505 (ca.Lck), in Jurkat cells, was also able to stimulate Rap1 GTPase activity but not Ras GTPase activity (Figure 2.9B). In contrast, ca.Fyn was unable to stimulate Rap1 GAP activity (Figure 2.9B). Therefore, although some Lck-dependent functions may be partially rescued by Fyn (Denny et al., 2000), Fyn does not appear to rescue Rap1 GAP activation by Lck. We next investigated the requirement of the SH3 domain of Lck for CD28's activation of Rap1GAP activity using JCaM1.6/LckWT or JCaM1.6/LckW97A cells. As shown in Figure 2.9C, JCaM1.6/LckWT cells demonstrated elevated Rap1GAP activity following CD28 stimulation to a significantly higher degree than did JCaM1.6/LckW97A cells. These data support a model where CD28's ability to stimulate Rap1 GAP activity is mediated by the last 16 residues of CD28 and the SH3 domain of Lck.

Mutation of an essential arginine within the catalytic active site of all GAPs produces an interfering mutant that can block Rap1GAP function (Hillig et al., 1999). T cells express specific Rap1 GAPs whose activity can be blocked by the expression of this interfering mutant of Rap1GAP1 (Reedquist et al., 2000). Here, the expression of this mutant Rap1GAP1-R289L/K285I/R286G (dn.Rap1GAP1) blocked CD28's augmentation of ERK activity (Figure 2.10A), suggesting that Rap1GAP1 (or a related Rap1-specific GAP) was responsible for CD28's augmentation of ERKs. In contrast, the expression of wild type Rap1GAP1 augmented CD3's activation of ERKs, to the level seen by co-stimulation, without increasing the activation of ERKs by CD28 (Figure 2.10A). Parallel Rap1 assays

confirmed that expression of Rap1GAP1 inhibited and expression of dn.Rap1GAP1 enhanced Rap1 activity (Figure 2.10B). Taken together, these data suggest that CD3 activation of Rap1 limit ERK signals whereas CD28 activation of a Rap1 GAP augments ERK signals (See Figure 2.11).

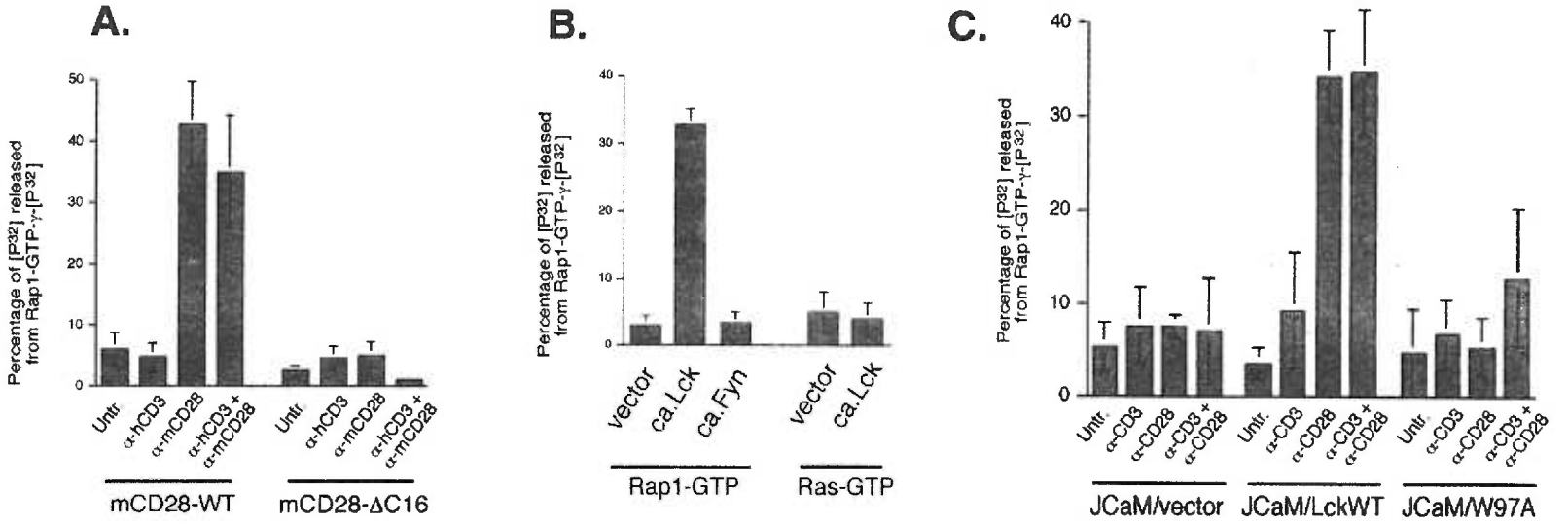

Figure 2.9 Involvement of Lck in CD28's enhancement of Rap1 GTPase activity. (A) The 16 carboxy-terminal amino acid residues of CD28 are required for stimulating Rap1GAP activity. Jurkat cells stably expressing full-length wild-type mCD28 (mCD28-WT) or Jurkat cells stably expressing mCD28-CΔ16 were treated with anti-human CD3 antibody (α-hCD3) and/or anti-mCD28 antibody (α-mCD28) or left untreated (Untr.) as indicated, and GTPase assays were performed as described in Materials and Methods. Percentages of release of [γ-32P]GTP are shown with standard errors (n=3). (B) Lck, but not Fyn, stimulates Rap1GAP activity. Jurkat cells were transfected with vector alone, ca.Lck, or ca.Fyn and incubated with recombinant [γ-32P]GTP-loaded Rap1 (left) or [γ-32P]GTP-loaded Ras (right) as indicated. Percentages of released [γ-32P]GTP are shown with standard errors (n=5). (C) JCaM/vector, JCaM/LckWT, or JCaM/LckW97A cells were incubated with anti-human CD3 antibody (α-CD3) and/or anti-human CD28 antibody (α-CD28) for 5 min. or left untreated as indicated. Cell lysates were prepared and assayed for Rap1 GAP activity as described for panel B. Percentages of release of [γ-32P]GTP are shown with standard errors (n=3).

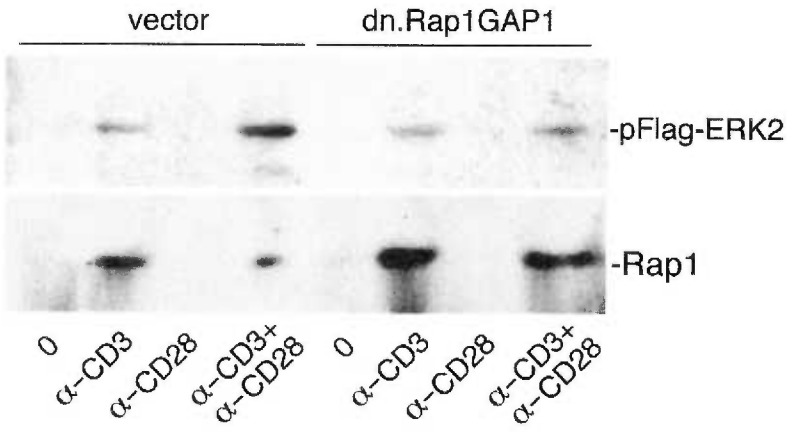

Figure 2.10 Interfering with Rap1 GAP blocks CD28 enhancement of ERKs. Jurkat cells were transfected with FLAG-ERK2 along with the vector and dn.Rap1GAP1 or the vector alone as indicated. Cells were treated with anti-CD3 antibody (α-CD3) and/or anti-CD28 antibody (α-CD28) or left untreated (lanes 0), as indicated. In the upper blot, the activation of FLAG-ERK2 was monitored using FLAG immunoprecipitation followed by pERK Western blotting. The position of pFLAG-ERK2 is shown. A representative gel is shown (n=4). In the lower panel, lysates prepared as described above were subjected to a GST-RalGDS pull-down assay and Rap1 Western blotting. The position of Rap1 is shown.

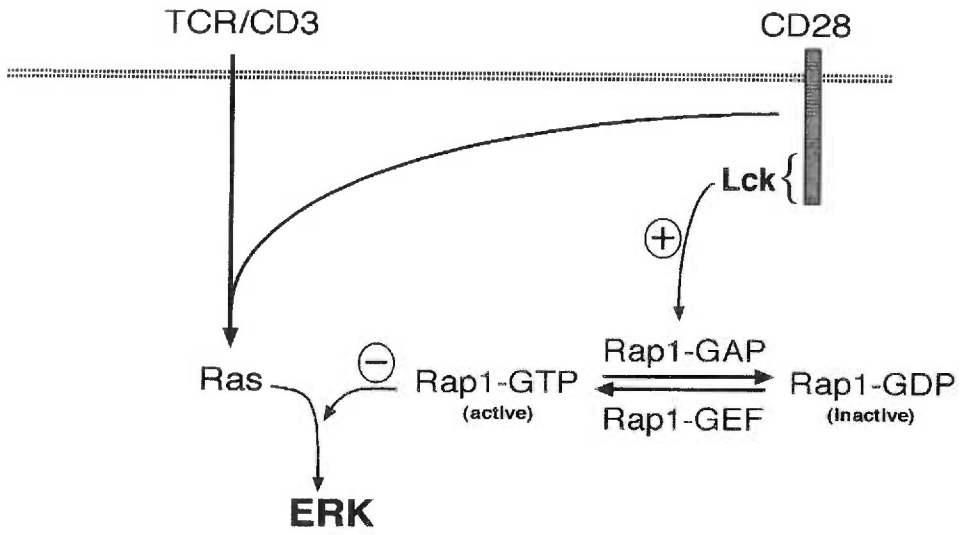

Figure 2.11: Diagram of Rap1 regulation by CD28/Lck. Rap1 is regulated by the balance between the action of Rap1 GEFs and Rap1 GAPs. During TCR-CD3 engagement, the activation of Rap1 limits signals generated by activated Ras. Co-stimulation of CD28 recruits Lck to its C terminus, where it can activate a Rap1 GAP. This activity functions to reverse the Rap1-dependent antagonism of Ras signaling to strongly potentiate Ras-dependent signals to Erks.

DISCUSSION

The small G protein, Rap1 has been proposed as an antagonist of Ras-dependent signaling pathways (Boussiotis et al., 1997; Kitayama et al., 1990; Schmitt and Stork, 2000; Vossler et al., 1997) in multiple cell types. However, a number of papers have also suggested that Rap1 may not limit Ras signaling (Busca et al., 2000; Zwartkruis et al., 1998). The data pesented here show that Rap1 limits CD3 signals to ERKs in Jurkat T cells. Moreover, we show that CD3-dependent activation of Rap1 is associated with the sequestration of Raf-1 away from Ras and propose that this provides a mechanism for Rap1 antagonism of Ras function. Furthermore, activated Rap1 is a target for regulation by additional intracellular signals. In particular, inhibition of Rap1 via CD28 co-stimulation enhances ERK activation. Therefore, Rap1 is a negative regulator of the ERK signaling pathway in T cells, and its inhibition by CD28 augments signals to ERK.

We show here that Lck is both necessary and sufficient for CD28's inhibition of Rap1 and its enhancement of ERKs. A structure/function analysis of CD28 revealed that the last 16 residues of the CD28 cytoplasmic domain were required for these effects. Contained within these residues is a proline-rich domain (PRD) capable of associating with the SH3-domain of Lck (Holdorf et al., 1999). Therefore, we tested whether constitutively active forms of Lck could also effect Rap1 activation. Constitutively activation of Lck strongly inhibited Rap1 activation by TCR engagement. Furthermore, interfering with endogenous Lck function blocked both CD28's inhibition of Rap1 and augmentation of ERKs.

Lck binding to CD28 is mediated by interactions between the Lck's SH3 domain and the PRD of CD28 (Holdorf et al., 1999). Interestingly, the SH3 domain of Lck has been shown to be important for Lck's activation of ERK (Denny et al., 1999), but not other actions of Lck, including activation and phosphorylation of ZAP-70 and LAT (Straus et al., 1996). Here, we show that Lck is also required for the inhibition of Rap1 activation by CD28. We propose that Lck's enhancement of ERK activity during CD28 co-stimulation is mediated by its inhibition of Rap1. We suggest that both the SH3 domain of Lck and the PRD of CD28 are required for this action. Since the activation of Rap1 is negatively regulated by Rap1-specific GAPs, Rap1 GAPs are potential targets of CD28 action. Indeed, measurements of Rap1 GAP activity in cell extracts after CD28 engagement revealed that CD28 engagement does result in significant stimulation of Rap1 GAP activity, and this requires both an intact PRD of CD28 and SH3 domain of Lck.

Despite extensive research into its mechanism of action, the exact function of CD28 remains unclear. Most current models propose that CD28 provides an essential second signal required for T cell activation. In these models, T cell activation requires two distinct signals for full activation (Guerder and Flavell, 1995; Lenschow et al., 1996). The first signal is transduced by the TCR while CD28 provides a second signal termed co-stimulation (Liu et al., 1992). However, the exact signal mediated by CD28 engagement has remained elusive. While it has been proposed that CD28 regulates signals like the activation of JNK kinase or NF–κB, these signals require co-engagement of the TCR; CD28 engagement by itself does not activate JNK or NF–κB (Su et al., 1994). Furthermore, JNK activation utilizes the

membrane-proximal region of CD28, a region that is dispensible for IL-2 production (Barz et al., 1998).

Recently, it was shown that CD28 engagement by itself can induce tyrosine phosphorylation as well as stimulate a c-fos reporter construct in an Lck-dependent fashion (Holdorf et al., 1999). This suggested that CD28 may not transduce a distinct signal, but rather functions to potentiate signals initiated by the TCR. In this model, although both TCR and CD28 can couple to Lck, CD28 also utilizes Lck in a distinct way to further augment TCR signaling (Dustin and Shaw, 1999; Shaw and Dustin, 1997). It has been proposed that Lck function is dictated not only through its activation but also by association with specific receptors. Here we extend those findings and demonstrate that Lck activation by CD28 results in the generation of a specific signal, activation of a Rap1 GAP, that is distinct from Lck's other functions, which are thought to be triggered by binding proteins assocaited with the TCR (Shaw et al., 1989; Straus et al., 1996). The findings presented here can potentially reconcile these two models and provide new insight into the function of CD28 co-stimulation.

In the absence of signaling by the TCR, CD28 is still capable of activating Lck resulting in Rap1 GAP activation. However, in the absence of Rap1 activation the stimulation of a Rap1 GAP is without effect on ERK signaling. This can explain why little to no signaling had been previously detected by CD28 engagement itself. In contrast, in the presence of Rap1 activation, CD28 engagement could have a significant effect. Consistent with previous reports, we found that TCR engagement is a potent activator of Rap1

(Boussiotis et al., 1997; Reedquist and Bos, 1998). Because Rap1 opposes the action of Ras, this suggests that the magnitude of signals transduced by the TCR is self-limiting in the absence of CD28. Thus, in the presence of activated Rap1, the stimulation of a Rap1 GAP could have a powerfully synergistic effect (See Figure 2.11) allowing ERK signaling to reach its maximal potential. This provides a model how co-engagement of CD28 with the TCR can have such a profound effect on T cell activation.

Rap1 activation is a mechanism utilized by multiple cells, including both T and B cells to modulate signals downstream of Ras (Boussiotis et al., 1997; Grewal et al., 2000b; Grewal et al., 1999; McLeod et al., 1998; Schmitt and Stork, 2000; Vossler et al., 1997). The ability of Rap1 to antagonize Ras-dependent actions requires Rap1 activation. This antagonism of signalings pathways to ERK contrasts the actions of Rap1 in other cell types that express the Raf-1 isoform B-Raf, a positive effector of Rap1 (Schmitt and Stork, 2000; Vossler et al., 1997). Neither peripheral lymphocytes nor Jurkat cells express B-Raf, and Rap1 antagonism of Raf-1 appears to be its major action in these cells. Rap1 activation is triggered by a growing family of Rap1-specific GEFs that can be activated by a diverse set of intracellular signaling pathways (de Rooij et al., 1998; Gotoh et al., 1995; McLeod et al., 1998; York et al., 1998). This activation of Rap1 may account for the ability of multiple intracellular signals like cyclic adenosine monophosphate (cAMP) to inhibit Ras-dependent pathways in lymphocytes (Hordijk et al., 1994; Lin and Abraham, 1997; Lingk et al., 1990), as well as other cell types (Burgering et al., 1993; Cook et al., 1993; Wu et al., 1993). cAMP can potently activate Rap1 in multiple cell types (Vossler et al., 1997; Wan and Huang, 1998), including lymphocytes (data not shown) (Wan and Huang, 1998), and the hydrolysis

of cAMP via CD28-regulated phosphodiesterases has recently been proposed as a mechanism for CD28 co-stimulation (Li et al., 1999).

Here, we identify Rap1 GAPs as novel targets of CD28 signaling. Dysregulation of Rap1 GAPs may underscore some of the signaling defects seen in states of T cell hyporesponsiveness, such as anergy (Boussiotis et al., 1997). Although largely unexplored, the recent identification of a growing family of Rap1-specific GAPs demonstrates that they are widely expressed in multiple cells types including T cells (Kurachi et al., 1997; Mochizuki et al., 1999). Some have a ubiquitous pattern of expression, while other Rap1 GAPs are restricted in their pattern of expression (Rubinfeld et al., 1991; Wienecke et al., 1995). For example, the Rap1 GAP, SPA-1, appears to be expressed only in lymphocytes (Kurachi et al., 1997). The structural diversity of these Rap1 GAPs suggest that they will have multiple mechanisms of regulation. At present, the mechanism of Rap1 GAP regulation is not well known (Polakis et al., 1992), however roles for heterotrimeric G proteins have been suggested (Jordan et al., 1999; Mochizuki et al., 1999). Our results using dn.Rap1GAP1 suggest that this protein (or a related GAP) may be the target of CD28's actions in Jurkat cells. Because CD28's ability to regulate both Rap1 and ERKs is common to both Jurkat and primary T cells, it is possible that regulation of Rap1GAP activity represents an important mechanism for modulating ERK activation *in vivo* as well.

The activation of Rap1 may play an important role in regulating signals transduced by the TCR. Rap1, which is activated by engagement of the TCR alone, is likely to provide a threshold that prevents activation of the T cell by non-specific ligands. This is important

because it can potentially explain how the TCR achieves its exquisite sensitivity and specificity (Lanzavecchia et al., 1999; Sloan-Lancaster et al., 1993). Although it is generally assumed that inhibitory signals function to terminate signaling processes, inhibitory molecules can also play important roles in shaping the character of the signaling response. Activation of inhibitory molecules during the signaling process can suppress weak signals, ensuring that non-specific ligands are unable to activate the T cell. The CD28/Lck signaling pathway allows the T cell a unique mechanism to amplify signals that overcome a specific threshold. CD28/Lck not only reverses the Rap1 signal, but may stimulate the Ras pathway on its own. This allows signaling by the TCR to achieve a "switch-like" character (Ferrell, 1996), where all signals below a specific threshold are suppressed and all signals above a specific threshold are amplified to their maximal potential. The requirement of a second molecule, CD28, to relieve the inhibition, allows the T cell a second layer of temporal control and may help explain the function of CD28 in T cell co-stimulation. It can also help explain the persistent activation of Rap1 in anergic cells (Boussiotis et al., 1997).

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CHAPTER THREE

The requirement of specific membrane domains for Raf-1 phosphorylation and activation

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ABSTRACT

Activation of Raf-1 by Ras requires recruitment to the membrane as well as additional phosphorylations, including phosphorylation at serine 338 (S338) and tyrosine 341 (Y341). In this study we show that Y341 participates in the recruitment of Raf-1 to specialized membrane domains called "rafts" which are required for Raf-1 to be phosphorylated on S338. Raf-1 has also been proposed to be recruited to the small G protein Rap1. However, this does not result in Raf-1 activation. We propose that this is because Raf-1 is not phosphorylated on Y341 upon recruitment to Rap1. Redirecting Rap1 to Ras-containing membranes or mimicking Y341 phosphorylation of Raf-1 by mutation converts Rap1 into an activator of Raf-1. In contrast to Raf-1, B-Raf is activated by Rap1. We suggest that this is because B-Raf activation is independent of tyrosine phosphorylation. Moreover, mutants that render B-Raf dependent of tyrosine phosphorylation are no longer activated by Rap1.

INTRODUCTION

The mitogen-activated protein (MAP) kinase family regulates diverse physiological processes including cell growth, differentiation, and death. Activation of one of these MAP kinases (the extracellular signal-regulated kinase, or ERK) is initiated by the recruitment of the MAP kinase kinase kinase Raf-1 to the small G protein Ras, a resident plasma membrane protein. Ras is tethered to the membrane via a carboxyl CAAX motif containing a cysteine (C) followed by two aliphatic amino acids (A) and a carboxyl-terminal amino acid (X), that directs the attachment of a farnesyl moiety (Hancock et al., 1991; Magee and Marshall, 1999). In addition to farnesylation, a second signal assists in correct membrane targeting. For Ha-Ras and N-Ras, this second signal is a palmitoyl moiety that is introduced on a neighboring cysteine. In Ki-Ras, this second site is a polybasic domain. The requirement of Ras' membrane localization for Raf-1 activation has been confirmed by mutating the terminal cysteine in a constitutively active Ras mutant, RasV12, resulting in a mutant that can not activate Raf-1 (Kato et al., 1992).

Membrane regions rich in cholesterol and sphingolipids, termed "rafts" or detergent-insoluble glycolipid-enriched complexes have been proposed to participate in signaling events by organizing additional molecules, such as c-Src, G protein subunits, and phospholipases, into discrete membrane domains (Anderson, 1998). Recent attention has focused on the role of these specialized microdomains in Ras signaling (Furuchi and Anderson, 1998; Mineo et al., 1996; Prior et al., 2001; Roy et al., 1999). A number of groups have shown that both Ras isoforms Ki-Ras (Kranenburg et al., 2001) and Ha-Ras are targeted to rafts (Chen and Resh, 2001; Furuchi and Anderson, 1998; Liu et al., 1996; Mineo et al., 1996; Rizzo et al., 2001; Roy et al., 1999). Others have suggested that Ha-Ras, but not Ki-Ras, can be targeted to raft microdomains (Carozzi et al., 2002; Prior et al., 2001). Targeting of Ras isoforms to specific membrane domains may be determined by the characteristics of the lipid modifications on Ras, as well as other sequences found within the hypervariable region (Prior et al., 2001). Localization of Ras isoforms to distinct membrane microdomains may influence selectivity of signaling among the Ras isoforms (Prior and Hancock, 2001). For example, Ki-Ras is thought to couple well to

Raf-1 but, unlike Ha-Ras, couples poorly to PI3-K (Jaumot et al., 2001; Yan et al., 1998). Differences between Ha-Ras and Ki-Ras in their promotion of cell survival have also been noted (Walsh and Bar-Sagi, 2001), suggesting that distinct localization of Ras isoforms dictate signaling pathways, as recently proposed (Chiu et al., 2002).

Raf-1 recruitment to the membrane can be achieved independently of Ras by the addition of Ras carboxyl-terminal sequences to the carboxyl-terminus of Raf-1. The addition of twenty amino acids from the carboxyl-terminus of Ki-Ras onto Raf-1 (Raf-KiCAAX) is sufficient to redirect Raf-1 to the membrane where it is constitutively active (Leevers et al., 1994; Stokoe et al., 1994). Maximal activity of this chimera, however, requires additional phosphorylation events (Marais et al., 1995; Mineo et al., 1997), consistent with the requirement of specific kinases for full activation for wild type Raf-1 (Dent et al., 1995a; Jelinek et al., 1996; Li et al., 2001a). In particular, two phosphorylations on S338 and Y341 have been shown to be required for full activity (Mason et al., 1999). Recently two additional sites within the kinase activation loop have also been shown to be required (Chong et al., 2001). Phosphorylation of serine 338 may be mediated by the serine/threonine kinase PAK (p21-associated protein) (Diaz et al., 1997) and phosphorylation of tyrosine 341 can be carried out by Src family tyrosine kinases (Fabian, 1993; Marais et al., 1995; Mason et al., 1999). The participation of specific membrane microdomains in these modifications is not known.

In this study, we examined the membrane requirements for the post-translational modification of Raf-1. In addition, we took advantage of chimeric Raf-1 molecules that are targeted to specific membrane domains to determine the specificity of these domains for raft localization, phosphorylation, and constitutive activation of Raf-1. Understanding the molecular basis for Raf-1 activation by Ras may also help explain the actions of the related small G protein Rap1, which recruits Raf-1, but unlike Ras, cannot activate it. In the present study we address whether membrane localization also plays a role in Rap1-mediated inhibition of Raf-1 activation by Ras. We found that Raf-1 phosphorylation was intimately linked to proper membrane targeting and that the ability

of Ras and Rap1 to support Raf-1 phosphorylation dictated the biochemical actions of both Ras and Rap1 on Raf-1.

MATERIALS AND METHODS

Cell Culture, Transfections, and Stimulations--Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transfected using LipofectAMINE 2000 according to the manufacturer's recommendations. Unless otherwise noted, cells were transfected with a total of 10 μg of plasmid DNA, with pcDNA3.1 (vector) used to adjust DNA amounts where necessary. After 24 hrs, transfected cells were switched to low serum containing medium and incubated for a further 12 hrs. Cells were stimulated with 50ng/ml epidermal growth factor (EGF) for the indicated times. For cholesterol depletion experiments, serum starved cells were preincubated for 1 hour with 2% methyl-β-cyclodextrin (Sigma) in DMEM prior to stimulation with EGF.

Plasmids--All cDNAs were tagged at the amino terminus with a 2xFlag epitope (Kodak) by PCR and introduced into the BamH1 and XbaI sites of pcDNA3.1 vector (Invitrogen), unless otherwise indicated. Raf-1 and B-Raf mutations were introduced by PCR using the QuickChange site-directed mutagenesis kit (Stratagene). The minimal membrane targeting domain of Ha-Ras (-hvr) (Hancock et al., 1990) was added to the 3' end of FlagRaf-1 by PCR using a primer with Raf-1 sequences along with an in-frame XhoI site (adding amino acids L and E) and the last nine amino acids of Ha-Ras (CMSCKCVLS), a stop codon and a XbaI site. This construct is designated Raf-HaCAAX(-hvr). Raf-HaRasCAAX(+hvr), containing both the minimal membrane targeting domain and the hypervariable domain, was constructed using a primer with an in-frame XhoI site, the carboxy-terminal 27 amino acids of Ha-Ras (IRQHKLRKLNPPDESGPGCMSCKCVLS), a stop codon and a XbaI site by PCR. Raf-Rap1CAAX was constructed using the same strategy with the carboxy-terminal 24 amino acids of Rap1b (LVRQINRKTPVPGKARKKSSCQLL) introduced into the Xho1-Xba1 site of Raf-Ha-RasCAAX(+hvr). B-Raf-HaRasCAAX, B-Raf-Rap1CAAX chimeras were constructed using a similar strategy. RapE63-HaRasCAAX(+hvr) was constructed by adding an in frame XhoI site by PCR into RapE63 at base pair 480 and cloning into the EcoR1-XhoI sites of FlagRaf-Ha-RasCAAX, thereby replacing the Rap membrane

targeting motif with the Ha-Ras hypervariable domain and CAAX motif. The full-length coding sequences of human Ha-Ras, bovine Rap1b, RapE63-Ha-RasCAAX, Raf-Rap1CAAX, and Raf(Y341D)-Rap1CAAX were introduced in-frame into the pEGFP-C1 cloning vector (Clontech).

Western blotting and immunoblotting--Cos-7 cells were stimulated and lysates prepared as described. Protein concentrations were determined by using the Bio-Rad protein assay dye reagent according to manufacturer's recommendations. Equal amounts of lysate were immunoprecipitated with either Flag M2 antibody coupled to agarose (Sigma) or anti-Myc antibody (9E10) coupled to agarose (Santa Cruz Biotechnology) where indicated and examined by western blot as previously described (Carey et al., 2000). Samples were separated by SDS-PAGE and transferred to PVDF membrane. Expression of Flag and Myc-tagged proteins was detected using monoclonal Flag M2 antibody (Sigma) or monoclonal anti-Myc 9E10 antibody (Santa Cruz Biotechnology). Rabbit polyclonal anti-phospho MEK1/2 antibody (Cell Signaling Technology) was used to detect activated GST-MEK1 (Upstate Biotechnology). Polyclonal anti-phospho ERK1/2 (Cell Signaling Technology) was used to detect activated MycERK2 (pMycERK2). Phosphorylation of Raf-1 at Ser338 or at Tyr341 was detected using anti-phosphoRaf-1 Ser338 or phosphotyrosine (pTyr, 4G10) (Upstate Biotechnology, Inc.). All experiments were repeated at least three times and representative blots shown.

Cell Fractionation--Cos-7 cells were washed twice in phosphate buffered saline (PBS) before scrapping into 0.5 ml of hypotonic lysis buffer (10 mM Tris pH 7.5, 5 mM Mg_2Cl, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium orthovanadate, and 1 mM PMSF). After 10 min., cells were homogenized at 4° C by 50 strokes in a tight-fitting Dounce homogenizer. Nuclei and unbroken cells were pelleted by centrifugation at 1500x g. Supernatants were then centrifuged at 100,000x g in a Beckman TLA 45 rotor at 4° C for 30 min. The supernatant was collected and designated the cytosolic fraction (S100) and the pellet was resuspended in 250 μl hypotonic lysis buffer and designated the membrane (P100) fraction.

Sucrose gradients--Transfected Cos-7 cells ($2x10^6$ cells) were rinsed twice in PBS and scrapped into 0.5 ml MES buffer (25 mM MES pH 6.5, 10 mM NaCl, 5 mM Mg_2Cl, 10 μg/ml aprotinin, 10 mg/ml leupeptin, 25 mM NaF, 25 mM β-glycerophosphate, 5 mM sodium orthovanadate). Cells were homogenized at 4° C by 30 passes through a 23 gauge syringe and sonicated on ice for 30 seconds at setting 2, 30 seconds at setting 3, and 30 seconds at setting 4 (Sonic Dismembrater, Fisher). The lysate was mixed with 0.5 ml of 90% sucrose in MES buffer and placed at the bottom of a 2.4 ml Beckman ultracentrifuge tube. The gradient was constructed by overlaying the 45% sucrose/lysate mixture with 1.2 ml of 35 % sucrose, 1 ml of 30% sucrose, 1 ml of 25% sucrose and 1 ml of 5% sucrose. The tubes were centrifuged at 4° C in a Beckman SW 55 rotor for 16 hrs at 48,000 rpm. Ten 0.4 ml fractions were collected from the top of the gradient and diluted with 1 ml MES buffer and the diluted fractions were re-centrifuged at 100,000 x g for 45 min. in a TLA 45 rotor. Pellets were resuspended in Laemmli buffer, separated by SDS-PAGE, and transferred to PVDF membrane for analysis by immunoblotting. Similar results were obtained for the Raf membrane targeted chimeras using the sodium carbonate method of raft preparation (Prior et al., 2001).

Immunofluorescence--Cos-7 cells were grown on glass coverslips and transfected with 100 ng of FlagRaf cDNAs using LipofectAMINE 2000. Cells were fixed in PBS containing 4% formaldehyde, permeablized in PBS containing 0.1%Triton X-100, and blocked with PBS/5% horse serum. Localization of transfected proteins was detected with a 1:2000 dilution of Flag M2 antibody (Sigma) in 5% horse serum/0.01% Tween followed by a 1:10,000 dilution of an anti-mouse-FITC conjugate in 5% horse serum/0.01%Tween. Cells were visualized using a Zeiss Axioplan 2 microscope.

Raf-1 kinase assays--Cos-7 cells were transfected, cells lysed and lysates prepared as described (Chong et al., 2001). FlagRaf proteins were immunoprecipitated from 500 μg of cell lysate with 30 μl of Flag M2 agarose (Sigma) at 4° C for 6 hrs. Immune complexes were washed twice with lysis buffer and once with kinase assay buffer (20 mM MOPS pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 μg/ml aprotinin). Pellets were resuspended in 40 μl of kinase assay buffer with 1.5 mM Mg_2Cl and 7.5 μM ATP along with 0.4 μg GST-MEK1 (Upstate Biotechnology) and the reaction was incubated for 30 min. at 30° C. The kinase reaction was terminated by adding 45 μl of 2x Laemmli buffer, boiled for 5 min., resolved by SDS-PAGE and transferred to PVDF membrane. Raf-1 activity was evaluated by immunoblotting with anti-phospho MEK1/2 antibody (Cell Signaling Technology).

PhosphoMycERK2 Assay--For MycERK2 assays, treated and untreated cells were lysed in ERK assay buffer and activation of MycErk2 was detected as described previously (Carey et al., 2000).

RESULTS

Raf-1 activation and S338 phosphorylation require intact raft microdomains. It has long been appreciated that Raf-1 recruitment to Ras is insufficient by itself to trigger Raf-1 activation and that additional post-translational modifications are required (Mason et al., 1999). The two best-studied modifications are serine phosphorylations on serine 338 and tyrosine 341. Figure 3.1A demonstrates this requirement. Constitutively active Ras (RasV12) activated wild type Raf-1 (Raf-1 WT), as measured by *in vitro* Raf-1 assay. However, Raf-1 that was mutated at either S338 to alanine (RafS338A) or Y341 to alanine (RafY341A) could no longer be activated by RasV12 (Figure 3.1A).

Activation of Raf-1 by Ras-dependent signals is associated with a redistribution of Raf-1 from the cytoplasm to the cell membrane, where it associates with Ras (Morrison and R. E. Cutler, 1997). Recent studies suggest that Ras may be localized to specific cholesterol-rich membrane microdomains called rafts (Kranenburg et al., 2001), and upon Ras activation Raf-1 may also be recruited to rafts (Rizzo et al., 2001). The localization of Ras proteins to rafts is not completely understood and some controversies remain (White and Anderson, 2001), with some groups showing that Ha-Ras, but not Ki-Ras, requires raft localization for full activity (Carozzi et al., 2002; Roy et al., 1999). Proteins that localize to cholesterol-rich microdomains (rafts) can be detected within low density fractions of sucrose density gradients (Brown and London, 1998; Chen and Resh, 2001; Prior et al., 2001). One of these proteins, caveolin-1, was used to identify the general density of these cholesterol-rich raft domains (Figure 3.1B, top panel) (Anderson, 1998). Using this technique, we show endogenous Raf-1 was excluded from raft microdomains in untreated cells (Figure 3.1B, second panel) and EGF treatment induced the redistribution of endogenous Raf-1 into raft domains (Figure 3.1B, third panel). A significant fraction of Raf-1 protein was also detected at higher densities within the gradient. Recruitment of transfected wild type Raf-1 to raft microdomains was also growth factor dependent (Figure 3.1C, top and second panel). Raft domains can be disrupted by the cholesterol-depleting agent methyl-β-cyclodextrin (CD) (Kranenburg et al., 2001). In the presence of CD, EGF recruitment of Raf-1 to low density fractions was

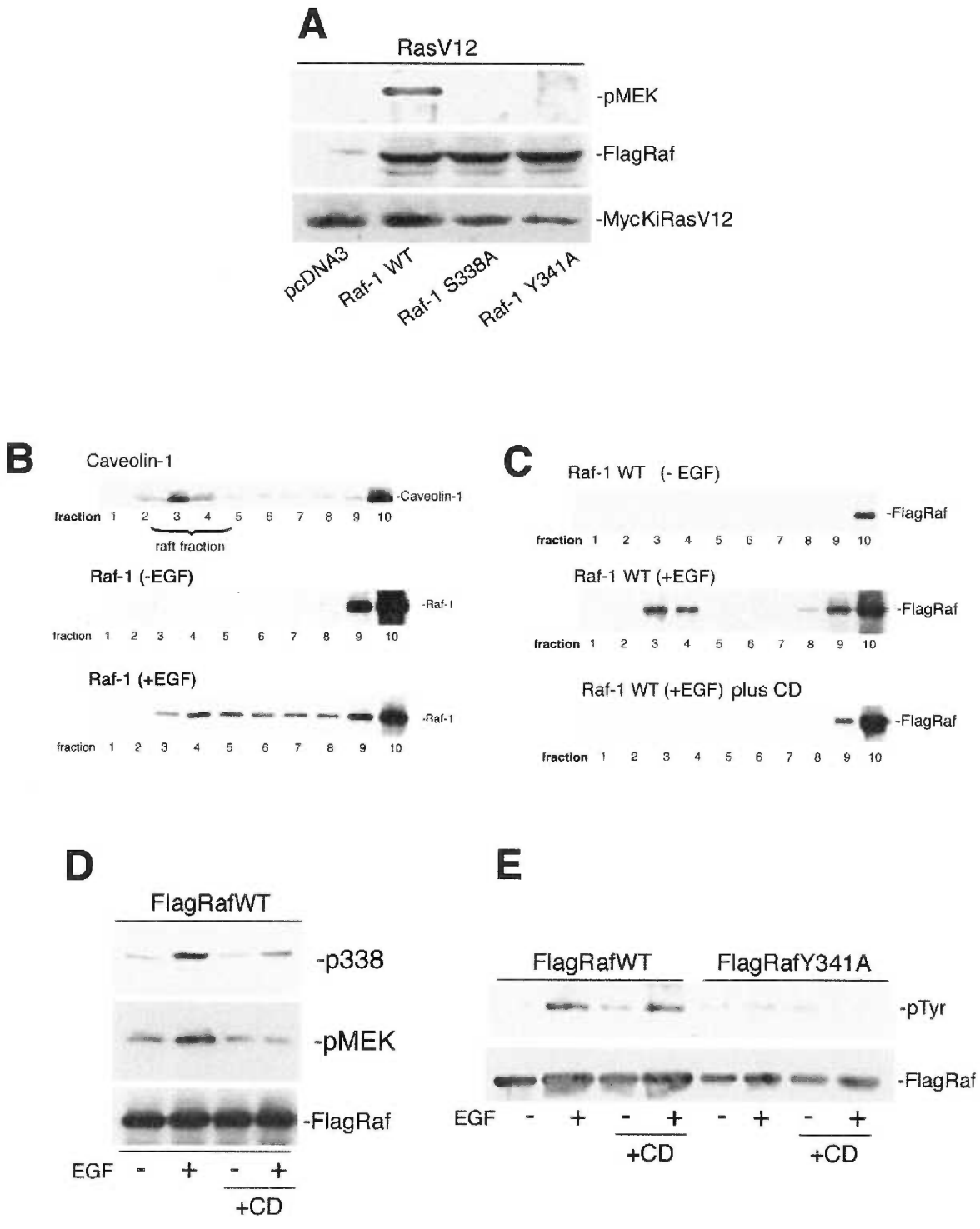

Figure 3.1 S338 phosphorylation and activation requires intact raft domains

FIG. 1. S338 phosphorylation and activation requires intact raft domains.
(A) Raf-1 kinase assays. RasV12 and either Flag-tagged Raf-1, RafS338, RafY341A, or pcDNA3 vector was transfected into Cos-7 cells. Equivalent amounts of Raf protein were immunoprecipitated using the Flag antibody (Flag I.P.) and assayed for the ability to phosphorylate MEK (pMEK) *in vitro* (top panel). The levels of FlagRaf (middle panel) and MycRasV12 (lower panel) are shown. (*B*) Raf-1 is recruited to lipid raft membrane microdomains. Cells were treated with or without EGF as indicated, and membrane proteins separated into the indicated fractions by sucrose density gradients. The position of caveolin-1 within the gradient is shown in the upper panel using calveolin-1 antisera. The presence of endogenous Raf-1 within each gradient is shown in the lower two panels, using an anti-Raf-1 antibody. (*C*) Raf-1 recruitment to low density sucrose fractions is cholesterol dependent. Cells were transfected with Flag-tagged Raf-1 WT, treated with or without EGF or CD as indicated, and membrane proteins separated into the indicated fractions by sucrose density gradients. The presence of FlagRaf within each gradient was detected using Flag antibody. (*D*) Cells were transfected with Flag Raf-1 wild type (WT) and treated with EGF and/or CD as indicated. Lysates were subjected to Flag I.P. and assayed for p338 (upper panel) and the ability to phosphorylate MEK (pMEK) *in vitro* (middle panel). The levels of FlagRaf-1 expression are shown in the lower panel, using Flag antibody. (*E*) Cells were transfected with Flag Raf-1 wild type (WT) or Raf-Y341A, and treated with EGF and/or CD as indicated. Lysates were subjected to Flag I.P. and assayed for tyrosine phosphorylation with pTyr antibody (pTyr) (upper panel). The levels of FlagRaf-1 expression are shown in the lower panel, using Flag antibody.

inhibited (Figure 3.1B, fourth panel), confirming that these low density fractions represented cholesterol-rich membrane microdomains.

Raft microdomains were required for phosphorylation on S338, since CD inhibited EGF-induced phosphorylation of Raf-1 on S338 (p338) and Raf-1 activation (Figure 3.1D). These data suggest that targeting to raft domains was required for full Raf-1 activation and phosphorylation at S338. In contrast, tyrosine phosphorylation of Raf-1 WT was not affected by CD (Figure 3.1E, upper panel, lanes 1-4). The absence of phosphorylation of Y341 in Raf-Y341A is provided as a negative control (Figure 3.1E), upper panel, lanes 5-8).

Y341 is required for targeting to raft microdomains and phosphorylation of S338. We next examined the requirement of S338 and Y341 in Raf-1 localization. EGF was able to direct RafS338A into rafts (Figure 3.2A, upper panel), but was not able to direct RafY341A into rafts (Figure 3.2A, lower panel). Neither Raf mutant RafS338A nor RafY341A entered rafts in the absence of EGF stimulation (data not shown). This suggests that phosphorylation of Y341 participates in localization of Raf-1 to lipid rafts, while S338 phosphorylation occurs once proper localization has been achieved. In Figure 2B, we show that the tyrosine at residue 341 was essential for EGF and RasV12 to phosphorylate S338 (Figure 3.2B, upper panel) and activate Raf-1 (Figure 3.2B, middle panel). Mimicking phosphorylation of Y341 by replacing tyrosine with aspartate in the mutant Raf Y341D did not effect EGF's actions (Figure 3.2B).

The requirement of Y341 phosphorylation for Raf-1 activation can be overcome by targeting to raft domains--Raf-1 can be constitutively targeted to the membrane following the attachment of a Kirsten Ras carboxy-terminal domain (Raf-KiCAAX) (Leevers et al., 1994; Stokoe et al., 1994). Here, we examined the localization of a related chimera created by fusing the Raf-1 protein to the carboxyl 27 amino acids of

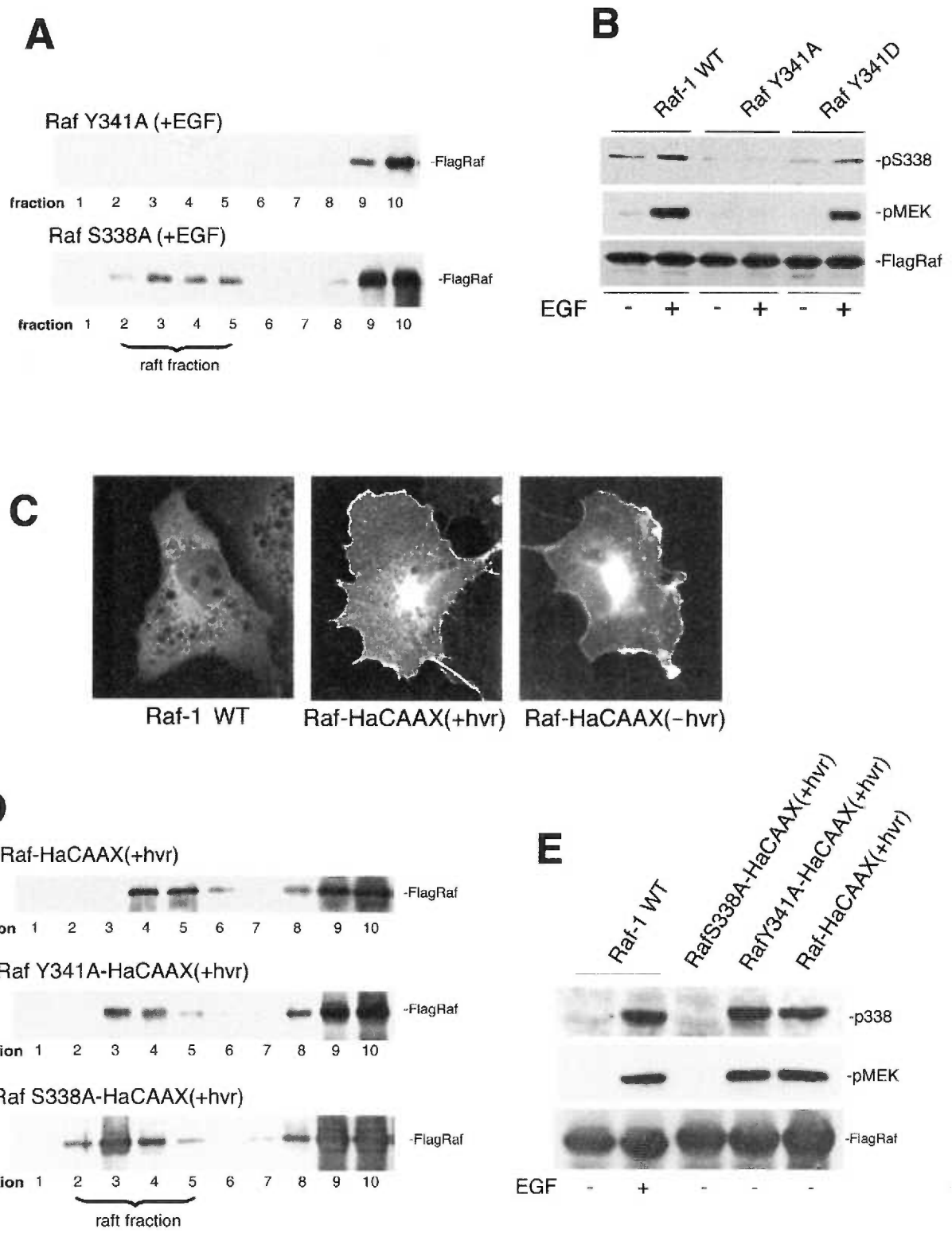

Figure 3.2 The requirement for Y341 can be overcome by targeting Raf-1 to raft domains

FIG. 2. The requirement for Y341 can be overcome by targeting Raf-1 to raft domains. (*A*) Sucrose density gradients of membrane fractions containing Raf-1 mutants. Cells were transfected with Flag-tagged RafY341A, and RafS338A, treated with EGF as indicated, and membrane proteins separated into the indicated fractions by sucrose density gradients, as in Fig. 1*B*. The presence of FlagRaf constructs within each gradient is shown in both panels, using Flag antibody. (*B*) Phosphorylation and activation of Raf-1 mutants by EGF. Flag-tagged Raf-1, RafY341A, or RafY341D were transfected into Cos-7 cells, and cells were either treated with EGF. Lysates were subjected to Flag I.P. and assayed for p338 (upper panel) and the ability to phosphorylate MEK (pMEK) *in vitro* (middle panel). The levels of FlagRaf proteins are shown in the lower panel, using Flag antibody. (*C*) Immunofluorescence of Raf-Ras chimeras. Cos-7 cells were transfected with Flag-tagged Raf-1 WT, Raf-HaCAAX(+hvr), and Raf-HaCAAX(-hvr), as indicated. Cells were prepared for epifluorescent microscopy as described in Experimental Procedures, and representative cells are shown. (*D*) Sucrose density gradients of Raf-Ras chimeras. Cells were transfected with Flag-tagged Raf-HaCAAX(+hvr), Raf Y341AHaCAAX(+hvr), and Raf S338AHaCAAX(+hvr), and left untreated. Membrane proteins were separated as in Fig. 1B and the presence of the chimera within each fraction is shown, using Flag antibody. (*E*) Lack of requirement of Y341 for S338 phosphorylation in targeted chimeras. Cells were transfected with Raf-1 WT, Raf-HaCAAX(+hvr), RafS338A-HaCAAX(+hvr), or RafY341AHaCAAX(+hvr) as indicated, and assayed for p338 (upper panel), or Raf-1 kinase activity (pMEK, middle panel). The levels of Flag-containing proteins are shown in the lower panel.

HaRas including both the CAAX domain and the hypervariable domain; Raf-HaCAAX(+hvr) (Jaumot et al., 2001). As expected, wild type Raf-1 was located within the cytoplasm of resting cells (Figure 3.2C, left panel), and the chimera Raf-HaCAAX(+hvr) was present on the plasma membrane (Figure 3.2C, middle panel). The chimera Raf-HaCAAX(-hvr) that lacked hvr sequences was also present on the plasma membrane (Figure 3.2C, right panel).

Raf-HaCAAX(+hvr) is constitutively localized within raft domains (Figure 3.2D, upper panel). Unlike Raf Y341A, the introduction of Y341A into Raf-HaCAAX(+hvr) (RafY341A-HaCAAX(+hvr)) did not prevent raft localization (Figure 3.2D, middle panel), constitutive phosphorylation of S338 or activation of Raf-1 (Figure 3.2E). These data demonstrate that phosphorylation of Y341 is required for raft localization, S338 phosphorylation and activation of wild type Raf-1, but that Y341 phosphorylation is not required if Raf-1 is constitutively targeted to rafts. In contrast, mutating S338 to alanine (A), in the chimera RafS338A-HaCAAX(+hvr), completely abolished activation of Raf-1 (Figure 3.2E), without affecting raft localization (Figure 3.2D, lower panel), demonstrating the requirement of pS338 for kinase activation, but not raft localization.

In addition to the CAAX domain, Ha-Ras contains a hypervariable (hvr) region that influences specific membrane localization (Prior et al., 2001). Here we examined chimeras either containing the Ha-Ras hypervariable region (+hvr) or lacking these sequences (-hvr). Like Raf-HaCAAX(+hvr), Raf-HaCAAX(-hvr) was present within the particulate (P100) fraction (Figure 3.3A), and was detected on the plasma membrane (Figure 3.2C, right panel). Unlike Raf-HaCAAX(+hvr), Raf-HaCAAX(-hvr) was excluded from low density gradient fractions (Figure 3.3B, upper panel), suggesting that despite its membrane localization, the chimera was targeted differently than Raf-HaCAAX(+hvr). Raf-HaCAAX(+hvr) gradients were included as a control (Figure 3.3B, lower panel).

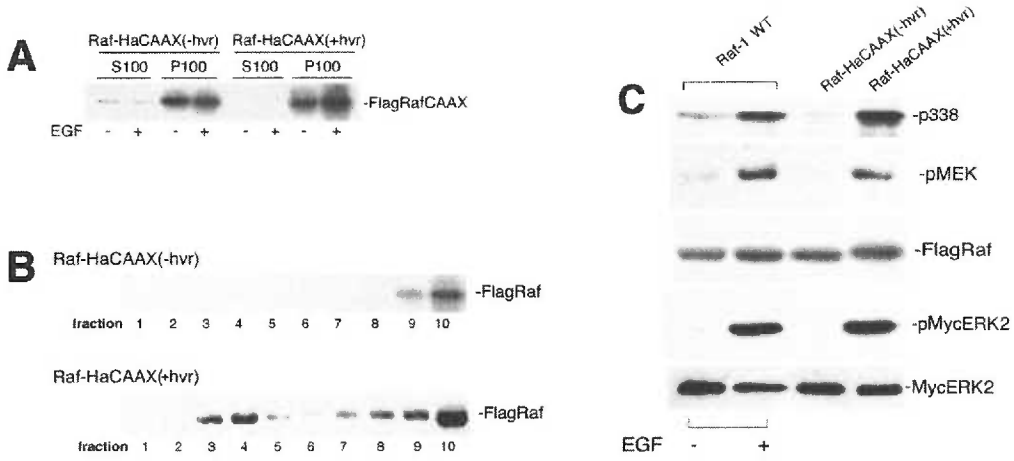

Figure 3.3 Ectopic Targeting of Raf-1 to raft domains is required for constitutive S338 phosphorylation, activation of Raf-1, and activation of Erks. (A) Localization of Raf-Ras chimeras. Cells were transfected with Flag-tagged Raf-1 WT and the Raf-Ras chimeras as indicated and fractionated into S100 and P100 fractions, and Flag-containing proteins detected by western blot. (B) Sucrose density gradients of Raf-HaCAAX(-hvr). Cells were transfected with Flag-tagged Raf-HaCAAX(-hvr), and left untreated. Membrane proteins were separated as in Fig. 1B and the presence of the chimera within each fraction is shown (upper panel), using Flag antibody. Gradients of lysates expressing Raf-HaCAAX(+hvr) are shown as a control (lower panel). (C) Raft-targeted chimeras show constitutive activity. Cells were transfected with Myc-ERK2 and individual Raf-1 chimeras. Cells transfected with wild-type Raf-1 were also treated with EGF as indicated. Flag-containing proteins were recovered by I.P. and examined for S338 phosphorylation (p338, first panel), and phosphorylation of MEK in vitro, as in Fig. 1B (pMEK, second panel). The position of Flag Raf proteins is shown in the third panel (FlagRaf). In the lower two panels, the lysates were subjected to Myc I.P. and the recovered MycERK2 examined for phosphorylation (pMycERK) or total MycERK levels (MycERK)

Only Raf-HaCAAX(+hvr), but not Raf-HaCAAX(-hvr) displayed constitutive phosphorylation of S338 (Figure 3.3C, p338, first panel), and Raf-HaCAAX(+hvr), but not Raf-HaCAAX(-hvr), was active in Raf-1 kinase assays *in vitro* (Figure 3.3C, pMEK, second panel) and ERK activation assays *in vivo* (Figure 3.3C, pMycERK2, fourth panel). As a control, EGF stimulation of wild type Raf-1 was included, which resulted in phosphorylation of S338 and activation of both Raf-1 and ERK. High levels of activation of Raf-HaCAAX(+hvr), but not Raf-HaCAAX(-hvr), were also seen in coupled *in vitro* kinase assays (data not shown). Therefore, both S338 phosphorylation and biochemical activity of these chimeras paralleled raft localization.

Y341D restored raft localization and activity to Raf-HaCAAX(-hvr)--For Raf-HaCAAX(-hvr), membrane targeting was not sufficient to trigger S338 phosphorylation and Raf-1 activity. Since Y341 phosphorylation potentiates the localization of Raf-1 within rafts, which may be required for subsequent S338 phosphorylation, we tested whether the Y341D mutation could restore S338 phosphorylation in the HaCAAX(-hvr) chimera. Indeed, this mutant, RafY341D-HaCAAX(-hvr), but not RafS338D-HaCAAX(-hvr), was capable of entering raft domains (Figure 3.4A). Moreover, RafY341D-HaCAAX(-hvr) was phosphorylated on S338 and showed constitutive activity to levels similar to those seen with Raf-HaCAAX(+hvr) (Figure 3.4B). Furthermore, the introduction of Y341D into Raf-HaCAAX(+hvr) did not significantly increase Raf-1 activity (Figure 3.4B,C). These data suggest that one of the functions of Y341 is to localize Raf-1 to specific membrane microdomains permitting efficient phosphorylation of S338 and coupling to downstream effectors.

Rap1 is unable to activate Raf-1 because it can not induce Y341 phosphorylation—The ability to recruit Raf-1 to the membrane is not sufficient for full activation of Raf-1 (Mineo et al., 1997). Another small G protein that can associate with Raf-1 in an activation-dependent manner is Rap1. Upon GTP loading, activated Rap1 (RapE63) binds to Raf-1 but cannot activate it (Schmitt and Stork, 2001). Unlike Ras, which is located at the plasma membrane, Rap1 is located in vesicular membranes (Mochizuki et al., 2001; Pizon et al., 1994; York et al., 2000). This localization is

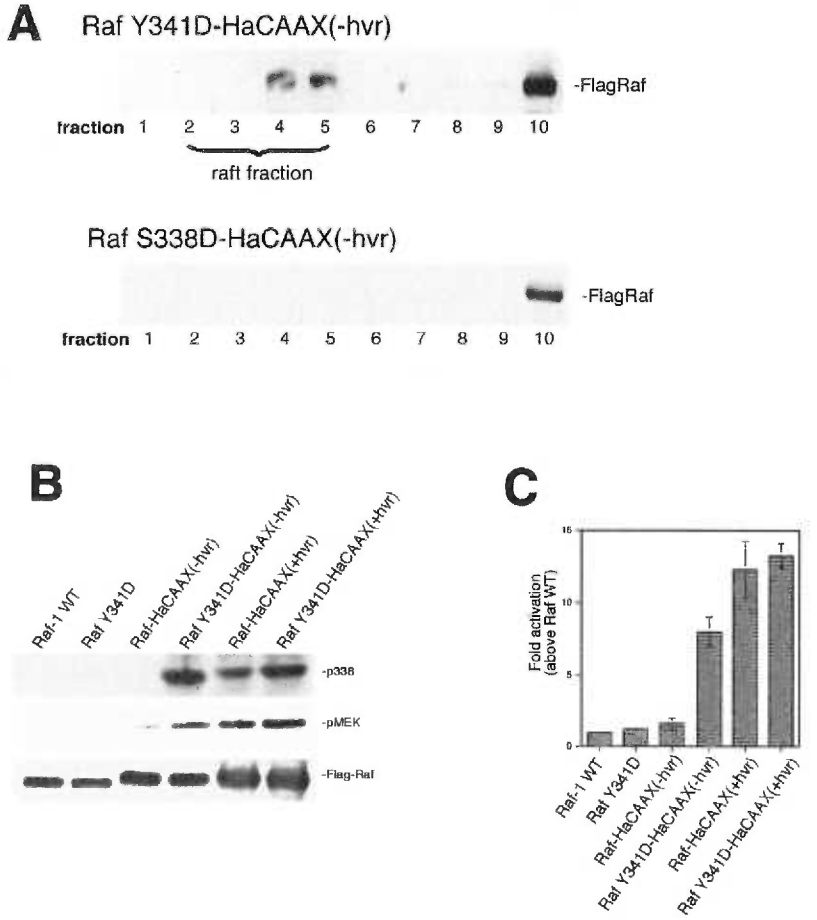

Figure 3.4: Mutation of Y341D restores phosphorylation of S338, activation and raft localization of inactive Raf-HaRasCAAX(-hvr) chimeras. (A) Raft localization of RafY341DHaCAAX(-hvr). Cells were transfected with RafY341DHaCAAX(-hvr) and membranes fractionated as in Fig. 1B (upper panel). RafS338D-HaCAAX(-hvr) is shown as a control (lower panel). The presence of Flag-containing proteins within each fraction is shown. (B) S338 phosphorylation, and Raf-1 activity assays. Cells were transfected with either Flag-tagged Raf-HaCAAX(-hvr), RafY341DHaCAAX(-hvr), Raf-HaCAAX(+hvr) or RafY341DHaCAAX(+hvr) and immunoprecipitated using Flag antibody and assayed for S338 phosphorylation (upper panel) and the ability to phosphorylate MEK (pMEK) in vitro (middle panel). The levels of FlagRaf-1 expression are shown in the lower panel, using Flag antibody. (C) Raf-1 assays from three independent experiments, as in Fig. 4B. The data are shown as fold activation above that seen for Raf-1 WT, with standard error.

directed by carboxy-terminal sequences of Rap1 that contain a distinct CAAX motif that regulate the attachment of geranyl modifications that direct Rap1 to vesicular membranes (Cox et al., 1992; Zacharias et al., 2002). This could be shown using green fluorescent protein (GFP) fusions to the RapE63 protein (GFP-Rap) which, unlike GFP alone (Figure 3.5A, a), was localized to perinuclear vesicles within the cytoplasm (Figure 3.5A, b). In contrast, GFP-HaRasV12 was detected at the plasma membrane, consistent with recent reports (Chiu et al., 2002) (Figure 3.5A, c). The chimera GFP-RapE63-HaRasCAAX was also present on the plasma membrane, confirming that the HaRasCAAX could redirect ectopic proteins (Figure 3.5A, d).

Activated Rap1 does not activate Raf1 (Okada et al., 1999). Rap1's inability to permit S338 phosphorylation and activation of Raf-1 could be partially overcome by swapping Rap1's CAAX domain with that of Ras (RapE63/HaRasCAAX) (Figure 3.5B). Although RapE63 could not activate wild type Raf-1, it could activate RafY341D, as measured by S338 phosphorylation and kinase activation (Figure 3.5C). These data suggest that the inability of RapE63 to activate Raf-1 was due to the inability of Raf-1 to be correctly phosphorylated when recruited by Rap1, since Rap1 was capable of supporting S338 phosphorylation in the Y341D mutation. These data also suggest that the inability of Rap1 to activate Raf-1 is not just a consequence of the interaction between Rap1 and Raf-1, as has been proposed (Okada et al., 1999), but may also be dictated by the localization of Rap1. However, HaRasV12 was better than RapE63-HaCAAX(+hvr) in activating both Raf-1 and RafY341D (Figure 3.5B,C), suggesting that sequences within Ras distinct from the carboxy-terminal membrane-targeting domain are critical for maximal activation of Raf-1.

To examine the effect of relocalizing Raf-1 to Rap1-containing membranes, we generated chimeras of Raf-1 fused to the Rap1 carboxy-terminal CAAX motif (Raf-Rap1CAAX) (Figure 3.5D). Raf-Rap1CAAX was not constitutively active, and could not be activated (Figure 3.5D, middle panel) or phosphorylated on S338 by EGF (Figure 3.5D, upper panel). These data suggest that Raf-1 needs to be targeted to specific

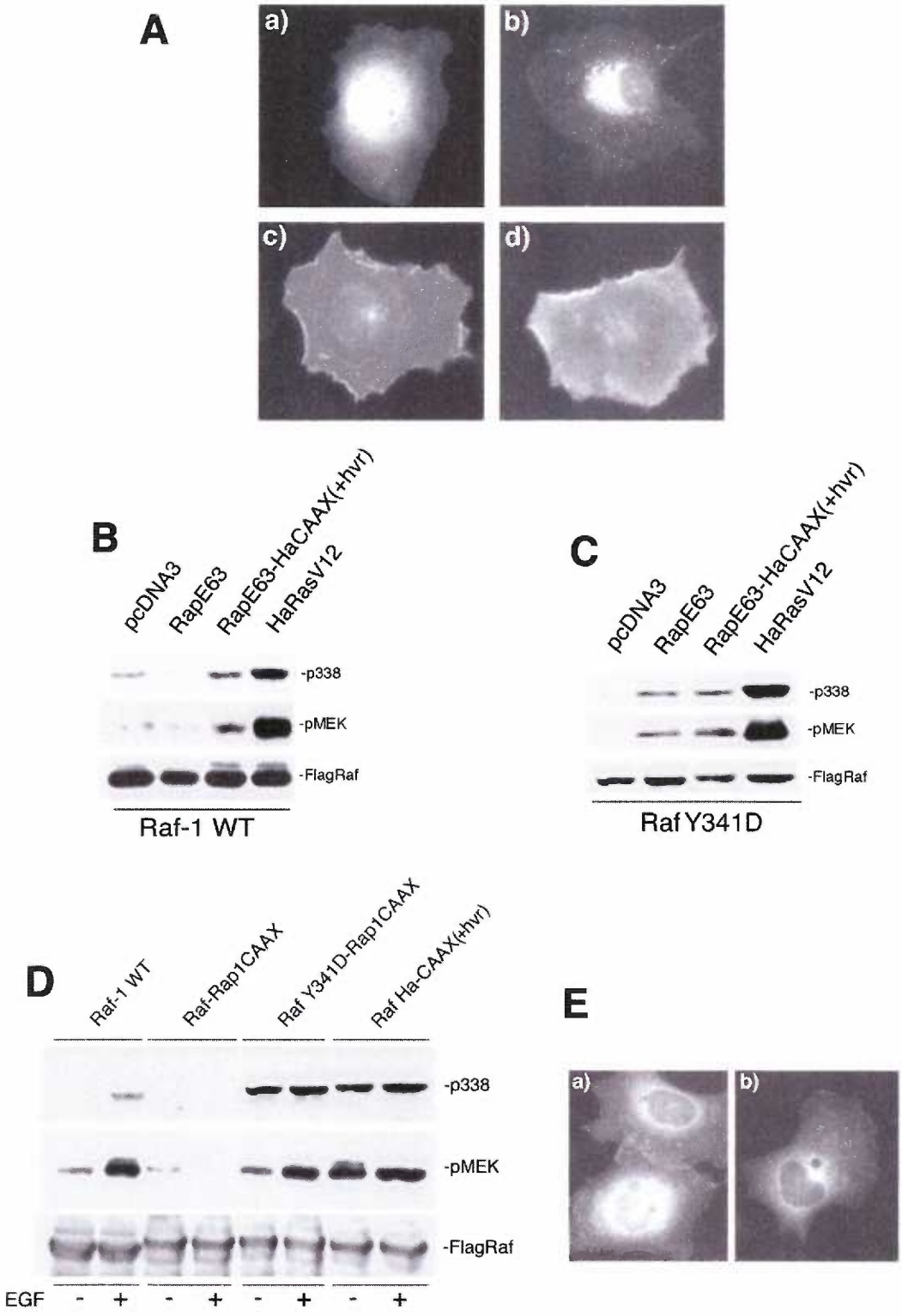

Figure 3.5 The inability of Rap1 to activate Raf-1 is due to the inability of Rap1 to induce S338 phosphorylation.

FIG. 5. The inability of Rap1 to activate Raf-1 is due to the inability of Rap1 to induce S338 phosphorylation. (*A*) GFP epifluorescence. Cells were transfected with GFP (a), GFP-RapE63 (b), GFP-RasV12 (c), and GFP-RapE63-HaRas-CAAX (d). The locations of the transfected proteins were examined by epifluoresent microscopy, and representative cells shown. (*B*) Rap1 supports neither Raf-1 activation nor S338 phosphorylation. Cells were transfected with Raf-1 WT along with pcDNA3, RapE63, RapE63-HaCAAX(+hvr) or HaRasV12 and examined for S338 phosphorylation (p338) and Raf-1 activity (pMEK). (*C*) S338 phosphorylation and activation of RafY341D by Rap1. Cells were transfected with RafY341D along with either pcDNA3, RapE63, RapE63-HaCAAX(+hvr) or HaRasV12 and examined for S338 phosphorylation (p338) and kinase activity of Raf-1 (pMEK) as in Fig. 5*B*. (*D*) Raf-Rap1 chimeras are not constitutively active. Cells were transfected with either Flag Raf-1 wild type (WT), Raf-Rap1CAAX, RafY341D-Rap1CAAX or Raf-HaCAAX(+hvr), and immunoprecipitated using Flag antibody and assayed for both S338 phosphorylation (upper panel) and Raf-1 activity *in vitro* (middle panel). The levels of FlagRaf expression are shown in the lower panel, using Flag antibody. (*E*) GFP epifluorescence. Cells were transfected with GFP (a), GFP-Raf-Rap1CAAX (a) or GFP-RafY341-Rap1CAAX (b). The locations of the transfected proteins were examined by epifluoresent microscopy, and representative cells are shown.

membranes in order to be activated and that Raf-1 targeting to Rap1-specific membrane domains does not support S338 phosphorylation or activation. The inability of Rap1 to direct the proper phosphorylation of Raf-1/Rap chimeras could be overcome by introducing negative charges into Raf-1 at Y341. Mutation of Y341 to aspartate to generate RafY341D-RapCAAX increased the basal levels of both phosphorylation of S338 and Raf-1 activation compared to Raf-Rap1CAAX (Figure 3.5D), and these werenot further increased by EGF. In Figure 3.5E, we show the subcellular localization of GFP-fusion proteins, GFP-Raf-Rap1CAAX (Figure 3.5E,a), GFP-RafY341-Rap1CAAX (Figure 3.5E,b). Both chimeras are largely localized to perinuclear regions, with little or no staining detected at the cell surface.

Rap1 activation of B-Raf requires aspartic acid at residues D447/D448--The Y341D mutation in Raf-1 resembles the naturally occurring sequences within the Raf isoform, B-Raf. B-Raf lacks tyrosines at the site corresponding to Y341 in Raf-1 (448 in B-Raf) . Instead, it contains aspartic acids at residues 447 and 448, that appear to mimic phosphorylation at these sites (Mason et al., 1999). Moreover, B-Raf was phosphorylated at the serine corresponding to S338 in Raf-1 (S445 in B-Raf) in resting cells (Figure 3.6A, lane 1, upper panel), and remained phosphorylated at this residue in the presence of the raft-disrupting agent, CD (data not shown).

Unlike Raf-1, B-Raf can be activated by the small G protein Rap1 (Okada et al., 1999). Because of this, Rap1 can activate MEK and ERK in B-Raf-expressing cells (Dugan et al., 1999; Vossler et al., 1997; Wan and Huang, 1998). The ability of Rap1 to activate B-Raf is shown in Figure 3.6A. Both constitutively active mutants of Ras (RasV12) and Rap1 (RapE63) could activate wild type B-Raf (B-RafWT) (Figure 3.6A). In contrast to B-RafWT, expression of a mutant B-Raf where the aspartic acid residues were mutated to the corresponding tyrosines residues in Raf-1 (B-RafYY) showed no basal phosphorylation on S445 and was no longer activated by constitutively active Rap1

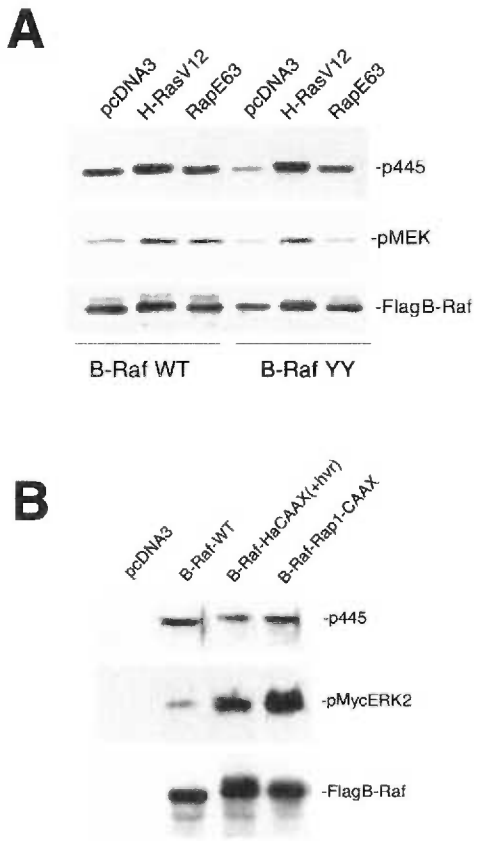

Figure 3.6: The ability of B-raf to be activated by Rap1 requires aspartic acid residues at positions D447/D448 in B-Raf. (A) p445 phosphorylation and B-Raf activity assays. Cells were transfected with FlagB-Raf wild-type (B-RafWT) or FlagB-RafYY (B-RafYY) along with either pcDNA3.1, HaRasV12, or RapE63, as indicated. Lysates were subjected to Flag I.P. and assayed for both p338 (upper panel) and Raf-1 activity in vitro (pMEK, middle panel). The levels of FlagB-Raf expression are shown in the lower panel, using Flag antibody. (B) ERK activation by B-Raf requires targeting to either Rap1- or Ras-containing membranes. Cells were transfected with MycERK2 and either FlagB-Raf wild-type (WT), B-Raf-HaCAAX(+hvr), or B-Raf-Rap1CAAX and immunoprecipitated using Flag antibody and assayed for S445 phosphorylation (p445, upper panel). In the middle panel (pMycERK2), the total lysates were assayed for phosphorylation of MycERK2. The levels of FlagB-Raf expression are shown in the lower panel, using Flag antibody.

(RapE63), as measured by both S445 phosphorylation and kinase activity (Figure 3.6A). HaRasV12 stimulated S445 phosphorylation and activity of the B-RafYY mutant. When Rap1-CAAX sequences were coupled to B-Raf, the resulting chimera, B-Raf-Rap1CAAX, was phosphorylated on p445 and activated ERKs to a similar degree as B-Raf-HaCAAX(+hvr) (Figure 3.6A). Although wild type B-Raf was constitutively phosphorylated on S445 and displayed detectable constitutive kinase activity against MEK *in vitro* (Figure 3.6A), it could not activate ERKs unless it was targeted to Ras or Rap1 (Figure 3.6B), reflecting the requirement of specific membrane targeting for B-Raf's activation of MEK/ERK *in vivo*. Therefore, we propose that B-Raf's ability to mimic phosphorylation at residues 447 and 448 is critical for its ability to be activated by Rap1.

DISCUSSION

S338 phosphorylation of Raf-1 requires targeting to raft microdomains--Recent studies suggest that the ability of small G proteins to regulate signaling cascades is dictated not only by the specificity of effector utilization, but also by their subcellular localization (Prior and Hancock, 2001). Differences in the localization of specific Ras isoforms within rafts has been reported by some (Prior and Hancock, 2001), but not others (Kranenburg et al., 2001). Paradoxically, disruption of rafts by CD can completely inhibit coupling to downstream effectors, while actually increasing the GTP loading of selected Ras isoforms (Kranenburg et al., 2001). This may reflect the need for selected Ras isoforms to shuttle in and out of the raft (Niv et al., 2002; Prior et al., 2001). In this study, we focused our attention on the requirement of raft localization not on Ras activation but activation of the proximal downstream effector Raf-1. We show that one of the functions of raft localization is that it permits phosphorylation of Raf-1 on S338.

Localization of Raf-1 to rafts appears to be required for full activation of ERKs (Rizzo et al., 2001). Using the cholesterol-depleting agent Methyl-β-cyclodextrin (CD), we and others (Kranenburg et al., 2001; Liu et al., 1996) have shown that disruption of raft microdomains interferes with signaling of Raf-1 to ERKs. Raf-1 activation also requires phosphorylation at serine 338. This activating phosphorylation occurs within the plasma membrane for Raf-1, subsequent to Raf-1's recruitment to Ras (Mason et al., 1999). Using CD to disrupt rafts, we show that intact rafts are required for proper phosphorylation at 338. Therefore, the requirement of raft localization for full Raf-1 activity is coupled to S338 phosphorylation, extending previous studies showing that the membrane-localized S338 kinase was required for Raf-1 activation by oncogenic Ras (Diaz et al., 1997). A candidate kinase, PAK, has been proposed (King et al., 2001; Sun et al., 2000; Zang et al., 2002), however, its role has been challenged (Chiloeches et al., 2001).

In this study we compared the ability of distinct CAAX motifs to potentiate the phosphorylation and activation of a variety of chimericRaf-1/CAAX proteins whose

carboxyl-terminal domains were derived from Ha-Ras or Rap1. We show that a Raf-1 chimera that included the complete carboxyl-terminal membrane targeting domains from Ha-Ras was localized to rafts, showed both constitutive activity and phosphorylation of S338, and activated ERKs. Chimeras containing only the minimal membrane-targeting motif [Raf-HaCAAX(-hvr)], however, had no basal activity. We suggest that the lack of activity of this chimera was a direct consequence of its inability to be phosphorylated on serine 338. The ability of Y341D to restore the raft localization and S338 phosphorylation of Raf-1-HaCAAX(-hvr) and kinase activity argues that localization to specific raft microdomains may be necessary and sufficient for S338 phosphorylation and activation of Raf-1. Recent studies have proposed that hvr sequences help shuttle HaRas out of the rafts in a GTP-dependent fashion (Prior et al., 2001) and cooperate in effector utilization (Jaumot et al., 2001). Differences in the localization of mutant Ha-Ras proteins and Raf/Ras chimeras may be due to the influence of sequences in Ras mutants that are absent from the Raf/Ras chimeras. It is also possible that activated Ras shuttles Raf-1 into the raft where it is phosphorylated on S338 and subsequently exits the raft, as suggested by recent studies (Niv et al., 2002; Prior et al., 2001).

Phosphorylation of Y341 is required for proper raft localization and subsequent phosphorylation of S338—Upon EGF stimulation, RafS338A was localized to a raft microdomain. Moreover, RafS338A-HaCAAX(+hvr) was constitutively localized to a raft domain. These data demonstrate that S338 phosphorylation was not required for raft localization, but likely occurs subsequently. This is consistent with a model that Raf-1 activation by Ha-Ras requires post-translational modifications, including S338 phosphorylation that occur within specialized microdomains.

We show here that Y341 phosphorylation of Raf-1was a prerequisite for S338 phosphorylation, consistent with previous results (Mason et al., 1999). Marais and colleagues also showed that tyrosine phosphorylation by Src enhanced S338 phosphorylation of Raf-1 (Mason et al., 1999). The data presented here suggest that one of the consequences of Y341 phosphorylation may be the repositioning of Raf-1 near potential S338 kinases. The requirement of Y341 in Raf-1 activation and S338

phosphorylation, however, could be overcome by membrane targeting, suggesting that one of the functions of Y341 phosphorylation is to facilitate proper membrane localization. Indeed, RafY341A mutants were unable to enter rafts upon EGF stimulation, unless linked to ectopic raft-targeting domains.

One explanation for the increased phosphorylation on S338 seen in Y341D mutants is that this reflects the strong cooperativity between the phosphorylations of these sites (Zang et al., 2002). However, this was not the case, at least in studies examining the ability of PAK to phosphorylate Raf-1 *in vitro* (King et al., 2001). Another explanation is that Y341D mutants relocalize Raf-1 to sites of S338 phosphorylation. Phosphorylation of Y341 has been proposed to function in concert with pS338 to provide a negatively charged surface on the Raf-1 protein (Mason et al., 1999). We suggest that one additional function of phosphorylation of Y341 that is distinct from that of S338, is to target Raf-1 to specific membrane sites that participate in subsequent phosphorylations.

Rap1 association with Raf-1 is not sufficient for the phosphorylation of Y341--

The inability of some small G proteins that bind Raf-1 to activate Raf-1 despite recruiting it to the membrane also suggests that recruitment to the membrane is not sufficient for Raf-1 activation. One small G protein that binds Raf-1 without activating it is Rap1 (Okada et al., 1999). Chimeric Ras/Rap1 proteins that replace Ras membrane targeting domains with those of Rap1 are growth inhibitory (Cox et al., 1992), but this inhibition can be relieved by constitutively active Raf-1, suggesting that the inhibitory effects of this chimera were due to impaired Raf-1 activation. However, why relocalizing Ras to Rap1 microdomains inhibited Ras function was not examined.

One proposed function for Ras is the displacement of the 14-3-3 protein from its binding site on residue 259 within Raf-1 (Morrison and R. E. Cutler, 1997). The inability to displace 14-3-3 from Raf-1 may explain the inability of selected G proteins to activate Raf-1 (Light et al., 2002). However, for Rap1, such a model has been ruled out (Light et

al., 2002). This suggests that other mechanisms account for the inability of Rap1 to activate Raf-1.

Studies have demonstrated that activation of endogenous Rap1 limits Ras activation of Raf-1 (Carey et al., 2000; Okada et al., 1999; Schmitt and Stork, 2001). It has been proposed that Rap1 interferes with Ras by trapping the Ras/Raf-1 complex in an inactive conformation (Hu et al., 1997; Wittinghofer and Herrmann, 1995). However, recent studies have demonstrated that Ras and Rap1 occupy distinct subcellular regions (Pizon et al., 1994; York et al., 2000; Zacharias et al., 2002), even following Rap1 activation (Mochizuki et al., 2001).

In part because of its distinct location, Rap1 has been proposed to inhibit Ras activation of Raf-1 by sequestering Raf-1 from Ras. This is consistent with studies showing a loss of Ras/Raf-1 association (and a parallel increase in Rap1/Raf-1 association) upon Rap1 activation (Schmitt and Stork, 2001). Data presented here suggest a possible explanation for the inability of Rap1 recruitment of Raf-1 to activate Raf-1--the inability of Rap1 to support Raf-1 phosphorylations. First, Raf-1 chimeras that were targeted to Rap1-containing membranes via Rap1CAAX motifs were neither activated nor phosphorylated on S338. Second, retargeting Rap1 by swapping in HaRasCAAX sequences allowed Rap1 to activate Raf-1 and to phosphorylate S388. Mutation of Raf-1 to mimic Y341 phosphorylation (Y341D) resulted in a Raf-1 protein that could be activated and phosphorylated on S338 following Rap1 activation, suggesting that the phosphorylation on Y341 can partially overcome Rap1's inability to activate Raf-1. This may be due to the lack of specific Y341 kinases within Rap1 domains. Therefore, we propose that Rap1 prevents Raf-1 activation by positioning it away from tyrosine kinases that are required for Y341 phosphorylation. One of the functions of Y341 phosphorylation might be to provide a regulatable interaction with proteins or lipids to participate in proper targeting of Ras/Raf-1 (Hekman et al., 2002; Sternberg and Alberola-Ila, 1998).

B-Raf's lack of dependence on tyrosine phosphorylation accounts for its activation by Rap1--The Y341D mutation in Raf-1 resembles the naturally occurring sequences within the Raf isoform, B-Raf. B-Raf lacks a tyrosine at the site corresponding to Y341 in Raf-1 (448 in B-Raf). Like Raf-1Y341D, B-Raf is constitutively phosphorylated on the nearby serine (S338 in Raf-1, S445 in B-Raf). Although B-Raf was constitutively active in *in vitro* kinase assays, we show that membrane recruitment was required to permit B-Raf to activate MEK and ERKs *in vivo*. Moreover, targeting of B-Raf chimeras via either Rap1-CAAX or Ras-CAAX was sufficient. The ability of B-Raf-Rap1 chimeras to activate ERKs confirms that the requirement for membrane localization for B-Raf activation by small G proteins is less stringent than that of Raf-1. The mutant of B-Raf in which D447D448 was replaced by tyrosines (B-RafYY) behaved like Raf-1; it was no longer activated by Rap1, but retained the ability to be activated by Ras. The unique specificity of Rap1 for B-Raf activation, but not Raf-1 activation, can be largely explained by the distinct requirements of each kinase for specific membrane targeting for phosphorylation and activation. Future studies examining the ability of Rap1 to support additional critical phosphorylations, including T491 and S494 in Raf-1 (T598 and S601 in B-Raf) (Chong et al., 2001; Zhang and Guan, 2000) may be informative as well.

It has been proposed that sequences within the cysteine-rich domain (CRD) of Raf-1 and B-Raf dictated the contrasting actions of Rap1 on each Raf isoform (Okada et al., 1999). However, the ability of Rap1 to activate RafY341D, as well as the ability of Rap/Ras chimeras to activate wild type Raf-1, both argue strongly that the interactions between Rap1 and Raf-1 are not the only determinants of Raf-1 inhibition. It should be noted that Rap1/Ras chimeras were not as effective as Ras in activating/phosphorylating Raf-1, suggesting that Ras also provides an activation function that is distinct from localization (Light et al., 2002; Mineo et al., 1997; Roy et al., 1997). Furthermore, the lack of activation of B-RafYY by Rap1 suggests that interactions between the B-Raf CRD and Rap1 are also not sufficient to promote activation, although they may be important (Okada et al., 1999). We propose that the carboxy-terminal domain of Rap1

provides specificity to Rap1 signaling in addition to that provided through the interaction between Rap1's effector loop and the Raf CRDs.

In conclusion, we show that Raf-1 phosphorylation at S338 requires membrane targeting of Raf-1 to specific raft microdomains. We propose that tyrosine phosphorylation of Y341 potentiates S338 phosphorylation by facilitating proper membrane localization. This two-step mechanism is outlined in Fig. 7 and may explain the contrasting actions of Ras and Rap1 on Raf isoforms.

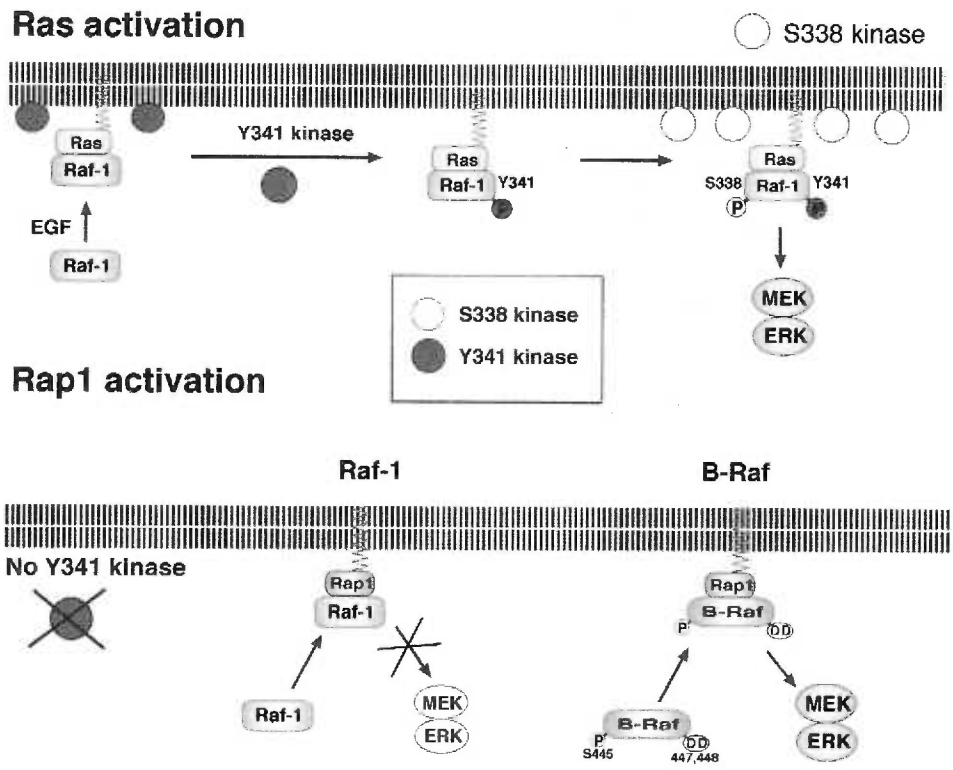

Figure 3.7: A model of sequential phosphorylation of Raf-1 for full activation. EGF induces the recruitment of Raf-1 to plasma membrane-bound Ras. Following this association of Ras with Raf-1 two sequential modifications occur. The first modification is the phosphorylation of Y341 by membrane-bound Y341 kinases, whose activities are induced by EGF and/or Ras activation. This phosphorylation mat relocalize the Ras/Raf-1 complex within specialized plasma membrane microdomains where a second phosphorylation on S338 can occur that renders the Raf-1 molecule competent in phosphorylating downstream effectors like MEK/ERK. Rap1 signaling is depicted in the lower portion of the figure. Rap1 activation does not lead to phosphorylation of Raf-1 or activation of MEK/ERK, although Raf-1 is recruited to Rap1-containing membranes. In contrast, B-Raf is constitutively phosphorylated on S445 (S338 in Raf-1), and the adjacent tyrosines in Raf-1 are replaced with aspartate residues (447D,448D), and therefore does not need to be phosphorylated upon recruitment to Rap1. In this case, Rap1 is capable of coupling B-Raf to MEK?ERK signaling. We suggest that the lack of Y341 activity within Rap1 domains is the limiting step in Rap1's inability to activate Raf-1. Gray and white circles represent Y341 kinases and S338 kinases, respectively.

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CHAPTER FOUR

DISCUSSION

Rap1 is a regulator of ERK signaling

The Raf kinase family regulate a diverse array of cellular functions including cell proliferation, differentiation, and gene transcription by initiating the ERK kinase cascade. Mechanisms have evolved to regulate Raf family members in vertebrates to elicit cell-type specific responses to environmental cues. Here, we have explored the regulation of Raf kinase activation by Ras and Rap1, and how this influences ERK regulation. In cells that express B-Raf, Rap1 activates ERKs. However, T cells only express Raf-1 and activation of Rap1 by the T cell receptor results in ERK inhibition. We show that this is because Rap1 is not able to activate Raf-1. We demonstrate that Raf kinase activation is dependent on its recruitment to different subcellular compartments by Ras and Rap1 where post-translational events are regulated that are required for the initiation of the ERK cascade. This may explain why, that despite interacting with an overlapping set of proteins, Ras and Rap1 differentially regulate signaling pathways in a cell-type specific manner. The findings of this thesis suggest that Rap1 sequesters Raf-1 to a membrane microdomain that does not support required activating phosphorylation events needed for Raf-1 kinase activation. However, Rap1 is able to activate B-Raf due to two key amino acid differences between Raf-1 and B-Raf. The cellular response to the environment is therefore dependent on the dynamics of Ras and Rap1 activation, and to the differential expression of Raf1 and B-raf (Figure 4.1).

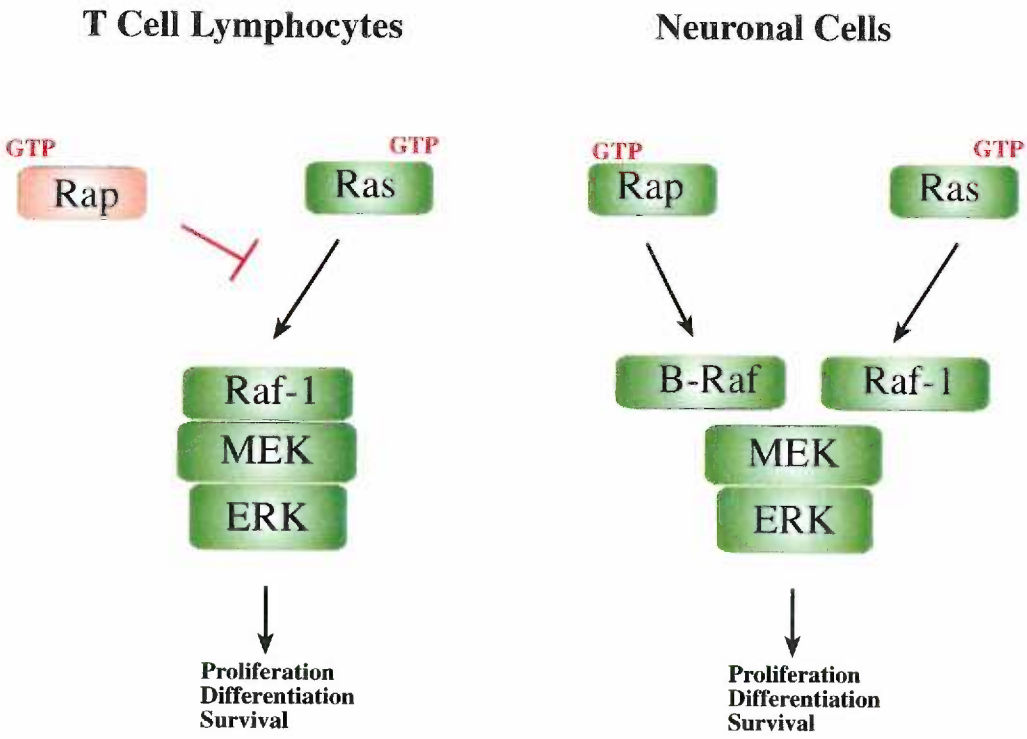

Figure 4.1 Rap1 signaling is cell-type specific. In neuronal cells, Rap1 activation results in proliferation, transcription, and differentiation. However, in T cell lymphocytes, Rap1 activation inhibits Ras-dependent ERK activation and inhibits proliferation. This cell-type specific response is due to the differential expression of Raf-1 and B-Raf. Rap1 is able to activate B-Raf kinase activity, but cannot activate Raf-1.

Rap1 inhibits ERKs in T cells

Engagement of the T cell receptor initiates multiple intracellular signals that can lead to T cell activation and cellular proliferation provided co-stimulatory receptors are also activated. The biochemical mechanisms that couple receptor binding to activation of signaling pathways is tightly regulated to insure a proper immune response to antigen. In the first part of this thesis, we have explored the biochemical mechanisms regulating the ERK cascade in T cells by Ras and Rap1. In chapter 2 of this thesis we demonstrate that Rap1 antagonizes Ras-dependent ERK signaling in T cells by directly competing for a shared effector, Raf-1, as has been observed in other systems (Cook et al., 1993) (Okada et al., 1998; Schmitt and Stork, 2001). Consistent with other studies, we find that T cells activate both Ras and Rap1 in response to TCR activation (Katagiri et al., 2002; Reedquist and Bos, 1998). Several studies have shown that activation of the T cell receptor alone causes minimal ERK activation, but CD28 co-stimulation is able to augment TCR-dependent ERK activation (August and Dupont, 1995; Li et al., 2001b; Nunes et al., 1996; Nunes et al., 1994). We found that overexpression of a constitutively active form of Rap1, RapE63, reduced TCR/CD28 ERK activation while overexpression of a Rap1GAP inhibited Rap1 and augmented TCR-dependent signals to ERK that are comparable to that seen by TCR/CD28 co-stimulation. Therefore, Rap1 is an antagonist of ERK signaling and control of Rap1 activation may set a threshold to prevent T cell activation in response to improperly stimulated T cells.

Previous studies have demonstrated that Rap1 is a negative regulator of T cell activation (Boussiotis et al., 1997). However, Rap1 may not regulate ERK activation in

all cell types, and the role of Rap1 in regulating ERKs has been challenged (Bos et al., 2001; Zwartkruis et al., 1998). The Jurkat model system allowed us to test the *in vivo* role of activated Rap1 because CD28 activation can block TCR dependent Rap1 activation (Reedquist and Bos, 1998). The development of affinity based methods to detect endogenous Ras and Rap1 activation in cells has made it possible to directly test the hypothesis that Rap1 is an antagonist of ERK signaling in T cells (see Appendix 1). We were able to examine TCR- and TCR/CD28-dependent activation of endogenous Ras and Rap1 and compare this to ERK activity using Jurkat cell lines where mutant mouse CD28 receptors are expressed and have been used to investigate the role of co-stimulation on gene transcription (Holdorf et al., 1999). These cell lines allowed us to identify what part of the intracellular domain of CD28 is responsible for Rap1 regulation. We determined the mechanism of CD28-dependent Rap1 inhibition was via the activation of an endogenous RapGAP. This system allowed us to regulate endogenous Rap1 activation without having to resort to overexpression of Rap and Ras inhibitors that may have indirect effects on other signaling pathways. Using antibodies that activate only the mutant mouse CD28 receptors, we found that Rap1 activation correlated with ERK inhibition. While these studies were performed in Jurkat T cells, a transformed cell line that may have mechanisms that are distinct from primary lymphocytes, our results confirm that Rap1 is able to antagonize Ras-dependent ERK signaling. The regulation of RapGEFs and RapGAP activities represents a mechanism to regulate ERK signaling in T cells and possibly other cell types. A model for T cell regulation by Rap1 is shown in Figure 4.2.

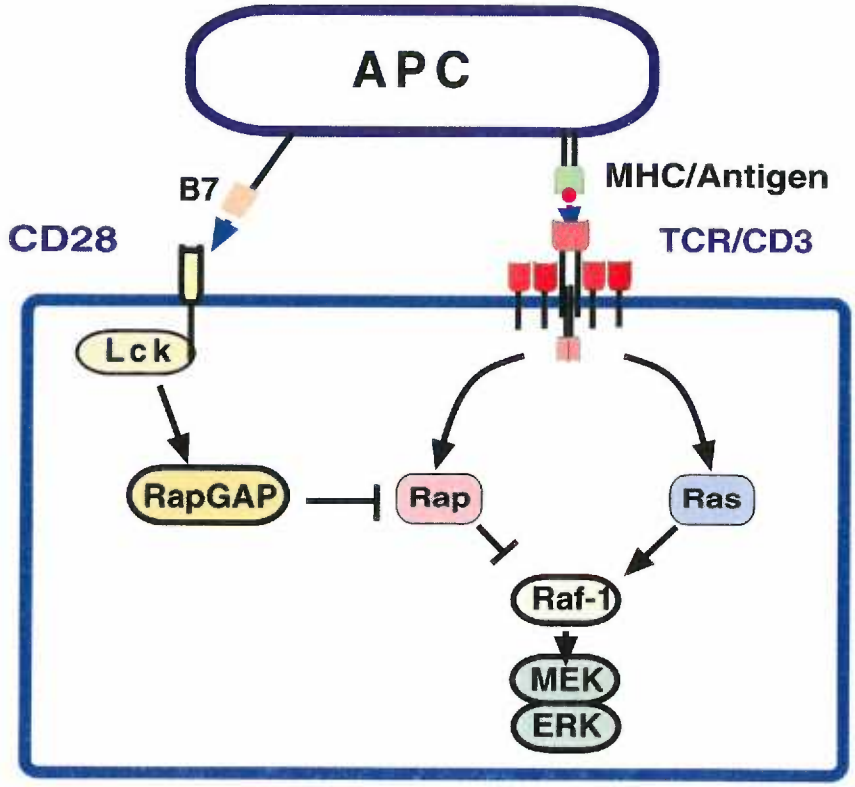

Figure 4.2 Rap1 regulates ERK activation in T cells. TCR activates both Ras and Rap1 but does not stimulat ERK activation. TCR/CD28 co-stimulation augments TCR activation of ERKs in T cells by inhibiting Rap1 activation via the Lck-dependent activation of a RapGAP. Rap1 may limit TCR-dependent activation of ERK1,2 by competing with Ras for the Ras-binding domain of Raf-1.

While Rap1 inhibition may account for the role of CD28 on co-stimulation of ERKs in Jurkat cells, there is evidence to suggest that this is not the only limiting step in T cell activation. Mice that lack expression of CD28 have a normal T cell repertoire and decreased overall responses to antigenic stimuli, but are still capable of proliferating and producing IL-2 (Green et al., 1994; Shahinian et al., 1993). Mice lacking both CD28 and CD2, another costimulatory receptor, have a more severe phenotype, showing reduced viability with age, cytokine production, and proliferative responses (Green et al., 2000). It is possible that CD2 may also regulate RapGAP activity, or regulate other pathways that influence Rap1 activation. Many other co-stimulatory receptors have been identified, suggesting that there are multiple mechanisms involved in T cell co-stimulation. Our laboratory has developed several lines of transgenic mice that overexpress Rap1GAP and a constitutively activated form of Rap, RapE63. These animals will be invaluable for examining the physiological role of Rap1 in T cell signaling.

Rap1 activation in T cells is regulated by tyrosine kinases

The Src family kinases Lck and Fyn play key roles in T cell activation by initiating the activation of downstream signaling pathways (Sefton and Taddie, 1994). However, genetic studies in mice suggest these kinases have only partially overlapping functions, as Fyn deficient mice, show only minor TCR activation defects while Lck null mice have significantly reduced T cell numbers and have diminished T cell responses (Appleby et al., 1992; Molina et al., 1993; Molina et al., 1992). In chapter two of this

thesis, we demonstrate opposing roles for Fyn and Lck in regulating Rap1 activation. Constitutively active Fyn is able to activate Rap1, while activated Lck mediates CD28 dependent Rap inhibition. Our data support a role for Fyn to positively regulate Rap1 activation, while Lck can activate an extrinsic RapGAP activity. Other studies in T cells show Fyn can activate Rap1 by phosphorylation of Cbl resulting in the recruitment and activation of C3G, a Rap1 GEF (Boussiotis et al., 1997; Reedquist et al., 1996). This pathway has been suggested to regulate Rap1 activation in anergic T cells (Appleman et al., 2001; Boussiotis et al., 1997), and is required for Rap1 activation by the B cell receptor (McLeod et al., 1998). Src has been shown to regulate Rap1 activation in other cell types as well, suggesting that tyrosine phosphorylation of adapters may be a general mechanism for Rap1 activation (Li et al., 2002; Schmitt and Stork, 2002; Xing et al., 2000). Our data suggests an opposite role for Lck, the activation of a RapGAP. Dominant negative Lck blocked the ability of CD28 to activate RapGAP activity, while dominant negative Fyn did not inhibit the ability of CD28 to activate Rap GAP activity. Furthermore, our experiments using a cell line that does not express functional Lck protein demonstrate that Lck is also required for both Rap activation and RapGAP activation, suggesting that Lck may have multiple roles in T-cell activation events. The implication that Lck can mediate both Rap1 inhibition and activation suggests that Lck regulation in T cell activation may be complex involving both late and early TCR signaling events each representing a crucial control point in T cell activation. The CD28 receptor may then be a switch to regulate the outcome of Lck activity. Recent studies demonstrate a direct association of Lck with the intracellular domain of co-stimulatory

receptors, an interaction that enhances kinase activity (Cho et al., 2000; Holdorf et al., 1999; Holdorf et al., 2002).

Like other Src family kinases, Lck contains a C-terminal kinase domain, a single Src homology 2 domain(SH2) a Src homology 3 domain (SH3), and an N-terminal region that is distinct from other family members (Brown and Cooper, 1996). SH3 domains are able to mediate protein interactions by binding proline-rich amino acid sequences (Ren et al., 1993), and a number of proteins have been reported to bind to the Lck SH3 domain including c-Cbl, CD2, and CD28 (Bell et al., 1996; Holdorf et al., 1999; Reedquist et al., 1996). The interaction of Lck with the proline-rich motif that is part of the intracellular domain of CD28 has been reported to play an essential role in CD28 costimulation (Holdorf et al., 1999). Using Jurkat cell lines that express a form of the mouse CD28 receptor that lacks this proline-rich motif, CD28 was no longer able to block endogenous Rap activation, or activate endogenous RapGap activity. CD28 cross-linking also lacked the ability to co-stimulate ERK activation in these cells. The SH3 domain of Lck has been shown to be required for TCR-dependent ERK activation and gene transcription (Denny et al., 2000). Using a cell line with a mutant Lck that lacks the SH3 domain, we found that CD28 is no longer able to inhibit Rap. Taken together, these findings support a role for the SH3 domain of Lck in providing the specificity for regulating Rap activition and CD28 costimulation of ERKs.

Lck may regulate RapGAP activity by direct phosphorylation. Rap1GAP is phosphorylated on multiple tyrosine, serine and threonine residues, although it is unclear

if these phophorylations regulate activity (Polakis and McCormick, 1992; Rubinfeld et al., 1992). Regulation of an extrinsic RapGAP activity has also been observed in platelets stimulated with epinephrine (Marti and Lapetina, 1992). In these cells, epinephrine couples to $G\alpha_i$ or $G\alpha_z$ signaling pathways via activation of the α_{2a}-adrenergic receptor, and $G\alpha_i$ activates Src family kinases (Lova et al., 2002; Ma et al., 2000). Alternatively, phosphorylation may regulate RapGAP activity indirectly via the regulation of an unkown protein-protein interaction. Support for either model exists from studies of another GAP, Ras-GAP, where it was observed that both the activity and recruitment to the adapter protein p62Dok is regulated by direct tyrosine phophorylation by Src (Druker et al., 1992) (Yamanashi and Baltimore, 1997). Mature T cells predominately express SPA-1, a recently identified RapGAP (Kurachi et al. 1997). SPA-1 contains a coiled-coil motif and is predominantly membrane bound (Kurachi et al., 1997). As Jurkat cells express SPA-1, Rap1GAP, and Rap1GAPII (Kurachi et al., 1997; Mochizuki et al., 1999), it will be necessary to identify which of these GAPs is regulated by Lck and CD28 to elucidate the mechanism of this enhancement of extrinsic RapGAP activity. Regulation of RapGAPs provides the cell additional ways to regulate Rap1 both spatially and temporally. In T cells, and other cell types, regulation of RapGAP activity could regulate the level of activated Rap1 thereby regulating the level of ERK activation.

Our data suggest that the outcome of T cell receptor signaling may depend on the relative activities of Fyn and Lck, and that this is through the regulation of Rap1 activation. Interestingly, anergic cells which have high levels of active Rap1 (Boussiotis et al., 1997), contain increased Fyn kinase activity and decreased Lck activity (Quill et

al., 1992). Recent studies suggest that the relative activity of Lck also influences the activation of immune synapse formation, and this is through the recruitment of Lck via the SH3 domain to the CD28 receptor (Holdorf et al., 2002). It will be interesting to determine the location and kinetics of Rap1 activation during T cell activation. Recent advancements in cellular imaging using FRET to localize Rap1 activation in real time will be invaluable in future studies (Mochizuki et al., 2001).

Rap1 and adhesion

Rap1 may also play a role in other signaling cascades that regulate T cell activation. Recent data from a number of labs have suggested that Rap1 plays a role in regulating T cell adhesion by influencing the avidity of the LFA-1-ICAM-1 integrin interaction by an unknown mechanism (Katagiri et al., 2002; Tsukamoto et al., 1999; Bachmann et al., 1997; Katagiri et al., 2002; Reedquist et al., 2000). This has important ramifications for T cell activation, since the T cell must remain in contact with the antigen presenting cell in order to form the immune synapse, a structure that is required to initiate proliferation and cytokine production (Dustin and Shaw, 1999). This would suggest that Rap1 plays a positive role in regulating T cell activation. Integrin avidity and function has been reported to be negatively regulated by Ras and constitutively active Raf (Hughes et al., 1997). Rap1 activation has recently been shown to regulate integrin signaling in fibroblasts where adhesion can be regulated by Raf-1 dependent pathways (Li et al., 2002). This has created some confusion in the field, since Rap antagonism of Ras is thought to be a negative regulator of lymphocyte signaling while Rap activation may actually be required for T cell activation (Sebzda et al., 2002). The ability of active

Rap to inhibit ERK signaling may therefore play a positive role in T cell activation by regulating the formation of the immune synapse. It will be interesting to examine the role of Rap1 in integrin-dependent adhesion using transgenic mice recently generated in our lab which overexpress an endogenous inhibitor of Rap, Rap1GAP. Preliminary data suggest that we can inhibit TCR dependent Rap activation and it will be interesting to determine if these animals are defective in synapse formation, adhesion, and ERK activation.

Rap1 and Costimulation

Costimulation was originally defined as a distinct signal required in conjugation with the signal transduced by the TCR for the initial activation of naïve T cells (Harding et al., 1992). Antibodies that block CD28 activation by its ligand, B7, induce an anergic like phenotype (Harding et al., 1992). Rap1 is constitutively activated in anergic T cells, and has been hypothesized to negatively regulate cellular proliferation and IL-2 gene transcription (Boussiotis et al., 1997). It would be interesting to determine if anergic cells have elevated Rap1 levels because of altered regulation of RapGEFs, or if there is misregulation of a RapGAP activity. This may have therapeutic value in certain autoimmune disorders. Our findings suggest that CD28 can inhibit Rap1 activation independently of TCR signals by the activation of a RapGAP. However, it has also been suggested that CD28 can regulate the levels of p27Kip1, and the expression of a transcriptional repressor, TOB (Appleman et al., 2001). A recent observation has been reported where mice deficient in SPA-1, a Rap specific GAP, have defective ERK activation and reduced proliferative responses (Katagiri et al., 2001). However, there is

also evidence that inhibition of ERKs does not play a significant role in anergy (DeSilva et al., 1998). Thus it is unclear if ERK inhibition is merely a result of anergy, or together with other pathways it contributes to anergy. Other studies from our laboratory using transgenic animals that overexpress B-Raf, have demonstrated that converting Rap1 into a positive regulator of ERKs is not sufficient to rescue the anergic phenotype (Dillon et al., 2001). These findings suggest that Rap1 may play a role in anergy, but is not sufficient to induce an anergic response. Future work using transgenic mice that overexpress a Rap1GAP that have been developed in our laboratory will be useful for determining the role of Rap1 in anergy.

Subcellular localization and Rap1 signaling specificity

In the second part of this thesis, we have determined a mechanism for the inhibition of ERK activation by Rap1. In chapter two, we showed that Rap1 is able to physically interact with Raf1 upon TCR activation. TCR/CD28 co-stimulation inhibits Rap1 via activation of a RapGAP thereby preventing the ability of Rap1 to bind Raf-1 enhancing ERK activation. Rap1 has been shown to form a stable complex with Raf1 in anergic cells, where Rap1 is constitutively activated (Boussiotis et al., 1997). Activated Rap1 may compete with Ras for binding to Raf-1, preventing Raf-1 activation and inhibiting ERK activation in anergy. This mechanism was observed in fibroblasts stimulated with the β_2-adenergic receptor agonist isopeteranol and may explain the ability of cAMP to inihibit Raf-1 activation and EGF-dependent proliferation (Schmitt and Stork, 2001). Another study has shown a stimulus dependent interaction between Rap1 and Raf-1 in Chinese hamster ovary cells (Okada et al., 1998). However, Rap1 localizes

to a subcellular compartment that is distinct from Ras and the ability of Rap1 to bind Raf-1 *in vitro* may not be physiologically significant (Mochizuki et al., 2001; Chiu et al., 2002).

Activation of Ras and Rap1 results in a conformational change in these proteins that allow a direct binding interaction between the effector loop domain and target protein (Nassar et al., 1995). In addition to the effector loop, the switch 1 region of Ras makes additional contacts with the cysteine-rich domain of Raf-1 that are required for Ras to fully activate Raf-1 kinase activity (Briva et al., 1995; Roy et al., 1997). It has been proposed that sequence differences between Rap1 and Ras and their ability to interact with the cysteine-rich domain of Raf isoforms are the determining factor for effector activation (Okada et al., 1999). In addition to a direct binding mechanism, Ras has been suggested to regulate effector function by membrane localization (Prior and Hancock, 2001). In support of this view, targetting of Raf-1 to the inner leaflet of the plasma membrane with a Ras CAAX motif is sufficient to activate Raf-1, suggesting a direct Ras-Raf interaction is not required (Leevers et al., 1994; Stokoe et al., 1994). As discussed in the introduction, Ras and Rap1 localize to different membrane compartments. We hypothesized that the localization differences between Rap1 and Ras may also contribute to their role in signaling. In the second part of this thesis, we found that Rap1 localizes to a membrane domain that is unable to support Raf-1 kinase activation.

ERK activation requires Raf-1 localization to lipid rafts

Signal transduction pathways depend on the localization of proteins to their sites of action (Pawson and Scott, 1997). Organizing signaling complexes onto scaffolds, recruitment to activating kinases, and mechanisms that regulate membrane targetting provide both specificity and influence the magnitude and duration of several signaling pathways (Teruel and Meyer, 2000; Teruel and Meyer, 2002). Emerging evidence suggests that distinct membrane microdomains that are enriched in cholesterol and sphingolipids act as organizers of signaling cascades, especially for T cell activation (Viola et al., 1999; Dykstra et al., 2001; Sedwick and Altman, 2002). In the second part of this thesis, we demonstrate that growth factor and Ras-dependent activation of Raf-1 kinase requires the recruitment to a distinct membrane domain that fits the operational definition of a lipid raft. Support for lipid rafts participating in the regulation of ERK signaling has been demonstrated from numerous other studies using cholesterol depleting agents that disrupt lipid rafts (Furuchi and Anderson, 1998; Kranenburg et al., 2001; Mineo et al., 1996; Prior et al., 2001; Roy et al., 1999). However, there is evidence to support both a positive and negative role for lipid rafts in regulating Raf-1 kinase activation depending on the stimulus (Rizzo et al., 2001; Furuchi and Anderson, 1998; Liu et al., 1996; Prior et al., 2001). Moreover, a recent study refutes the hypothesis that lipid rafts regulate ERK signaling due to the non-specific effects of methyl-β-cyclodextrin on other signaling pathways (Chen and Resh, 2001). The strategy we used in this thesis directly tested the requirement of lipid rafts for Raf-1 kinase activation by localizing Raf-1 to lipid raft and non-raft membrane microdomains by constructing chimeras attaching different CAAX motifs from H-Ras, K-Ras and Rap1B to the C-

terminus of Raf-1. This strategy avoided the potential non-specific effects in the use of cholesterol depleting agents, and the technical problems involved in the isolation of lipid rafts (White and Anderson, 2001). The data presented in chapter 3 of this thesis support a model that Raf kinase activation requires recruitment to lipid rafts.

Role of tyrosine phosphorylation in Raf-1 activation

The mechanism of Raf-1 kinase activation is poorly understood. A novel finding of this thesis is that phosphorylation of Raf-1 on tyrosine residue 341 (Y341) regulates the movement of Raf-1 into lipid rafts. Previous studies have demonstrated that phosphorylation of Y341 is absolutely required for Raf1 kinase activation (Chong et al., 2001; Fabian, 1993; Li et al., 2001a; Mason et al., 1999), although the functional role for this site has never been identified. However, phosphorylation of Y341 has been suggested to be required for the subsequent phosphorylation of serine residue 338 in Raf-1 (Mason et al., 1999). Using Raf-1 chimeras that localize to lipid rafts constitutively, we find that tyrosine phosphorylation is no longer required for kinase activation and these chimeras are constitutively phosphorylated on serine 338. Interestingly, we found that substituting an alanine residue for tyrosine at position 341 prevents EGF induced recruitment of Raf-1 into a lipid raft fraction. However, serine 338 phosphorylation is still required for kinase activity of these membrane targeted Raf1 chimeras, consistent with prior published studies requiring that this site is phosphorylated for kinase activation (Diaz et al., 1997; Mason et al., 1999; Sun et al., 2000). Taken together, our data suggests that tyrosine phosphorylation is required for entry into lipid rafts, where Raf-1 is phosphorylated on serine 338. Our data suggests that a kinase, which phosphorylates

residue 338 in Raf-1, is constitutively active and localized to lipid rafts. The role of tyrosine phosphorylation may be to interact with a protein that facilitates entry into a raft, or to relieve some form of allosteric inhibition or lipid that prevents Raf-1activation. Future efforts in our laboratory will be to identify this factor. The role of Ras may be to recruit Raf-1 to a membrane domain that enables tyrosine phosphorylation that may be required for the Ras/Raf-1 complex to enter a lipid raft where serine 338 kinases reside. This model is shown in Figure 4.3.

The Raf-1 Y341 kinase has not been identified. Raf-1 is a substrate for Src family kinases and overexpression of v-Src can augment Raf-1 kinase activity (Mason et al., 1999). However, there is evidence that Src phosphorylates Raf-1 on Y340, and not Y341 (Fabian et al., 1996). Interestingly, a Y340D mutation is sufficient to render Raf-1 constitutively active and oncogenic independent of Ras (Fabian et al., 1996; Diaz et al., 1999). However, a Y341D mutation is not sufficient to activate Raf-1 (Chong et al., 2000). This suggests that these tyrosine residues have distinct functions in regulating Raf-1 kinase activation and downstream signaling specficity. Our data suggests that the Y341 site serves a regulatory function in the recruitment to lipid rafts. The identification and localization of the Y341 kinase will be useful in extending these studies. We have developed an assay to detect serine and tyrosine phosphorylation of Raf-1 using isolated membranes and lipid raft fractions. This assay may be useful in the isolation and purification of the kinase. Identification of the kinases involved in tyrosine phosphorylation of Raf-1 may provide targets for the development of therapeutic agents in the treatment of proliferative disorders such as cancer.

A mechanism for Raf-1 kinase regulation by Rap1

Our data comparing the localization and kinase activities of Raf1-CAAX chimeras support a role for subcellular localization differentially regulating Raf-1 activation. Raf1-RapCAAX chimeras are not phosphorylated on S338 and have no detectable basal kinase activity in our assays. Our finding that tyrosine phosphorylation is required for Raf-1 kinase localization to lipid rafts and S338 phosphorylation suggested that the Raf1-RapCAAX chimeras is due to the inability for Rap1 membrane domains to activate tyrosine phosphorylation. Since we had found a role for tyrosine phosphorylation in the regulation of Raf-1 recruitment to lipid rafts, we introduced a Y341D mutation into our Raf1-RapCAAX chimeras and tested their ability to become activated. We observed that both kinase activation as well as serine 338 phosphorylation could be restored in these mutants. These studies were complemented using chimeric RapRas constructs, which redirect Rap to a membrane domain that can now support Raf-1 activation, converting Rap to a Raf-1 activator. Therefore, our findings support a model in which the mechanism of Rap1 inhibition of ERK activation is due, in part, to the sequestration of Raf1 to a membrane domain that does not support kinase activation.

The inability of Rap to activate Raf-1 has been attributed to differences in the way Ras and Rap interact with a domain of Raf-1 known as the cysteine-rich domain (CRD) (Okada et al., 1999). While we cannot exclude a role for this model, our findings suggest that the inability of Rap1 to activate Raf-1 is due to localization. The binding model would predict that any Rap/Ras chimera would not be able to activate Raf-1. However, our results demonstrate that a Rap-RasCAAX chimera is able to induce Raf-1 serine 338

phosphorylation and RapE63 is able to activate Raf-1 Y341D. However, our data demonstrates that the level of 338 phosphorylation and ERK activation by the Rap-RasCAAX chimera was not as robust compared to that observed for RasV12 overexpression, suggesting that both mechanisms may play a role. The inability to completely rescue Raf-1 activation by the Rap-RasCAAX chimeras suggests that tyrosine phophorylation may be just one of several phosphorylation events that are absent in Rap1 subcellular compartments, since multiple activating phosphorylation events are required for Raf-1 kinase activation (Chong et al., 2001). This hypothesis is supported by the observation that Raf-RapCAAX Y341D is as phosphorylated on serine 338 to the same extent as Raf-RasCAAX, since the Y341D mutation may allow for entry into a lipid raft. Identifying additional regulatory phosphorylation events that are regulated by Rap1 will help to resolve which mechanism, binding or recruitment, plays the most crucial role in Raf-1 kinase regulation by Rap1. Multiple levels of control would ensure that an effector would only become activated by the correct G protein. We propose that Rap1 sequesters Raf-1 to a membrane compartment that does not contain the tyrosine kinase that is required for Y341 phosphorylation (Figure 4.3).

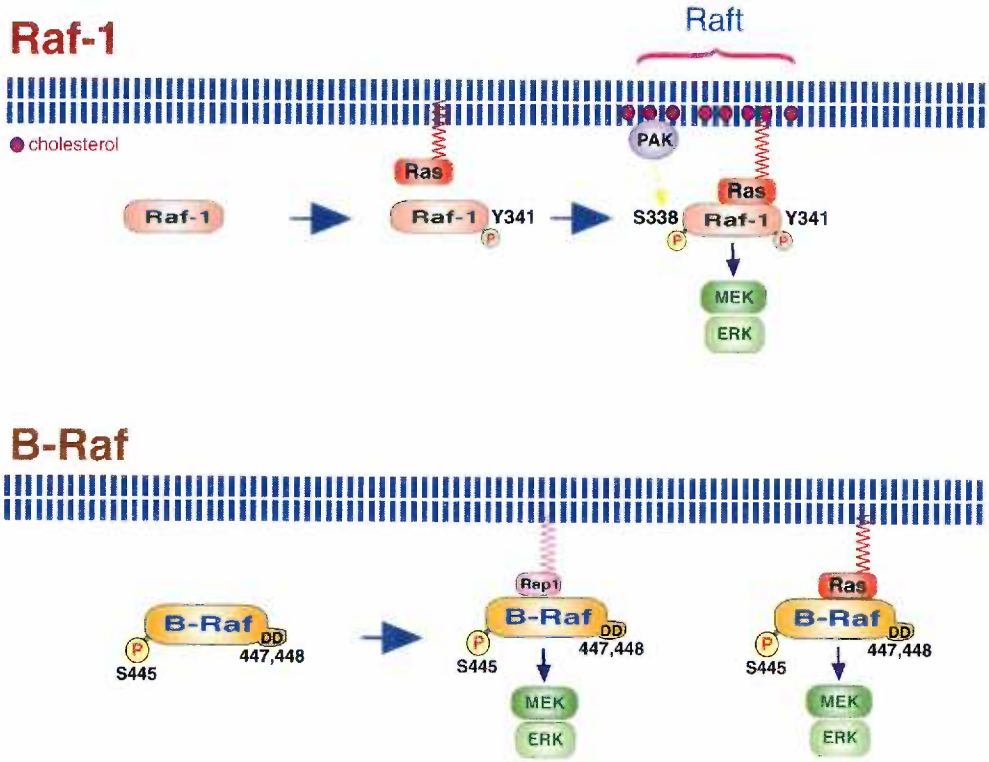

Figure 4.3 A model for Ras and Rap1 regulation of Raf-1 and B-Raf. Activated Ras recruits Raf-1 to a membrane-bound kinase that phosphorylates Raf-1 on tyrosine Y341. This phosphorylation event is required to relocalize the Ras/Raf-1 complex to a lipid raft membrane microdomain where a second phosphorylation on S338 occurs. These sequential phosphorylation events facilitate Raf-1 kinase activation. Rap1 membrane microdomains are unable to support Raf-1 Y341 or S338 phosphorylation. This may be due to the absence of the regulatory kinases. However, B-Raf is constitutively phosphorylated on S445 (S338 in Raf-1) as a result of the aspartate residue substitutions at residues 447 and 448 (Y340, Y341 in Raf-1). Therefore, B-Raf does not require tyrosine phosphorylation upon recruitment to Rap1. Thus, Rap1 is capable of coupling B-Raf to MEK and ERK. The lack of Y341 activity within Rap1 membrane domains is a key factor in preventing Rap1-dependent activation of Raf-1.

Rap1 activation of B-Raf

In the course of these studies, we have identified a mechanism for Rap1-mediated activation of B-Raf. Both Ras and Rap1 can activate B-Raf *in vitro* and *in vivo* (Ohtsuka et al., 1996; Vossler et al., 1997). In chapter 3 of this thesis, we show that this is because there are two key amino acid mutations that differ between Raf-1 and B-raf. B-raf substitutes two aspartate residues at positions 445 and 446, which correspond to tyrosine 340 and 341 in Raf-1. These substitutions represent a naturally occurring mutant form of Raf-1 Y341D, which we demonstrated can be activated by Rap1. Substituting these sites with tyrosine permitted the activation of B-raf by Ras, but this mutant was no longer activated by Rap1. These findings support our model for a requirement for Raf kinase isoforms to be recruited to membrane compartments that permit kinase activation by phosphorylation.

CONCLUSIONS

The goal of these studies was to understand the biochemical mechanisms of Rap1-dependent ERK signaling. In this thesis, we have demonstrated that Rap1 function is determined by three different, but equally important factors, subcellular localization, the activities of GEFs and GAPs, and the expression of effectors. The ability of Rap1 to regulate ERK activation is cell-type specific and is dependent on the expression of effectors such as Raf-1 and B-Raf. Rap1 inhibits Raf-1 but activates B-Raf. We show evidence that this is because Rap1 localizes to a subcellular membrane compartment that is capable of only activating B-Raf. Rap1 signaling is also likely to be cell-type specific due to the regulation of RapGEFs and RapGAPs, which can regulate the duration of Rap1-dependent signaling events. These mechanisms are likely to be common among other small G proteins, and we have demonstrated the significance of the subcellular localization of Raf-1 by Ras. Control of both effector usage and kinetics of activation would allow growth factor specificity and the ability of the same growth factor to elicit the same biological response in different cell types. The regulatory mechanisms among the Ras G protein superfamily facilitates greater complexity in signaling outcomes by dynamically regulating multiple G proteins in different cellular compartments.

APPENDIX

Non-isotopic methods for detecting activation of small G proteins

By KENDALL D. CAREY and PHILIP J. S. STORK

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INTRODUCTION

Rap1 proteins are members of the Ras superfamily of small G proteins and cycle between the GDP and GTP bound state (Zwartkruis and Bos, 1999). In the GTP bound state, Rap1 is biologically active and is capable of binding with high affinity to several downstream signaling molecules, including Raf-1, B-Raf, and RalGDS (Bos, 1998; Vossler et al., 1997; Wittinghofer and Herrmann, 1995). Activated Rap1 regulates several signaling pathways, including the mitogen-activated protein kinase cascade (Kitayama et al., 1990; McLeod et al., 1998; Mochizuki et al., 1999; Vossler et al., 1997) and plays a role in regulating cellular adhesion (Buensuceso and O'Toole, 2000; Katagiri et al., 2000; Reedquist et al., 2000). A number of stimuli which may induce cell growth or differentiation utilize Rap1 signaling to achieve specific physiological effects, including T cell receptor activation (Carey et al., 2000), B cell receptor activation (McLeod et al., 1998), growth factor action (Yao et al., 1998; York et al., 1998) (Xing et al., 2000) and hormonal stimulation (Jordan et al., 1999; Mochizuki et al., 1999; Schmitt and Stork, 2000; Vossler et al., 1997). All these stimuli increase levels of GTP-bound Rap1 through the activation of Rap1-specific guanine nucleotide exchange factors (GEFs), many of which are themselves regulated by intracellular signals (de Rooij et al., 1998; Grewal et al., 1999). Therefore, the ability to measure Rap1 activation may provide a useful tool in the examination of a number of biological systems.

Several methods for studying the activation of small G-proteins have been described (DeClue et al., 1992; Downward, 1985; Gibbs et al., 1987; Gotoh et al., 1995; Satoh and Kaziro, 1995). These methods rely on the use of radiolabelled purified proteins to measure exchange activity *in vitro* or radiolabelling cells with orthophosphate and immunoprecipitating the G protein of interest to measure the GTP/GDP-bound ratio. However, for some G proteins, such as Rap1, good immunoprecipitating antibodies are

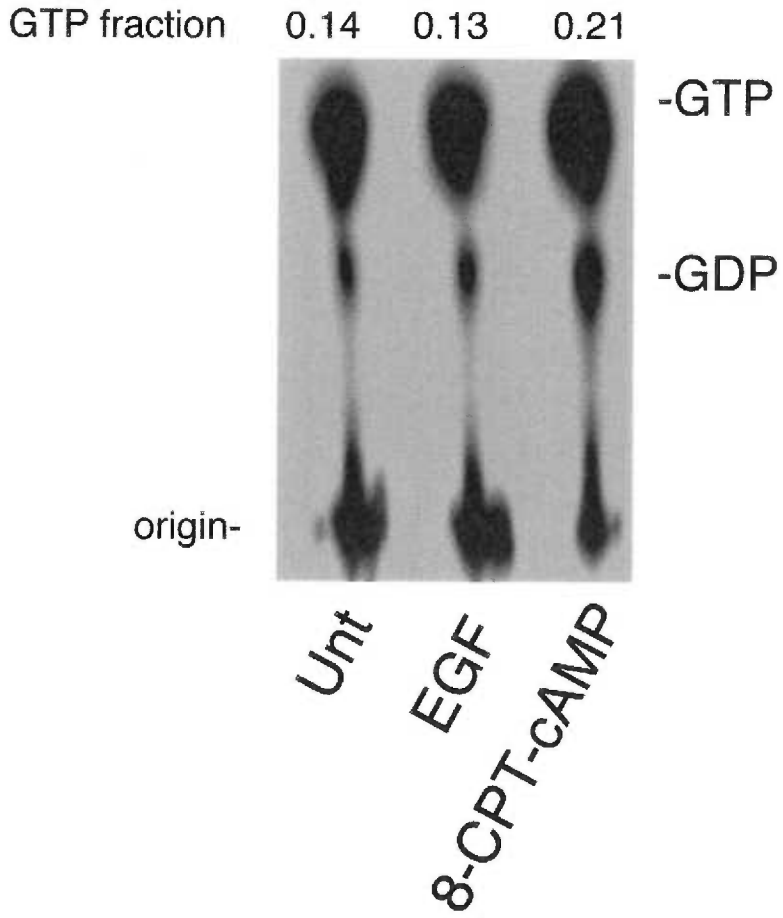

Figure A.1 GTP loading of Rap: Rap1 activation by 8-CPT. GTP loading was assayed in PC12 cells after transfection of His-Rap (30 μg). PC12 cells were left untreated (Unt), or were treated with EGF or 8-CPT-cAMP for 5 min. as indicated. His-Rap was precipitated with Ni-NTA-agarose and eluates were analyzed for GTP and GDP content by thin-layer chromatography. The GTP fraction of total guanine nucleotide is given above each lane.

unavailable, requiring transfection of epitope-tagged Rap1 or the use of purified proteins to study Rap1 activation (Altschuler et al., 1995; Gotoh et al., 1995; Vossler et al., 1997) (see Figure A.1). A major disadvantage of this method is the high level of radioactivity needed to metabolically label cells with orthophosphate, the technical difficulties of retaining *in vivo* GTP levels during steps *in vitro*. Recently, non-isotopic methods have been devised that enable the detection of Ras and Rap1 activation in treated cell lysates. All of the methods presented here take advantage of the ability of proteins to bind selectively to the GTP-bound state of the small G proteins Ras and Rap1. Some of these methods use epitope-tagged versions of Ras- or Rap1-binding proteins to selectively pull down GTP-bound Ras or Rap1 from treated cellular lysates. In this manner, activation of endogenous Rap1 or Ras can be detected directly through its ability to interact with downstream binding partners *in vitro*, such as the amino terminus of Raf-1, or the Ras binding domain of RalGDS, respectively (Franke et al., 1997; Zhang et al., 1993). Alternatively, transfected epitope-tagged Ras or Rap can be used to pull down endogenous downstream effectors such as Raf-1 or B-Raf *in vivo* (Carey et al., 2000; Schmitt and Stork, 2000). Examples of both of these methods are illustrated in Figure A.2. In Figure A.2A, Rap1 activation is assayed by measuring its binding to RalGDS *in vitro*. Purified GST-RalGDS is added to stimulated cellular lysates to pull-down endogenous GTP-bound Rap1, which is then detected by Western blotting using Rap1 antisera. In Figure A.2B, Ras activation is measured by examining the binding of Raf-1 *in vivo*. In this example, an epitope-tagged version of Ras (polyhistidine-tagged Ras, or His-Ras) is transfected into mammalian cells. Upon stimulation, His-Ras becomes GTP loaded via the activation of Ras exchange factors and binds with high affinity to Raf-1. Nickel-agarose beads are then used to selectively pull-down both activated and inactive His-Ras and levels of Raf-1 are evaluated by Western blotting using Raf1 antisera.. Since only activated Ras exists in a complex with Raf-1, the amount of Raf-1 detected by

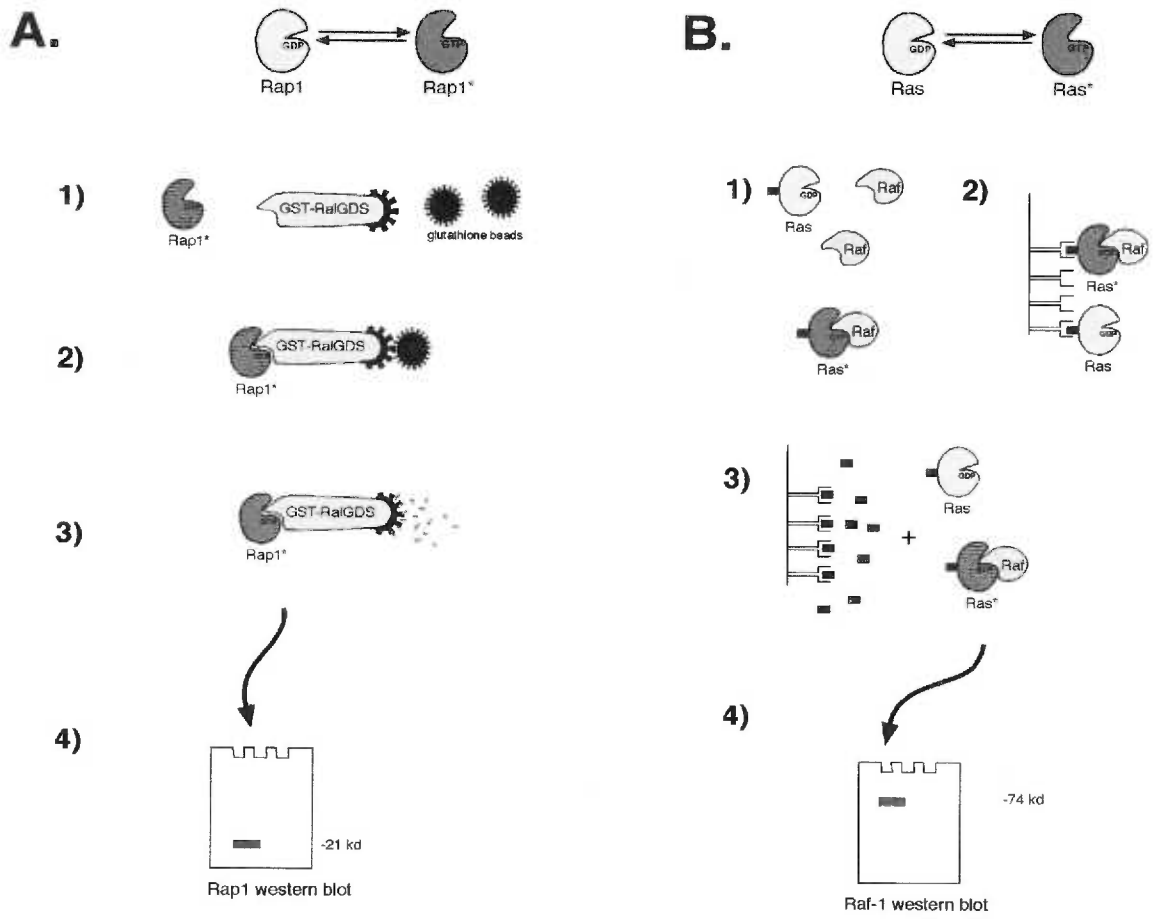

FIGURE A.2 Schematic of nonisotopic assays

FIGURE A.2 Schematic of non-isotopic assays.
(A) *In vitro* RalGDS assay. Rap1 exists in an equilibrium between GTP-bound (active) and GDP-bound (inactive) states. Effectors like B-Raf and RalGDS bind with much higher affinity to the GTP-bound (active) state of Rap1. This can be monitored *in vitro* using chimeric proteins that contain the Rap1 binding domain of such effectors. One useful tool is GST-RalGDS, a chimeric protein combining the Glutathione-S-transferase domain with the RalGDS domain. Ral is a small G protein that can be activated by a Guanine dissociation stimulator called RalGDS. This protein has been shown to bind to activated Rap1 with high affinity and can be used *in vitro* to select GTP-bound Rap1 molecules. 1) Cells are treated with potential Rap1 activators and lysed. Lysates are mixed with bacterially purified GST-RalGDS, at high concentrations to displace any bound endogenous effectors. Subsequently, glutathione-coupled beads are added to the mix. 2) Glutathione forms a tight association with the GST-RalGDS and bound active Rap1. Excess GST-RalGDS is free to bind to other molecules including GTP-bound Ras. 3) The addition of free glutathione displaces the beads from the GST-RalGDS complex. 4) SDS-PAGE separates out proteins within the GST-RalGDS complex and specific antibodies can be used in Western blotting to identify the relative amount of bound Rap1 (or Ras). Since very little GDP-bound Rap1 can bind GST-RalGDS, the level of Rap1 recovered provides a useful index of Rap1 activation. This same assay can be used simultaneously to examine multiple proteins that bind RalGDS including Ras. Other GST-fusion have been designed over the years that display high affinities for selected G proteins. These fusion proteins can be utilized is similar manners to examine the activation state of other G proteins including Ras, Rac, Ral, and Rho (see Table 1).
(B) *In vivo* pull down assay. In this example, we measure the activation of Ras. Ras exists is an equilibrium between GTP-bound (active) and GDP-bound (inactive) states. Effectors like Raf-1 and RalGDS (see A) bind with much higher affinity to the GTP-bound (active) state of Ras (or Rap1, see A). This can be monitored using epitope-tagged (black rectangle) Ras. Polyhistidine-tagged Ras (His-Ras) is used in this example, although other epitopes can be used as well. Cells are transfected with His-Ras and lysates prepared following stimulation, for example with growth factors, to activate Ras. 1) Active GTP-bound Ras will remain associated with effectors, where as inactive GDP-bound Ras will not. 2) Both active and inactive Ras and bound effectors can be immobilized using Nickel affinity column, and 4) both active and inactive Ras eluted with imidazole (black rectangles), which competes with Histidine for the Nickel binding sites. 4) SDS-PAGE separates out active and inactive Ras from any bound effectors, including Raf-1, as shown in this example. These associated proteins can be detected by Western blot. In this example, Raf-1 is examined usinf Raf-1 antidsera. Other Ras effectors can be examined from the same eluates, including B-Raf, RalGDS, PI3-K and others using the appropriate antibodies. In this way, the levels of effectors detected by Western blot provide an index of the activation state of Ras. This is largely because transfected His-Ras is in excess. Note that this assay measures Ras activation state rather than Raf-1 activation state. For Ras addition signals may be needed in addition to recruitment of Raf-1 to Ras to achieve full activation of Raf-1. This is particularly relevant for Rap1 pull down assays. Rap1/Raf-1 pull downs are effective for monitoring Rap1 activation but are not appropriate to measure Raf-1 activation, since Raf-1 binding to Rap1 does not result in Raf-1 activation (Okada et al., 1999; Vossler et al., 1997).

Western blotting reflects Ras activation. This method has also been used to detect other Ras and Rap1 effectors in treated cellular lysates, such as B-Raf (Seidel et al., 1999; Vossler et al., 1997; York et al., 2000; York et al., 1998) (Grewal et al., 2000b).

As mentioned above, the physiological actions of both Ras and Rap1 require activation, or GTP-loading of the respective G proteins. Likewise, the association of RalGDS with Rap1 is dependent on the activation state of Rap1 itself. This is shown in Figsure A.3A and B. The GTP dependence of Rap1 association with the Ras binding domain of RalGDS is demonstrated in Fig. 3A. Here PC12 cells were transfected with cDNAs of an N-terminally FLAG-tagged version of Rap1 with or without a cDNA for Rap1GAP, a Rap1 specific GTPase-activating protein (Polakis et al., 1991; Quilliam et al., 1990). Rap1GAP1 can selectively reduce the levels of GTP-bound Rap1 when expressed ectopically within cells (Anneren et al., 2000; Reedquist et al., 2000; York et al., 2000). In this example, cAMP is used as a Rap1 activator (Altschuler et al., 1995) cAMP can activate Rap1 through specific cAMP-regulated exchangers or by PKA itself (de Rooij et al., 1998; Vossler et al., 1997). The actions of both cAMP and PKA can be mimicked by Forskolin, a potent activator of adenylyl cyclase. As shown in Figure A.3A, this activation can be measured by GTP loading assays (Vossler et al., 1997) and by RalGDS pull down assays. In this example FLAG-Rap1 is transfected into cells and the association of FLAG-Rap1 is monitored by Western blot using Flag antibodies. This use of epitope tagging to examine transfected Rap1 allows the simultaneous examination of co-transfected cDNAs. In this example, this approach is used to examine the action of Rap1GAP1 on Rap1 activation. When transfected cells are stimulated with Forskolin, intracellular cAMP levels are raised, leading to FLAG-Rap1 activation and increased association with GST-RalGDS (Figure A.3A). However, Rap1GAP1 blocks this association by stimulating the intrinsic GTPase activity of Rap1 thereby reducing the

level of Rap-GTP within the cell and decreasing the amount of Rap1 pulled down by GST-RalGDS. The GTP dependent association with RalGDS can also be shown directly using purified proteins *in vitro* (Figure A.3B). Purified HisRap1 associates with GST-RalGDS, but the amount of HisRap pulled down is enhanced when it is loaded with GTP-γ-S, a non-hydrolyzable analog of GTP. Since very little GDP-bound Rap1 binds GST RalGDS, the level of Rap1 protein that binds to RalGDS which is detected by Western blot provides a useful index of Rap1 activation, and provides a convenient non-isotopic method to examine stimuli that activate Rap1 *in vivo*. The purpose of this chapter is to provide protocols and points of discussion regarding the two non-isotopic methods outlined in Figure A.2 that are designed to detect Ras and Rap1 activation in mammalian cells.

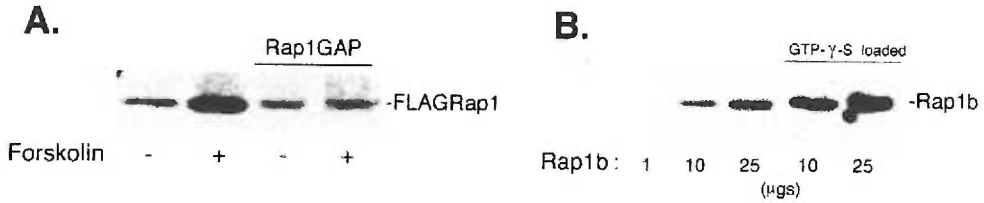

Figure A.3 GST-RalGDS binds GTP-bound Rap1. (A) GST-RalGDS as a measure of Rap1 activation. Untransfected PC12 cells or PC12 cells transfected with a cDNA encoding Rap1GAP were stimulated with forskolin, as indicated. This assay shows that the activation of Rap1 can be monitored by the GST-RalGDS assay. Moreover, the fold increase in Rap1 activation is blocked in cells expressing Rap1GAP. This demonstrates that GST-RalGDS is recognizing GTP-bound Rap1. The level of Rap1 detected in unstimulated cells is not a reflection of active Rap1 in the resting cells because this level is not further reduced by Rap1GAP. Rather, it reflects the low level of GDP-bound Rap1 that may be detected by GST-RalGDS. (B) The GST-RalGDS assay is sensitive to GTP loading. Baculovirally expressed Rap1b was purified and left unloaded, or loaded with GTPγS *in vitro*. The resultant protein was mixed with purified GST-ralGDS protein and the levels of Rap1 protein measured as described in Fig. A2. The data demonstrate that the loading of Rap1 with GTP increases the level of Rap1 recovered after the GST-RalGDS assay. Note that unloaded Rap1 does bind to a limited degree to GST-RalGDS. This may be because of the high levels of protein used in this assay, and may reflect the residual GTP-loading of Rap1b recovered from baculovirus.

Method 1: *In vitro* assay of small G proteins; Precipitating activated Rap by GST-RalGDS

Introduction

The method examined in this section is largely derived from the labs of J. Bos (Franke et al., 1997) and M. Gold (McLeod et al., 1998). Because this method relies on purified proteins that are not commercially available, we will discuss the bacterial purification of GST-RalGDS as well as its utilization in mammalian cell lysates.

Preparation of cell lysate

Subconfluent cultured cells that have been maintained in appropriate medium are serum starved in low serum containing medium (0.2-1% HS or FCS, depending on cell type) for up to 24 hours prior to stimulation. It is important to maintain cells at subconfluency, since Rap1 activation may be regulated by cell density (Posern et al., 1998). Cells are stimulated with the appropriate concentration of agonist for the desired time interval, rinsed twice in ice-cold (4°C) phosphate-buffered saline (PBS) on ice and then lysed in ice-cold (4°C) RalGDS lysis buffer (0.5ml/2x10^6cells) (50mMTris (pH=8.0) , 150mM NaCl, 1% (v/v) Non-idet P-40 (NP-40), 10% (v/v) glycerol, and 5 mM $MgCl_2$ with freshly added protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM aprotinin, 1μM leupeptin, 1mM sodium vanadate (Na_3VO_4), 10mM NaF, and 10μg/ml soybean trypsin inhibitor). Cell lysates are clarified by centrifugation for 2 minutes at 2000 rpm to remove insoluble debris. A small (1-10μl) aliquot is removed and protein levels for each condition are determined using the Bradford assay (Bradford, 1976). The cell lysates should be tested immediately. Also the amount of lysate needed

to detect Rap1-GTP varies depending on the strength of the signal induced by the stimuli being examined as well as the cell line used. This may not be only due to Rap1 protein levels within the cell, but may also be due to the particular Rap1 exchanger activated or the levels of endogenous Rap1GAP activity within the cell line examined. Because of these considerations, typically, experiments should begin with as much as 1 mg of total cell lysate.

Purification of RalGDS fusion protein

The Ras binding domain of RalGDS, consisting of amino acids (726-852) is expressed in DH5α bacteria as an N-terminally tagged GST fusion protein in pGEX-4T3. Ten mls of an overnight culture of pGEX-4T3-GST-RalGDS is added to 1 Liter of Luria broth (10gm tryptone, 5gm yeast extract, 10 gm NaCl per liter) with 50ug/ml ampicillin and grown to an OD_{600} of approximately 0.6 at 37°C. Expression of GST-RalGDS is induced by adding 1mM of isopropyl-β-D-thiogalactopyranoside, and the culture is incubated at 37°C for an additional 6 hours. The GST-RalGDS protein is stable using these conditions, although slightly higher yields are obtained by growing the cells at 30°C. for 8-12 hours.

The culture is harvested by centrifugation, and the bacterial pellets can be stored at -80°C. The bacterial paste is resuspended (0.1gm/ml) in ice-cold (4°C.) phosphate buffered saline containing 1mM dithiothreitol along with freshly added protease inhibitors (1mM PMSF, 1mM aprotinin, 5mM benzamidine, 10μM leupeptin, and 10μg/ml soybean trypsin inhibitor). Bacterial cell lysis is achieved by 3 passages through a french press, or by sonication (microtip, 10 times, 30 sec each). Triton X-100 is added to a final concentration of 1% (v/v) and the bacterial lysate is rocked at 4°C. for 30 minutes. Bacterial debris is pelleted at 12000xg for 10 minutes at 4°C., and the supernatant is transferred to a fresh tube. At this point, the GST-RalGDS can be

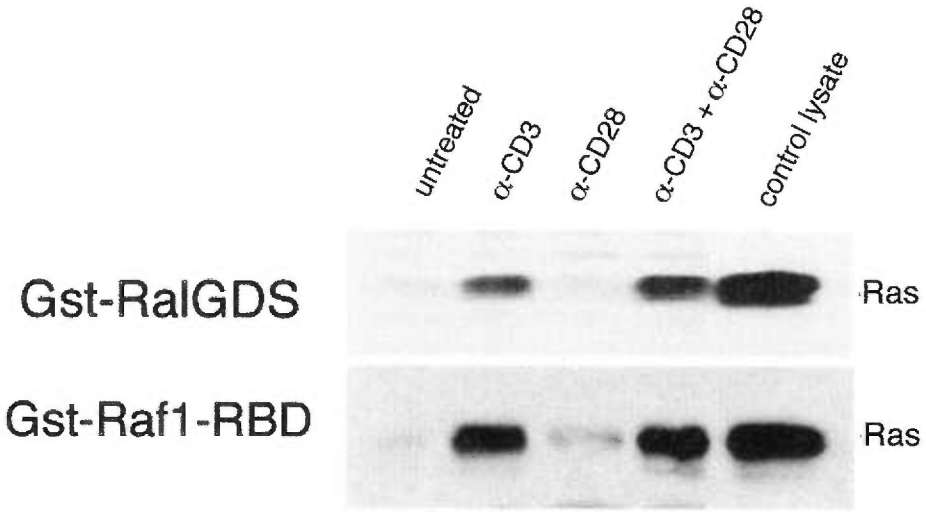

Figure A.4 Nonisotopic examination of Ras activation, using GST fushion proteins *in vitro*. Top: GST-RalGDS as a measure of Ras activation. Jurkat cells were incubated with anti-human CD3 (α-CD3) and/or anti-human CD28 (α-CD28) for 5 min. or left untreated as indicated. Lysates were prepared and assayed for Ras activation, using GST-RalGDS and Western blots performed with Ras antiserum, and the position of Ras is shown. Bottom: Raf1-RBD assay as a measure of Ras activation. Jurkat cells were incubated with anti-human CD3 (α-CD3), and/or anti-human CD28 (α-CD28), for 5 min. or left untreated as indicated. Lysates were prepared and assayed for Ras activation, using GST-Raf1RBD and Western blots performed with Ras antiserum. The position of Ras in control lysates before and after isolation of glutathione-bound proteins is shown. The data show that Ras is activated by cross-linking the T cell receptor (α-CD3) in the presence and absence of simultaneous CD28 activation. Equal amounts of control lysates serve to normalize the levels of Ras pulled down in this assay as compared with the GST-RalGDS assay shown (top). Note that the results using GST-Raf1RBD and GST-RalGDS are qualitatively similar.

separated from bacterial proteins by either affinity chromatography or by batch binding. For batch binding, 1ml/50ml of supernatant of a 1:1 slurry of glutathione-agarose is added and rocked at 4°C. for 1 hour. The GST-RalGDS/bead complex is pelleted at 2000rpm for 2 minutes. The beads are washed 3 times with ice-cold PBS with protease inhibitors, and the GST-RalGDS is eluted with 5mM reduced glutathione, 10mM Tris (pH 8.0), with protease inhibitors. The eluted GST-RalGDS is then dialyzed against PBS. The purity of the GST-RalGDS protein is estimated by Coomassie blue staining and should typically be between 85-95%. Typical yields range from 0.3-1mg of GST RalGDS per liter of bacteria. Some researchers have found that the GST-RalGDS protein stability can be increased if the aliquots of GST-RalGDS are maintained at high protein concentrations. Alternatively, aliquots of the unpurified bacterial extracts can be maintained in a frozen state. In this case, the bacterial extracts can be mixed directly with lysates from mammalian cells if necessary (personal communication, M. Gold (McLeod et al., 1998))

RalGDS assay

For each sample, add 40μg of purified GST-RalGDS protein to 0.5-1ml of cell lysate having 0.5mg-1mg of total cellular protein. We have found that using 40-60μg of purified GST-RalGDS protein works best for detecting Rap1 activation. This may be because the Ras binding domain of RalGDS will also efficiently pull down activated forms of other small G proteins which compete with Rap1 for binding to RalGDS, including Ras and Rap2 (Ohba et al., 2000; Reedquist and Bos, 1998) (Fig. 4B). Samples are rocked with the GST-RalGDS protein at 4°C. for 30 minutes. Add 30μl of a 1:1 (w/v) (beads:water) slurry of glutathione agarose beads (sigma) and continue to rock samples for an additional 30 minutes. Centrifuge samples for 2 minutes at 14,000xg to

pellet complexes. Rinse samples twice with ice-cold (4°C) RalGDS lysis buffer containing protease inhibitors as previously described. Proteins are eluted from the beads using 2x Laemmli buffer and applied to a 12% SDS polyacrylamide gel. Proteins are transferred to a PVDF membrane, blocked for 1 hour, and probed with a 1:1000 dilution of an anti-Rap1(Krev) antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA). overnight at 4°C., followed by an HRP-conjugated anti-rabbit secondary antibody. Rap1 is detected using enhanced chemiluminescence (DuPont-NEN). We find that it helps to run a non-stimulated total lysate control lane as a positive control for detection of Rap1 protein on Western blots.

Notes

1) An important variable to consider when designing experiments examining Rap1 activation is that the time course of Rap1 activation can vary depending both on the cell type and the stimulus. In PC12 cells, GTP loading of Rap1 is detectable when stimulated with NGF after 5 minutes, Rap1 activation peaks at 10-15 minutes, and slowly decreases thereafter (Figure A.5A). However, in Jurkat cells, Rap1 activation by stimulation with anti-CD3 antibodies is detectable after 30 seconds, is maximal at 2-5 minutes and returns to basal levels after 10 minutes (Reedquist and Bos, 1998). Both the amount of lysate to use and the time course of activation will depend on the cell type and the stimulus.

(A) This method can also be used to detect activation of epitope-tagged G proteins, like FLAG-Rap1, as shown in Figure A.3A. Technically, following transfection of FLAG-Rap1, cells are treated with specific stimuli and the activation state of FLAG-Rap1 is determined as before. It is important to note that GST-RalGDS will bind both endogenous GTP-bound Rap1 as well as GTP-bound FLAG-Rap1. To identify

transfected FLAG-Rap1 and endogenous Rap1, Western bloting is performed using an anti-FLAG antibody. Total Rap1 activation can also be monitored using antisera to Rap1, although the separation of endogenous Rap1 and FLAG-Rap1 on the Western blot may be technically demanding. As is shown in Figure A.5A, the time course of activation of Rap1 by NGF stimulation in transfected PC12 cells reflects the kinetics seen in endogenously stimulated cells (Anneren et al., 2000). These non-isotopic methods give similar results to exchange assays (Figure A.5B). However, it should be noted that exchange assays may not necessarily reflect the GTP-bound state of Rap1, since the GTP-bound state represents an equilibrium between exchange and GTPase action by Rap1 GAPs. These Rap1GAPs can be regulated independently by certain stimuli (Carey et al., 2000; Jordan et al., 1999; Mochizuki et al., 1999). Loading purified Rap1 with (3H)-GDP will ensure that endogenous GAP activity will not influence the GEF assay. In this case, the contribution of GAP activity to the activation state of Rap1 *in vivo* will not be measured.

(B) Although RalGDS binds tightly to Rap1, it has been suggested that it may be a physiological effector of Ras *in vivo* (Bos et al., 1997; Zwartkruis and Bos, 1999). Indeed, RalGDS binds to Ras, but with a lower affinity that it does to Rap1(Esser et al., 1998; Vetter et al., 1999). Because RalGDS can bind to multiple G proteins, these assays can be used simultaneously to examine the activation of other small G proteins that recognize the same effector proteins (see Figure A.4).

The peptide fragment of Raf-1 comprising the Ras binding domain of Raf-1 (Raf1-RBD) is now commercially available (Upstate Biotech, Inc, Lake Placid, NY) and provides a convenient non-isotopic method analagous to RalGDS for examining Ras activation.

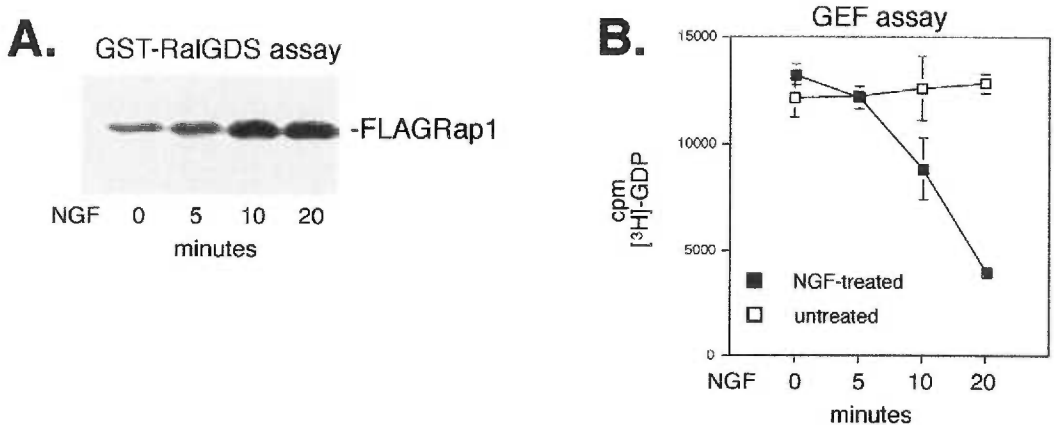

Figure A.5 Comparison of RalGDS assay and GEF assay. (A) PC12 cells transfected with FLAG-Rap1 were treated with NGF for the indicated times. RalGDS assays were performed on lysates and Western blots were probed for FLAG-Rap1, using anti-FLAG antibody. The position of FLAG-Rap1 is shown. (B) Untransfected PC12 cells were treated with NGF for the indicated times, lysates were prepared, and exchange assays were performed as described (Carey et al., 2000). Open squares represent unstimulated cells, and solid squares represent NGF-treated cells.

Method 2: *In vivo* Pull Down of Effectors

Introduction.

Generally, the activation of small G proteins can be monitored by the recruitment of effectors to the small G protein itself. This recruitment is essential for activation of the recruited molecule but is not necessarily sufficient (Avruch et al., 1994; Marais et al., 1995), and subsequent activation steps may be required (Marais et al., 1998). Indeed, not all molecules that are recruited to Rap1 are activated. For example, Raf-1 binds with high affinity to Rap1 but is not activated by Rap1 *in vivo* (Okada et al., 1999). In contrast, B-Raf binds with lower affinity to Rap1 than does Raf-1 (Okada et al., 1999), but is activated by Rap1 (Ohtsuka et al., 1996; Vossler et al., 1997). With this caveat, the recruitment of endogenous molecules like Raf-1 and B-Raf to Ras and Rap1 are powerful indicators of GTP-loading of Ras and Rap1, which provide an index for the activation state of Ras and Rap1 *in vivo*. In this section, the methods for examining Ras- or Rap1-associated proteins will be discussed.

Transfection and lysis.

Very little GDP-bound Rap1 associates with GST-RalGDS or Raf-1, even when Rap1 is overexpressed following transfection of His-Rap1 (see Figure A.3). This ability of transfected Rap1 proteins to retain the GTP-dependence of action forms the basis for another non-isotopic method used to detect Rap1 activation and Rap1 effectors.

For this assay, cells are transfected with epitope-tagged Rap1 using methods that have been optimized for that cell type. The choice of epitope depends on the method of recovery of tagged Rap1. Polyhistidine-tagged methods utilize metal-chelate resins, such

as nickel-nitrilotriacetic acid (Ni-NTA), from which associated proteins can be easily eluted for futher biochemical characterization (see Figure A.7). FLAG and myc epitopes have been successfully used for immunopreciptation. In both cases, the use of multiple (tandem) epitope tags increases the efficiency of FLAG immunoprecipitation. Subsequent to transfection, the cells are allowed to recover for 24 hours. It is important to have at least 30-50% transfection efficiency in order for this method to work well. Cells are rinsed twice with ice-cold PBS, and then lysed in ice-cold buffer containing 1% NP-40, 10mM Tris, pH 8.0, 20mM NaCl, 30mM $MgCl_2$, 1mM PMSF, 0.5mg/ml aprotinin, 0.1mg/ml leupeptin, 50mM NaF, 1mM Na_3VO_4 , and supernatant is prepared by low speed centrifugation. Lysates should be used immediatly.

Elution of Proteins associated with His-Rap1

For this example, we will utilize polyhistidine-tagged Rap1 (HisRap1) and recover His-Rap1 by Nickel affinity chromatography. Transfected His-tagged proteins are precipitated from supernatants containing equal amounts of protein using Ni-NTA agarose (Qiagen Inc. Chatsworth, CA.) and washed with 20mM imidazole in lysis buffer to remove non-specific binding interactions. Proteins are eluted with 500mM imidazole and 5mM EDTA in PBS. The eluates containing His-tagged proteins and effectors are separated on SDS-PAGE and endogenous Raf-1 proteins are detected by Western blotting. This protocol is designed for the detection of proteins that associate with His-tagged GTP-bound Rap1 or Ras, but it can be adapted for FLAG epitope-tagged proteins as well by using the RalGDS buffer and lysis conditions as described in the first section (Carey et al., 2000). Figure A.7 shows an application of this method for the detection of B-Raf association with His-tagged Rap1 in PC12 cells stimulated with agents that elevate intracellular cAMP (Vossler et al., 1997).

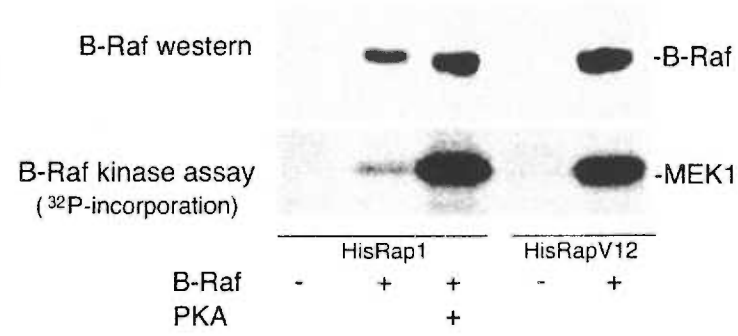

FIGURE A.6 Rap1 pull-down assay examining the association and activation of B-Raf with Rap1b. Using a pull-down assay with histidine-tagged Rap1 proteins, both the association of B-Raf as well as the kinase activity of associated B-Raf can be monitored. COS-7 cells were transfected with 10μg of His-Rap, 12 μg of B-Raf, 5 μg of PKA, or 10 μg of His-RapV12 as indicated. His-Rap proteins were precipitated with Ni-NTA-agarose and associated B-Raf was detected by Western blotting. The position of B-Raf is shown (top). In addition, associated B-Raf kinase activity was immunoprecipitated with B-Raf antiserum and kinase assays were performed with MEK1 as a substrate *in vitro* (bottom). The position of MEK-1 is shown. Note that in this example, the detection of B-Raf association with Rap1 is dependent on transfection of B-Raf, presumably because of the low levels of endogenous B-Raf in COS-7 cells. The data show the effect of transfection of the catalytic subunit of PKA on Rap1 activation. In addition, the GTP dependence of the association of B-Raf with Rap1 is examined by comparing the action of His-Rap1b (wild-type) with His-RapV12 (a constitutively activated mutant form of Rap1).

Notes

1) Endogenous G proteins can also be examined with the method if available immunopreciptating antibodies exist. While good antibodies are suitable for Ras (Y13-238) (Furth et al., 1982), currently there are no immunopreciptating antibodies for Rap1. Therefore, for Rap1, epitope-tagging provides the surest way to immunopreciptate proteins that bind GTP-bound Rap1.

2) The proteins that bind epitope-tagged Ras or Rap1 can be endogenous or transfected. Transfection allows the examinination of association with proteins that may not be expressed to high levels in the cell employed. This is the case in Fig. 7, where the association of B-Raf with Rap1 in COS-7 cells is dependendent on transfection of B-Raf (Vossler et al., 1997).

3) One advantage of the pull down assays for examining Ras and Rap1 function is that these methods examine the association of Ras or Rap1 with endogenous proteins *in vivo*. Because of this, additional biochemical studies can be performed on the eluates recovered from these pull downs. For example, the kinase activity associated with Rap1 can be examined. In Figure A.7, we show the examination of B-Raf activity within the eluates from a His-Rap1 pull down.

4) Because this method examines the association of endogenous proteins with transfected small G proteins, additional cDNAs can be transfected to examine the consequence of selected genetic manipulations on the recruitment of proteins to Ras or Rap1. The ability to co-transfect additional activators or interfering mutants can be very

informative. In Figure A.7, we show that Rap1 association with B-Raf can be enhanced by co-transfection of the cDNA encoding the catalytic subunit of PKA. Of the two techniques presented here, the RalGDS assay is primarily a method to examine endogenous Rap1 activation, although it can be modified to examine transfected Rap1 (see above). In the second pull down method, the examination of proteins that associate *in vivo* with transfected Rap1 works consistently in a variety of cells, and can be used to examine the association of transfected G proteins with endogenous or co-transfected proteins, as shown in Figure A.7.

Conclusions

We have described two simple non-isotopic methods for the detection of endogenous or transfected activated Rap1, which give similar results to isotopic methods. One method, the RalGDS assay allows examination of the activation state of endogenous Rap1, whereas the pull-down method is better suited for examination of epitope-tagged Rap1. These methods should be broadly applicable for the detection of the activation of a number of small G proteins.

Similar methods to the RalGDS method described are currently being utilized for other small G proteins, taking advantage of the selective binding of specific proteins to GTP-bound active forms of the G proteins. Using various epitope-tagged fusion proteins that display high affinities for selected G proteins, researchers have developed tools to examine the activation state of other G proteins including Ras, Rac, Ral, and Rho (Table 1). Like all Western blotting techniques, these methods are not rigourously quantitative. However, a recent report suggests an adaptation that can render the RalGDS method semi-quantitative (von Lintig et al., 2000).

TABLE 1

DETECTION OF SMALL G PROTEINS

USING EFFECTOR DOMAINS

G protein	Recognition Domain	Reference
Rap1a, Rap1b	Ras binding domain of RalGDS (aa 726-828)	(Franke et al., 1997)
Rap2	Ras binding domain of RalGDS (a.a. 726-828)	(Ohba et al., 2000) (Reedquist and Bos, 1998)
Harvey-Ras	N-terminus of Raf-1 (a.a. 1-147)	(Zhang et al., 1993)
Ral	Ral binding domain of RalBP1 (a.a. 397-518)	(Goi et al., 1999)
Rac1	p21-binding domain of PAK1 (a.a. 67-150)	(Benard et al., 1999) (Bagrodia et al., 1998)
Cdc42	p21-binding domain of PAK1 (a.a. 67-150)	(Benard et al., 1999) (Bagrodia et al., 1998)
RhoA	Rho-binding domain of Rhotein (a.a. 7-89)	(Ren et al., 1999)

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