MEMBRANE LOCALIZATION OF RASGRPs BY C1 DOMAINS

by

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ABSTRACT

Ras and Rap GTPases are membrane-bound activators of signal transduction pathways that regulate several cell processes including proliferation, apoptosis and adhesion. Guanine nucleotide exchange factors (GEFs) positively regulate Ras and Rap GTPases by exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP). In order to activate Ras and Rap GTPases, GEFs must be at the same membrane compartments where their target GTPases are located. The Ras guanine nucleotide releasing protein (RasGRP) family of four GEFs regulate both Ras and Rap GTPases, with differential specificities. All RasGRPs contain C1 domains, which have the potential to bind the lipid second messenger diacylglycerol (DAG) that is generated at membranes in response to the ligation of many cell surface receptors. Binding of their C1 domains to DAG could serve to co-localize RasGRPs with membrane bound Ras and Rap GTPases. While some evidence exists for each member of the RasGRP family being potentially regulated by their C1 domains binding to DAG, there is contradictory evidence for RasGRP2. My thesis research focused on C1 domain-mediated mechanisms of RasGRP membrane localization, with special focus on RasGRP2. I found that the C1 domains of RasGRP2 and the β splice variant of RasGRP4 do not bind either DAG or phorbol ester, a DAG analog. However, all RasGRP C1 domains were shown to bind anionic phospholipids. I determined that the C1 domain of RasGRP2 is required for constitutive plasma membrane localization in NIH 3T3 fibroblasts and T-cells, and also for translocation to the plasma membrane in SDF-1α-stimulated T-cells. I also identified a putative PDZ protein binding site which is required for RasGRP2 localization at the Golgi. My experiments showed that while RasGRP2 localization can occur at the plasma membrane and Golgi of NIH 3T3s, RasGRP2 mediated Rap1 activation at the plasma membrane via its C1 domain is required for changes in cell morphology that are induced by RasGRP2 expression. My thesis research has demonstrated that all four members of the RasGRP family utilize their C1 domains to localize to membranes, although in the case of RasGRP2 this occurs via a DAG-independent mechanism, which targets RasGRP2 to the plasma membrane.

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ADAP, adhesion and degranulation promoting adapter protein

APC, antigen-presenting cell

BCR, B-cell receptor

BMC, bone marrow cell

CFP, cyan fluorescent protein

CRD, cysteine rich domain

DAG, diacylglycerol

DMEM, Dulbecco's modified Eagle's medium

ECM, extra-cellular matrix

EGF, epidermal growth factor

ER, endoplasmic reticulum

ERK1/2, extracellular signal-regulated kinase 1/2

FRET, fluorescence resonance energy transfer

GAP, GTPase activating protein

GDP, guanine diphosphate

GFP, green fluorescent protein

GEF, guanine nucleotide exchange factor

GRP, guanine nucleotide releasing protein

GST, glutathione S-transferase

GTP, guanine triphosphate

HEV, high endothelial venules

HVR, hypervariable region

ICAM-1, intercellular adhesion molecule-1 (ICAM-1)

IP3, inositol trisphosphate

KSR, kinase suppressor of Ras

LAD, leukocyte adhesion deficiency

LFA-1, lymphocyte-function-associated antigen-1

LPA, lysophosphatidic acid

MAPK, mitogen activated protein kinase

MDCK, Madin Darby canine kidney

MEK1/2, MAPK/ERK kinase 1/2

MHC, major histocompatibility complex

PA, phosphatidic acid

PC, phosphatidylcholine

PDZ, post synaptic density protein (PSD95), drosophila disc large tumor suppressor (DlgA), and zonula occuldens-1 protein (Zo-1)

PDZB, PDZ-binding motif

PE, phorbol ester

PG, phosphatidylglycerol

PI3K, phosphatidylinositol 3-kinase

PIPs, phosphatidylinositol phosphates

PKC, protein kinase C

PLC, phospholipase C

PMA, phorbol 13-acetate

PS, phosphatidylserine

PT, plasma membrane targeter

RBD, Ras binding domain

RTK, receptor tyrosine kinase

SKAP55, Src kinase-associated phosphoprotein of 55 kDa

SDF-1α, stromal cell-derived factor-1α

SLV, sucrose-loaded large unilamellar vesicles

SPA-1, signal-induced proliferation associated gene 1

TCR, T-cell receptor

VCAM-1, vascular cell adhesion molecule-1

VLA-4, very late antigen-4

YFP, yellow fluorescent protein

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CO-AUTHORSHIP STATEMENT

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A version of *Chapter 3* will be submitted for publication: Goulding RE and Kay RJ. Localization of RasGRP2 is specified by the C1 domain and a potential binding site for PDZ proteins. All experiments in this chapter were carried out by Rebecca Goulding. Robert Kay made the following RasGRP2 constructs: GFP-tagged RG2, RG2ΔC1, RG2C1, RG2C1x2, RG2-PBμ, RG2-pr and RG2-1C1. Rebecca Goulding made the following constructs: GFP-tagged RG2-C-term and RG2-C1+C-term.

Rebecca Goulding .....

Robert Kay .....

CHAPTER 1: INTRODUCTION

1.1 COMPARTMENTALIZATION OF CELL SIGNALING AT MEMBRANES

Signal transduction pathways transform external stimuli from simple messages into complex internal cellular processes. These outside-in molecular communication strategies are commonly initiated via peripheral stimulation of cell surface receptors by specific ligands such as growth factors, cytokines and hormones. Following initial receptor stimulation, an ordered cascade of intracellular biochemical events, involving an array of signal transducers, transmit information and help to elicit a response. The organizational complexity of such a system is vast, and cell organelles and macromolecular components play a large role in governing signaling hierarchy. Lipid membranes act as effective signaling platforms because they are sites where signaling proteins can be anchored, concentrated and organized. Lipid second messengers are produced and retained at membranes from existing membrane components. The cell has a diversity of membranes including the plasma membrane, endoplasmic reticulum, Golgi apparatus and nuclear envelope. Specific signaling proteins can localize and concentrate at specific membranes; therefore membranes function to organize signal transduction pathways into separate compartments. Different cellular responses may be dependent on where particular signal transduction pathways are activated; therefore compartmentalization of signal transduction in a membrane-specific manner has the potential to govern the output of such signaling pathways.

1.2 COMPARTMENTALIZATION OF RAS GTPASE SIGNALING AT MEMBRANES

1.2.1 Ras and Rap GTPases are membrane bound signal transducers

Ras and Rap GTPases, which are members of the Ras subfamily of small GTPases, are examples of membrane localized signal transducers. GTPases function as molecular binary switches which cycle between GDP-bound (inactive) and GTP-bound (active) states. Ras subfamily GTPases play a central role in the direction of cell signaling pathways that are critical for cell processes including proliferation, differentiation, apoptosis, adhesion and locomotion (Figure 1.1). Ras, Rap and other Ras subfamily GTPases are constitutively localized at membranes. In their active GTP-bound state they localize and activate their

target effectors, which are themselves signal transducers [reviewed in (Mor and Philips, 2006)]. Ras GTPase isoforms (H-Ras, N-Ras and K-Ras4B; also known as "classical" Ras) can bind the same effectors, yet knockouts of the different isoforms lead to different phenotypes (Esteban et al., 2001; Johnson et al., 1997). It is still unclear how functional specificity of different isoforms can be achieved, however recent studies support the notion that different membrane localization of Ras isoforms in the cell may account for their specific signaling outputs.

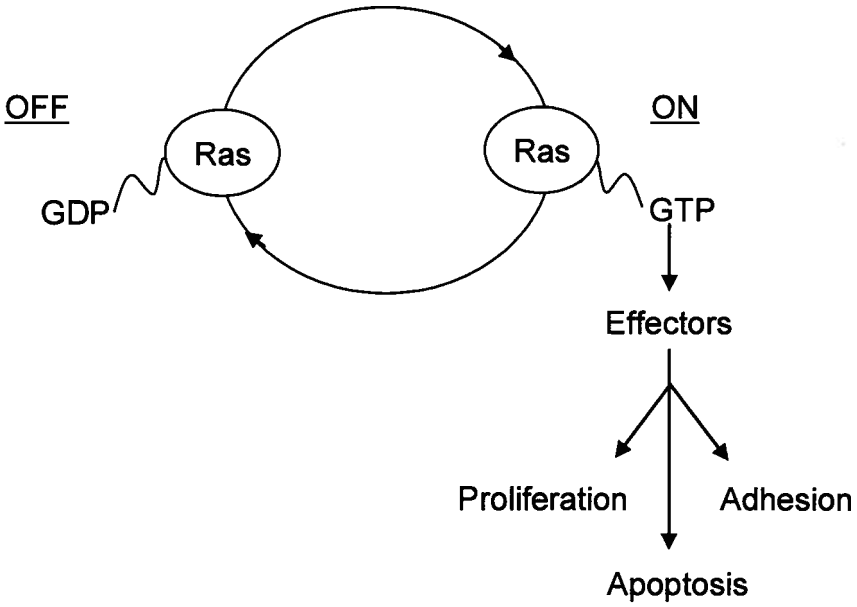

Figure 1.1 Ras GTPases function as a molecular binary switches

In its "OFF" form, Ras-GDP is thought of as inert. The "ON" and thus active form of Ras, Ras-GTP, activates effectors that are involved in several signal transduction pathways, including those that regulate proliferation, apoptosis and adhesion.

1.2.2 Mechanisms of Ras and Rap membrane localization

Membrane localization of classical Ras (H-Ras, N-Ras and K-Ras4B) and Rap (Rap1A and B; Rap2A, B and C) proteins is thought to be required for their biological activity [reviewed in (Colicelli, 2004; Takai et al., 2001)]. These GTPases have neither membrane localization sequences nor hydrophobic membrane spanning domains and are synthesized on free ribosomes as hydrophilic proteins. They achieve appropriate membrane localization through a number of post-translational lipid modifications [reviewed in (Mor and Philips, 2006)]. Classical Ras proteins exhibit close to 100% homology at their N-termini, but in contrast their C-termini are characterized by a hypervariable region (HVR) (Figure 1.2). Overall, Rap1 proteins are 97% homologous while Rap2 proteins are 90% homologous, and like Ras GTPases, Rap GTPases have regions of hypervariability at their C-termini. Within the HVR, Ras and Rap GTPases have a C-terminal CAAX prenylation signal (C = Cysteine, A = aliphatic amino acid, X = any terminal amino acid), which is prenylated in the cytosol by one of two soluble prenyltransferases. Prenyltransferases covalently attach a farnesyl or geranylgeranyl lipid by a stable thioether linkage to the CAAX cysteine. The substrate specificity for farnesyltransferase versus geranylgeranyltransferase depends on the X residue of the CAAX motif. Classical Ras proteins have the signature CAAX motif, where X = serine or methionine, which allows recognition by farnesyltransferase (see Figure 1.2), while Rap1A and Rap1B have CAAL (L= leucine) sequence at their C-termini, and are recognized by geranylgeranyltransferase [reviewed in (Zhang and Casey, 1996)]. Rap2A terminates in CAAQ (Q=Glutamine), and is farnesylated, while Rap2B terminates in CAAL and is geranylgeranylated (Farrell et al., 1993). Newly discovered Rap2C (Paganini et al., 2006) ends in CAAQ and therefore is predicted to be farnesylated (Guo et al., 2007). Prenylation is followed by -AAX proteolysis by Ras-converting enzyme (Rce1) and C-terminal carboxylmethylation, catalyzed by isoprenylcysteine methyltransferase (ICMT). Rce1 and ICMT are associated with the endoplasmic reticulum (ER), which is where both of these two processes take place. Together all three modifications, collectively termed carboxyl-terminal CAAX processing, give rise to a hydrophobic C-terminus that can insert into membranes.

Figure 1.2 Classical Ras and Rap GTPase hypervariable regions (HVRs)

Cysteine (C) palmitoylation and prenylation of the hypervariable regions function to target Ras and Rap GTPases to membranes. In the case of K-Ras4B, Rap1A and Rap1B, polybasic stretches of amino acid residues lysine (K) or arginine (R) are thought to interact with anionic phospholipids via electrostatic interactions, thereby stabilizing membrane binding. **K, R**: basic residues **C**: palmitoylated residues; ***C***: prenylated residues; CAAS/M/Q: motif is farnesylated; CAAL: motif is geranylgeranylated.

While CAAX processing targets Ras and Rap GTPases to the ER, these modifications are insufficient for plasma membrane targeting (Choy et al., 1999). In the case of H-Ras, N-Ras and Rap2 proteins, plasma membrane localization is achieved through further modification of the HVR. One (N-Ras) or two (H-Ras, Rap2) palmitoyl residues are added to cysteines adjacent to the farnesyl group in the HVR (Figure 1.2), in a reaction catalyzed by palmitoyl acyltransferases (PAT). Palmitoylation serves to further increase plasma membrane affinity of the hydrophobic CAAX processed C-termini, via insertion of the palmitoyl groups (Figure 1.3). Palmitoylation also allows H-Ras and N-Ras to be transported via Golgi to the plasma membrane, via normal secretory pathways (Choy et al., 1999), and the same may be true for Rap2 proteins. H-Ras and N-Ras can be subsequently depalmitoylated and then transported in retrograde fashion back to the Golgi [reviewed in (Mor and Philips, 2006; Omerovic et al., 2007)]. Rap2 proteins are predicted to be similarly transported back to the Golgi.

K-Ras4B and Rap1 (A and B) proteins do not undergo palmitoylation to achieve plasma membrane localization, but instead have a stretch of basic amino acids (polybasic domain) proximal to the CAAX motif, which bind to anionic phospholipids non-specifically

via electrostatic interactions (see Figure 1.2, Figure 1.3). K-Ras4B and Rap1 are transported from the endoplasmic reticulum to the plasma membrane by a distinct and as yet undetermined alternative transport mechanism. K-Ras4B can also be rapidly transported back to the Golgi, and this is thought to be facilitated by protein kinase C (PKC) phosphorylation of the C-terminal HVR (Mor and Philips, 2006). It is not clear whether Rap1 also undergoes retrograde transport from the plasma membrane to the Golgi.

While H-Ras and N-Ras can localize to both the plasma membrane and Golgi, N-Ras has been found to localize more strongly to the Golgi than the plasma membrane in Madin-Darby canine kidney (MDCK) cells (Rocks et al., 2005). This is thought to be due to the fact that N-Ras has only one palmitoylate modification, whereas H-Ras has two. In contrast, K-Ras4B is restricted to the plasma membrane in the same cells (Rocks et al., 2005), most likely because of its polybasic domain within the HVR. While Rap1 has also been reported to localize in several different cell lines and tissues at the Golgi (Beranger et al., 1991a; Nomura et al., 2004) and to the plasma membrane (Bivona et al., 2004), its localization at late endosomes, (Bivona et al., 2004; Pizon et al., 1994; York et al., 2000) differentiates it from other Ras GTPases. Rap2 GTPases have been found to localize at the plasma membrane, Golgi (Ohba et al., 2000b; Pizon et al., 1994) and ER (Beranger et al., 1991b).

In summary, lipid modification targets H-Ras, N-Ras, Rap1 and Rap2 GTPases to both the plasma membrane and Golgi, K-Ras4B to only the plasma membrane, while Rap1 is targeted to endosomes and Rap2 is targeted to the ER. Thus, Ras and Rap GTPases can be found localized at diverse membrane compartments.

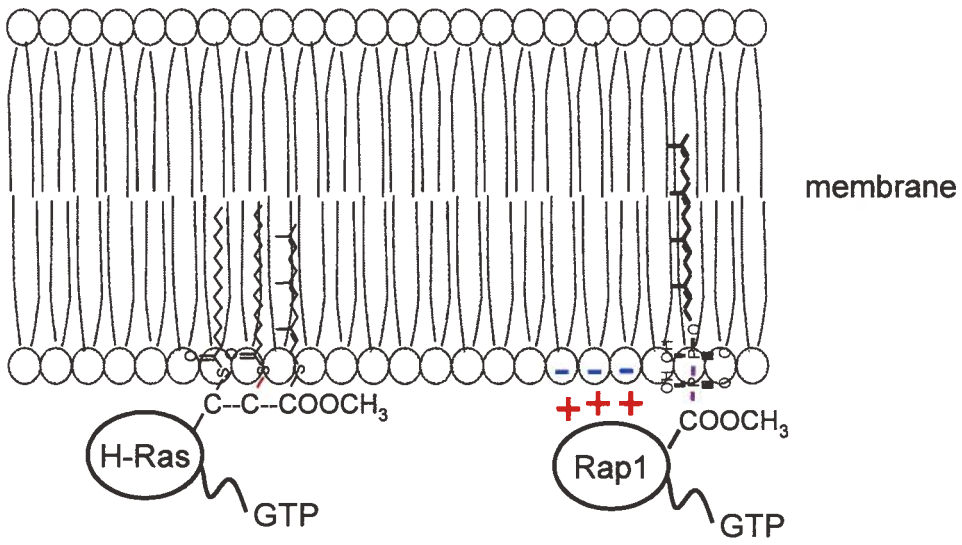

Figure 1.3 H-Ras and Rap1 GTPase membrane localization

H-Ras GTPase is targeted to membranes via farnesylation of the C-terminus, and palmitolaytion (double) of the hypervariable region. Rap1 GTPase is geranylgeranylated at its C-terminus and has a polybasic stretch of amino acids that interact with anionic phospholipids present in membranes.

1.2.3 Activation of Ras and Rap GTPases at specific membrane locations

The plasma membrane compartment was thought to be the definitive site for Ras and Rap activation due to the proximity of surface receptors capable of activating these GTPases. The advent of a method which detects activated Ras or Rap directly, by fluorescent microscopy, has allowed in depth analysis of where Ras and Rap protein activation really occurs. The Ras Binding Domain of Raf-1 (RafRBD) selectively binds activated GTP-bound Ras with an affinity 10^4 fold higher than for inactive GDP-bound Ras (Taylor et al., 2001). Philips' group created a green fluorescent protein (GFP) fusion protein with RafRBD (GFP-RafRBD) which they expressed in COS-1 cells and observed its membrane distribution in response to epidermal growth factor (EGF) stimulation (Chiu et al., 2002). This probe for GTP-bound Ras is distributed equally throughout the cell in serum-starved, unstimulated cells (with some concentration at the Golgi). In response to EGF stimulation, GFP-RafRBD first translocates to the plasma membrane after 3-10 minutes, and then relocates to the Golgi

after 10-20 minutes (Chiu et al., 2002). Using the same probe, Philips' group went on to discover that in response to T-cell receptor (TCR) stimulation of Jurkat T-cells, membrane specific Ras activation occurs exclusively at the Golgi (Bivona et al., 2003; Perez de Castro et al., 2004). This finding was further dissected by the same group using fluorescence resonance energy transfer (FRET) microscopy experiments co-expressing cyan fluorescent protein (CFP)-tagged Ras proteins with yellow fluorescent protein (YFP)-tagged RafRBD, in Jurkat T-cells and primary human T-cells. When CFP is in close proximity (less than 5 nm apart) to YFP and upon excitation of CFP at 436 nm (which would otherwise not excite YFP), a transfer of energy occurs from CFP (donor) to YFP (acceptor), leading to an emission from YFP at 535 nm, indicating co-localization of CFP and YFP. This study revealed that while H-Ras and N-Ras are localized at both plasma membrane and Golgi, they are only activated on the Golgi in response to TCR stimulation (Mor et al., 2007). In addition, under the same conditions, K-Ras localizes to and remains inactive at the plasma membrane. Remarkably, robust H-Ras, N-Ras and K-Ras activation at the plasma membrane was found to occur when cells were co-stimulated with TCR and the integrin molecule intracellular adhesion molecule-1 (ICAM-1), which binds and activates the integrin molecule lymphocyte-function-associated adhesion-1 (LFA-1). Co-stimulation also led to activation of H-Ras and N-Ras, but not K-Ras at the Golgi (Mor et al., 2007). Ras activation has also been demonstrated to occur at other membranes, for example on endosomes (Jiang and Sorkin, 2002) and the ER (Chiu et al., 2002). To summarize: specific signals can lead to membrane-specific Ras activation; for example, H-Ras is localized at both the plasma membrane and Golgi, but is only activated on the Golgi in response to TCR stimulation. Therefore membrane localization of Ras is not the only determinant that specifies activation. Work done in Jurkat T-cells showed that RNA silencing of CAPRI, a plasma membrane-localized negative regulator of Ras, led to H-Ras activation at the plasma membrane in response to antigen activation of the TCR (Bivona et al., 2003). Thus specific membrane-targeted negative regulators of Ras are able to downregulate activation of Ras at particular membranes. The same may also be true for positive regulators of Ras.

Although little is known about membrane-specific activation of Rap2, there is now considerable information about where Rap1 activation occurs, particularly in T-cells.

Analysis of Jurkat T-cells co-expressing fluorescently-tagged Rap1 and a fluorescently-tagged probe for activated Rap GTPases, the Rap binding domain of RalGDS (RalGDSRBD), revealed that despite the fact that expression of GFP-Rap1 was strong on intracellular membranes and weak on the plasma membrane, activation is only observed at the plasma membrane in cells grown in serum (Bivona et al., 2004). In addition, Rap1 is activated only at the plasma membrane of Jurkat T-cells in response to TCR stimulation (Bivona et al., 2004). Rap1 activation at the plasma membrane in response to TCR stimulation has been confirmed with similar experiments in other T-cells lines, although internal membrane activation of Rap1 was not detectable in the method of microscopy used (Medeiros et al., 2005). While Rap1 activation seems to only occur at the plasma membrane in response to TCR stimulation, disruption of cytosolic adapter protein complex of SKAP55 (Src kinase-associated phosphoprotein of 55 kDa) and ADAP (adhesion and degranulation promoting adapter protein) led to abrogation of Rap1 localization at the plasma membrane after TCR ligation, without affecting the overall activation of Rap1 (Kliche et al., 2006). These results suggest that the ADAP/SKAP55 complex is involved in recruiting activated Rap1 to the plasma membrane. Therefore the possibility remains that Rap1 could be activated elsewhere, e.g. at internal membranes and could subsequently translocate to the plasma membrane. There is also evidence of Rap1 activation at endosomes (Hisata et al., 2007; Wu et al., 2001; Wu et al., 2007), which may represent a pool of activated Rap that could play a role at this location, or be transported to the plasma membrane. Taken together, these studies show that Rap1 is similar to Ras GTPases in so much that activation occurs at selective membranes in response to signaling and that predominant sites of activation do not necessarily correlate with the predominant sites of steady state localization. This gives rise to the hypothesis that membrane targeted regulators are in part responsible for this pattern of activation. Once active, Ras and Rap GTPases can activate their target effector proteins, and thus propagate signal transduction pathways.

1.3 SIGNAL TRANSDUCTION FROM RAS AND RAP GTPASES

1.3.1 Ras activation of the Raf-ERK pathway

Signals transduced by Ras activation stimulate several effector pathways which critically regulate downstream cellular responses. The best characterized effector pathway activated by Ras signaling is the Raf-ERK (extracellular signal-regulated kinase) pathway [reviewed in (Leicht et al., 2007; Wellbrock et al., 2004)]. Raf phosphorylates and activates mitogen activated protein kinase (MAPK)/ERK kinase (MEK)1 and/or 2. Active MEK1/2 phosphorylates ERK1 and/or 2 (ERK1/2), which translocate to the nucleus to phosphorylate Ets family transcription factors, for example Elk-1. Raf knockout and transgenic mouse models as well as cell culture studies have revealed a role for the Raf- ERK pathway in a number of cellular processes such as proliferation, differentiation, survival and apoptosis [reviewed in (Galmiche and Fueller, 2007; Leicht et al., 2007)]. There are three Raf kinases, A-Raf, B-Raf and C-Raf, and knockout studies have shown that these Raf isozymes may have specialized function in normal cell processes [reviewed in (O'Neill and Kolch, 2004)]. For example, B-Raf may be the most potent promoter of proliferation as a MEK1/2 activator, while C-Raf may protect against apoptosis in a MEK1/2 independent manner (Huser et al., 2001; Mikula et al., 2001). H-Ras, N-Ras and K-Ras4B have differences in their binding affinities for Raf isozymes, which presents a potential for differential regulation (Weber et al., 2000).

1.3.2 Ras activation of the Raf-ERK pathway at specific membranes

In its GTP bound form, Ras binds to the Ras binding domain of Raf (RafRBD), thereby recruiting Raf from the cytosol to membranes (Figure 1.4). It is widely believed that the ability of Raf to activate MEK1/2 and then ERK1/2 is dependent on membrane localization; Hancock's group recently found that a constitutively activated form of Raf which is unable to bind Ras and remains in the cytosol, is incapable of activating the ERK pathway (Tian et al., 2007). Membrane localization of Raf and an auxiliary scaffold protein; kinase suppressor of Ras (KSR), facilitates the binding of MEK1/2, and this complex provides a platform for ERK1/2 binding to MEK1/2.

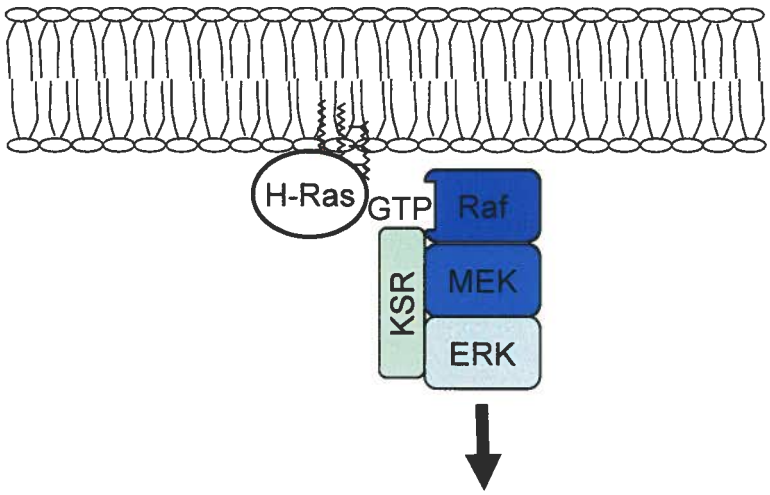

Figure 1.4 Ras signaling to the Raf-ERK pathway

When localized to membranes, GTP loaded H-Ras binds to the Ras binding domain (RBD) of Raf kinases (A-Raf, B-Raf and C-Raf), thus localizing these effectors to membranes where Ras is bound and activated. When active, Raf kinases can bind to and phosphorylate MEK1/2. KSR stabilizes the Raf:MEK:ERK complex. Activated ERK1/2 translocates to the nucleus to activate (phosphorylate) Ets transcription factors.

Membrane specific localization of Ras GTPases may serve to modulate Raf-ERK pathway outputs. Restriction of Ras to different internal membranes has been shown to affect Raf-ERK pathway activation (Chiu et al., 2002). Golgi targeting of a constitutively activated form of H-Ras (H-Ras61L), leads to strong activation of ERK1/2, whereas ER targeting results in weak ERK1/2 activation (Chiu et al., 2002). One explanation for this is that different membranes are sites of different levels and functional modes of regulators of the Ras to ERK pathway, such as levels of MEK1/2 or ERK1/2 deactivating phosphatases. The cytosol has increased levels of MEK1/2 and ERK1/2 dephosphorylation compared to the plasma membrane (Harding et al., 2005), and the same may be true when comparing membrane sites.

Membrane specific restriction of Ras activation has also been shown to affect other Ras effector signaling pathways. Golgi targeting of an activated H-Ras, also gives rise to

strong activation of the phosphatidylinositol 3-kinase (PI3K) pathways, while ER targeting leads to weak PI3K pathway activation (Chiu et al., 2002). Thus, signaling output from Ras activation of effector pathways can be dependent on which membrane these signaling events occur. Because of this, regulatory events that determine selective membrane activation of Ras have the potential to affect cellular responses to Ras-activating signals.

1.3.3 Rap1 activation and inhibition of the Raf-ERK pathway

Rap1 also couples to the Raf-ERK pathway. Rap1 has an identical effector domain to Ras, and is able to bind Raf kinases. Rap1 binds and activates B-Raf, leading to downstream ERK1/2 activation in response to a number of extracellular signals (Vossler et al., 1997; York et al., 1998). Paradoxically, several groups showed that ectopic Rap1 expression can interfere with Ras-mediated ERK1/2 activation *in vitro* (Boussiotis et al., 1997; Cook et al., 1993; Dillon et al., 2005; Mochizuki et al., 1999). These opposing effects appear to be due to differences in the interaction strength between Rap1 and the two Raf cysteine rich domains (CRD) (Okada et al., 1999). The interaction with Ras with Raf proteins is mediated by the Ras binding domain (RBD) and CRD of Raf, and binding of Ras to both regions is required for full Raf activation. In the case of B-Raf, Rap1 binds CRD with a similar affinity as does Ras, and is also able to activate this Raf. B-Raf activation can thus then be carried out by both Ras and Rap1. However, Rap1 binds the C-Raf CRD with much higher affinity than Ras (Okada et al., 1999), and thereby inhibits Ras binding to C-Raf. In addition, Rap1 is unable to activate C-Raf and so Rap1 binding to the CRD of C-Raf inhibits its activation. While B-Raf is selectively expressed in neuronal and hematopoietic cells (Eychene et al., 1995), C-Raf is ubiquitously expressed. Therefore the ability of Rap1 to activate or inactivate the Raf-ERK pathway is thought to depend on which Raf it interacts with, and thus is cell type specific.

Further insight into how Rap1 differentially regulates the Raf-ERK pathway in different cell types came from experiments with mice deficient for SPA-1 (signal-induced proliferation associated gene 1), a GTPase activating protein (GAP), which negatively regulates Rap. $SPA^{-/-}$ mice develop latent myeloproliferative stem-cell disorders, and a majority of these mice developed aggressive lethal leukemia with abundant blast cells of

either a myeloid or an erythroid lineage (Ishida et al., 2003a). Preleukemic bone marrow cells (BMCs) demonstrated increased Rap1GTP levels as well as constitutive ERK activation, but not Ras activation. Given that B-Raf is expressed in myeloid precursors and plays a pivotal role in myelopoiesis (Kamata et al., 2005), deregulated Rap1 activation of ERK may occur via B-Raf in preleukemic bone marrow cells (BMCs), and this may in part lead to an enhanced expansion of $SPA^{-/-}$ hematopoietic progenitors. During blast crisis however, Rap1GTP, RasGTP and phosphorylated ERK levels are all greatly increased. Reconstitution of a cell line generated from a $SPA^{-/-}$ mouse with CML in crisis with wild type SPA-1, reduced Rap1 activation but not RasGTP or phosphorylated ERK levels, suggesting that Rap1 activation and Ras-ERK1/2 activation are independent at this stage of disease. Another interesting finding was that $SPA^{-/-}$ mice exhibit an age-dependent progression of T-cell unresponsiveness (Ishida et al., 2003b). In this study, stimulation of T-cells from SPA-/- mice lead to persistent Rap1 activation, but showed defective Ras-ERK1/2 activation. For the most part T-cells do not express B-Raf (Kometani et al., 2004) and TCR-induced anergy (an irreversible inactive state resulting from lack of co-receptor signaling during T-cell receptor stimulation) is prevented in cells ectopically expressing B-Raf (Dillon et al., 2003). The T-cell anergy observed in $SPA^{-/-}$ mice is likely to result from TCR-induced Rap1 inhibition of C-Raf-ERK1/2 signaling and these results are supported by earlier findings from several *in vitro* studies indicating that Rap1 inhibition of the Raf-ERK pathway plays a role in T-cell anergy (Boussiotis et al., 1997; Reedquist and Bos, 1998). Of further interest is the possibility that the resultant T-cell immunodeficiency may lead to an impaired immune surveillance which could be linked to the onset of the overt myeloid leukemia in $SPA\text{-}1^{-/-}$ (Kometani et al., 2004).

1.3.4 Rap1 activation of the Raf-ERK pathway at specific membranes

Recent research has shown that Rap1 activation at the plasma membrane results in robust ERK1/2 activation, whereas activation of Rap1 at internal membranes is incapable of activating ERK1/2 (Wang et al., 2006). This suggests that like Ras, membrane specific activation of Rap has consequences with respect to downstream effector activation. Thus, like Ras, signaling output can be determined by membrane compartmentalization of Rap

activation and membrane localized regulators of Rap GTPases are possible candidates that can achieve this signaling segregation.

1.4 RAP GTPASES AND ADHESION

1.4.1 Role of Rap GTPases and integrin mediated adhesion

While Rap1 has received attention for its role in activation and inhibition of the Raf-ERK pathway, this GTPase has also been found to play a central role in a number of processes regulating cell adhesion, including integrin mediated cell adhesion and cadherin mediated cell-cell junction formation [reviewed in (Bos, 2005; Kinashi, 2005; Kooistra et al., 2007)]. While their role is less defined, Rap2 GTPases are believed to play a role in lymphocyte cell migration (McLeod et al., 2002), and integrin mediated adhesion in B lymphocytes (McLeod et al., 2004) and neutrophils (Jenei et al., 2006).

Integrins are cell surface receptors that mediate adhesion of cells to other cells or the extra-cellular matrix (ECM). Lymphocyte stimulation by antigen receptors (e.g. T-cell receptor or B-cell receptor) or chemokine receptors (e.g. CXCR4) leads to integrin activation by a process called inside-out signaling, which ultimately changes both the structural conformation of the integrin molecule from a low to a high affinity state, and increases the integrin valency (also known as clustering or density). Together, these two outcomes of inside-out signaling result in increased avidity of integrins for their adhesion molecule counterparts expressed on the surface of other cells or present in the ECM [reviewed in (Kinashi, 2005)].

In lymphocytes, Rap1 is involved in the regulation of several inter-related integrin mediated adhesion processes including migration, cell spreading, cell polarity and immune synapse formation [reviewed in (Bos et al., 2001)]. Expression of a constitutively active mutant of Rap1, Rap1V12 (in which Rap1 has a valine residue at position 12), was shown to increase adhesion of lymphocytes, by augmenting the avidity of the very late antigen-4 (VLA-4) integrin molecule for its integrin counterpart molecule, vascular cell adhesion molecule-1(VCAM-1) (Sebzda et al., 2002).

Furthermore, as previously mentioned with Ras GTPases, activated Rap1 increases the affinity of binding of the integrin molecule LFA-1 to its ligand, intercellular adhesion molecule-1 (ICAM-1) and induces the conformational changes that are associated with increased affinity (Katagiri et al., 2000; Reedquist and Bos, 1998). Rap1A and Rap1B have been shown to have different roles in integrin mediated adhesion. Rap1A knockout mice have mild defects in T- and B-cell integrin mediated cell adhesion (Duchniewicz et al., 2006) whereas Rap1B deficient mice exhibit reduced integrin mediated platelet adhesion in response to agonists (Chrzanowska-Wodnicka et al., 2005) and have also been shown to have defects in B-cell adhesion and chemotaxis (Chen et al., 2008).

1.4.2 Role of Rap1 in antigen receptor induced integrin mediated adhesion

T-cells detect antigen by forming immune synapses with antigen presenting cells (APCs). Immune synapses are facilitated by interactions between T-cell receptor (TCR) and antigen bound to major histocompatability complex (MHC), and are dependent on adhesive interactions between integrin molecules (e.g. LFA-1, VLA-1) and their counterpart integrin molecules (e.g. ICAM-1, VCAM-1). TCR stimulation leads to integrin inside-out stimulation, which ultimately increases adhesion of integrin molecules to their counterparts, and thus intensifies the T-cell:APC interaction. Rap1 has been shown to positively regulate LFA-1 mediated adhesion to ICAM-1 in response to TCR stimulation [reviewed in (Menasche et al., 2007b)]. Rap1 is activated at the plasma membrane in response to TCR antigen ligation (Bivona et al., 2004) and expression of SPA-1 abrogates TCR mediated Rap1 activation, and LFA-1 mediated adhesion to ICAM-1 (Katagiri et al., 2002). Following TCR stimulation, active Rap1 associates with RapL, which localizes to small vesicles near perinuclear regions. RapL then forms a complex with LFA-1 and initiates LFA-1 translocation from perinuclear regions to the peripheral boundaries of the immonuolgical synapse (Katagiri et al., 2003). Rap1 also regulates RIAM (Rap1-interacting adaptor molecule) which interacts with actin regulating proteins such as talin and the VASP (vasodilator-stimulated phosphoprotein) family. Recently, RIAM has been shown to interact with the ADAP/SKAP55 module, and this complex acts as a scaffold to recruit Rap1 to the plasma membrane in response to TCR, in order to facilitate adhesion (Menasche et al.,

2007a). Other regulators of the actin cytoskeleton that are required for adhesion include the Vav GEF family, and Vav1 and Vav2 have recently been shown to regulate B-cell receptor-dependent adhesion, immune synapse formation and activation of Rap1 (Arana et al., 2008). In a complementary study, using overexpression of the Rap1 and Rap2 negative regulator RapGAP, Rap activation was shown to be required for the B-cell immune synapse formation, the formation of F-actin-rich sites at locations of B-cell interaction with antigen and also BCR mediated cell spreading (Lin et al., 2008).

1.4.3 Role of Rap1 in chemokine induced integrin mediated adhesion

Leukocytes emigrate from blood venules into inflamed tissues in a process dependent on adhesion. Prior to emigration, rolling lymphocytes are induced to tether to high endothelial venules (HEVs) via increased LFA-1 adhesion. Chemokines localized to the apical surface of the epithelium, or in soluble form (e.g. stromal cell-derived factor-1, SDF-1α), bind to the chemokine receptors (G-protein coupled receptors) on rolling lymphocytes, and stimulate a cascade of signaling events that lead to an increase in the avidity of LFA-1, and thus adhesion to ICAM-1, which acts to stop the rolling lymphocyte in its tracks. Rap1 is activated in response to the chemokine SDF-1α and inhibition of Rap1 activation in lymphocytes by ectopic SPA-1 (a RapGAP) expression eliminates SDF-1α induced adhesion of LFA-1 to ICAM-1 (Shimonaka et al., 2003). Recently, basal and SDF-1α-stimulated activation of Rap1 was discovered to be defective in Epstein-Barr-virus-transformed lymphocytes derived from a patient with leukocyte adhesion deficiency (LAD) (Kinashi and Katagiri, 2004). LAD patients are characterized by defective integrin-mediated adhesion of leukocytes, and are thus immunodeficient. In other LAD patients, defective adhesion can be explained by mutations in integrin molecules, but this and other similar cases (Alon et al., 2003) express normal integrins. Therefore, these cases are categorized as LAD-III, since inside-out signalling is impaired rather than the integrins themselves. RapL also plays a role in chemokine-induced adhesion via Rap1. RapL associates with Rap1 in response to SDF-1α stimulation and RapL deficient T- and B-cells are defective in chemokine induced adhesion, which is dependent on LFA-1 (Katagiri et al., 2004).

1.4.4 Role of Rap1 in cadherin mediated adhesion

Rap1 is also an important regulator of cadherin based cell-cell junction formation [reviewed in (Kooistra et al., 2007)]. Cadherins are cell-cell adhesion molecules which mediate cell-cell adhesion through homophilic interactions at the cell surface. The cytoplasmic tails of cadherins bind to several proteins to form a connection with the actin cytoskeleton. Studies of the conditional *rap1* deficient mutant in *Drosophila melanogaster* first demonstrated a role for Rap1 GTPase in cadherin regulated cell-cell adhesion (Knox and Brown, 2002). Cell clones that are *rap1*-deficient in the wing have a different morphology, disperse into the surrounding tissue, and have an uneven distribution of DE-cadherin (Drosophila endothelial-cadhein), all indicators of defective cell-cell junction formation. The atypical Rap activator DOCK4, a potent tumor suppressor, was shown to be a regulator of cell-cell junctions. An osteocarcinoma cell line lacking DOCK4 was found to lack cell-cell junctions, and reformed on the transduction of wild-type DOCK4 or an active form of Rap1 (Yajnik et al., 2003). Further studies showed that adhesion of ovarian carcinoma cells to E-cadherin (endothelial-cadherin) is inhibited by expression of a dominant negative form of Rap (Price et al., 2004) and in epithelial cells ligation of E-cadherin leads to Rap1 activation, which is itself required for appropriate targeting of E-cadherin to maturing cell-cell contacts (Hogan et al., 2004). Rap1 has also been shown to play a role in the regulation of VE-cadherin (vascular endothelial-cadherin) in endothelial cells, via the cyclic adenosine monophosphate (cAMP) -responsive Rap activator Epac1 (Kooistra et al., 2005). In the regulation of E- and VE-cadherin-mediated-adhesion, Rap1 effectors are thought to recruit junctional proteins to sites of developing cell-cell contacts in order to stabilize the association between actin cytoskeleton and the junctional complex (Glading et al., 2007; Hoshino et al., 2005; Kooistra et al., 2007).

1.5 GUANINE NUCLEOTIDE EXCHANGE FACTORS: POSITIVE REGULATORS OF RAS AND RAP

1.5.1 Regulation of Ras and Rap GTPases by membrane localization of GEFs

Ras subfamily GTPases are activated via the exchange of GDP for GTP. Ras activation requires guanine nucleotide exchange factors (GEFs) to promote the release of

GDP and thereby enable binding of GTP, which is at a higher cellular concentration. All GEFs contain a homologous catalytic domain which is known as a GEF domain. As positive regulators of Ras and Rap, GEFs counteract negative regulation by GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity of Ras and Rap GTPases (Figure 1.5).

Ras and Rap GTPases are activated by distinct GEFs. Ras GTPases are activated by three GEF protein families; Son-of-sevenless (Sos1 and 2), Ras guanine releasing factor (RasGRF1 and 2) and Ras guanine nucleotide releasing protein (RasGRP1, 2, 3 and 4). Sos is the most well characterized RasGEF family; both Sos1 and 2 are ubiquitously expressed and are localized in the cytosol of resting cells (reviewed in Mor and Philips, 2006). In response to growth factor stimulation - commonly via receptor tyrosine kinases (RTKs) - these GEFs translocate to the plasma membrane, via interaction with the adapter protein Grb2 and E3b1 [reviewed in (Nimnual and Bar-Sagi, 2002)] and via localization through binding of the pleckstrin homology (PH) domain of Sos1/2 to phosphatidic acid (PA), which is generated by phopholipase D 2 (PLD2) [(Zhao et al., 2007); reviewed in (Hancock, 2007)]
.

In contrast to Sos1/2, RasGRF1 is constitutively localized at membranes via interactions with its N-terminal PH domain. RasGRFs are activated through calcium signaling, via the coordinated interaction of its PH, coiled coil and calcium-dependent calmodulin-binding IQ domains (Buchsbaum et al., 1996). Of the RasGRP family members, RasGRP1, 3, 4 and a human splice variant of RasGRP2 have been shown to activate Ras GTPases. RasGRPs are also known as CalDAG-GEFs, since as a family they are believed to be regulated by the second messengers calcium and diacylglycerol (DAG). Membrane localization and activation of RasGRP family members will be discussed in detail in the sections that follow.

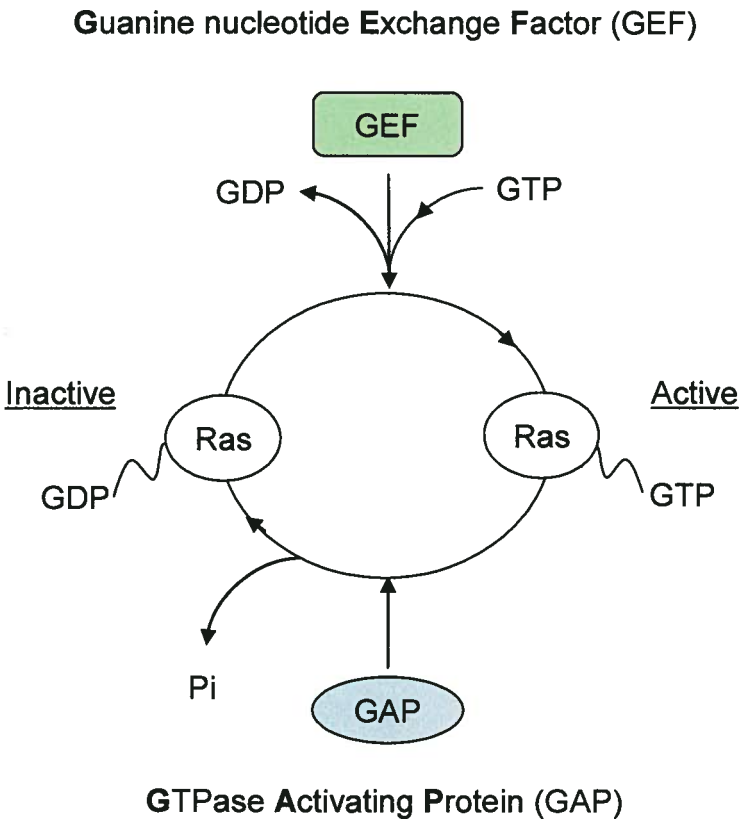

Figure 1.5 GEF and GAP regulation of Ras GTPases

Guanine nucleotide exchange factors (GEFs) activate Ras GTPase by releasing GDP, and allowing the binding of GTP, which is in greater concentration (10X) in the cell. GTPase activating proteins (GAPs), stimulate the intrinsic GTPase activity of Ras GTPases (GAPs).

Rap GTPases are regulated by a variety of distinct GEF proteins that have different membrane targeting strategies [reviewed in (Stork, 2003)]. In general, all Rap GEFs appear to have specificity for both Rap1 and Rap2 (Ohba et al., 2000b). These Rap GEFs include C3G, Epacs/cAMP-GEFs (Epac1 and 2), PDZ-GEFs (PDZ-GEF1 and 2, MR-GEF) and RasGRPs (RasGRP2 and 3). C3G (Crk SH3 domain guanine nucleotide exchanger), the first Rap GEF discovered (Gotoh et al., 1995), is recruited to the plasma membrane from the cytosol through binding to the SH2 domain of the adapter protein Crk in response to receptor tyrosine kinase stimulation (Feller, 2001; Okada et al., 1998). C3G may also be recruited to the plasma membrane through the action of cAMP-dependent protein kinase A (PKA) (Radha et al., 2004; Schmitt and Stork, 2002; Wang et al., 2006). Epacs (Exchange proteins directly activated by cAMP) are also known as cAMP-GEFs, and as their name implies are activated through direct interaction with the second messenger cAMP (de Rooij et al., 1998; Kawasaki et al., 1998b).

Epac1 and 2 are localized to perinuclear regions, the nuclear membrane and mitochondria and their localization is thought to be regulated during the cell cycle (Qiao et al., 2002). PDZ-GEFs contain a PSD-95/DlgA/ZO-1 (PDZ) domain, which regulates membrane localization [reviewed in (Ehrhardt et al., 2002)]. PDZ-GEF2 and MR-GEF also contain Ras-associating domains that specifically bind to M-Ras (another Ras subfamily GTPase) at the plasma membrane, which serves to localize these GEFs at locations where they can activate Rap GTPases (Gao et al., 2001). As already mentioned RasGRP2 activates Rap GTPases and RasGRP3 activates both Ras and Rap GTPases, and as such acts as a dual Ras/Rap activator. RasGRPs are regulated by membrane localization mechanisms that bring them in close proximity to their substrates (either Ras or Rap GTPases). RasGRPs are thought to be localized at membranes through binding of their C1 domain with DAG which is produced in membranes by phospholipase Cs which are activated in response to tyrosine kinase and antigen receptor stimulation. I will next discuss the structure, function and regulation of RasGRPs in more detail.

1.6 RASGRP FAMILY OF GEFS

1.6.1 RasGRP family protein structure

The RasGRP family has four members, RasGRP1, 2, 3 and 4 (Stone, 2006). They display a high degree of conservation, with RasGRP1 and RasGRP3 exhibiting the greatest similarity (Yamashita et al., 2000). All RasGRPs have a Ras exchange motif (REM) and a GEF domain (Figure 1.6) that together constitute the catalytic region responsible for GDP to GTP exchange on Ras and Rap GTPases. In addition to the catalytic region, RasGRPs contain either one or two EF hands. EF hands are calcium binding domains, and RasGRPs are thought to be regulated by calcium signaling, although minimal direct evidence exists for this hypothesis. RasGRPs also have a C1 domain that is homologous to protein kinase C (PKC) C1 domains that bind lipid second messenger diacylglycerol [reviewed in (Colon-Gonzalez and Kazanietz, 2006)]. Membrane localization of RasGRP1 is also regulated by its C-terminal plasma membrane targeter (PT) domain (Beaulieu et al., 2007).

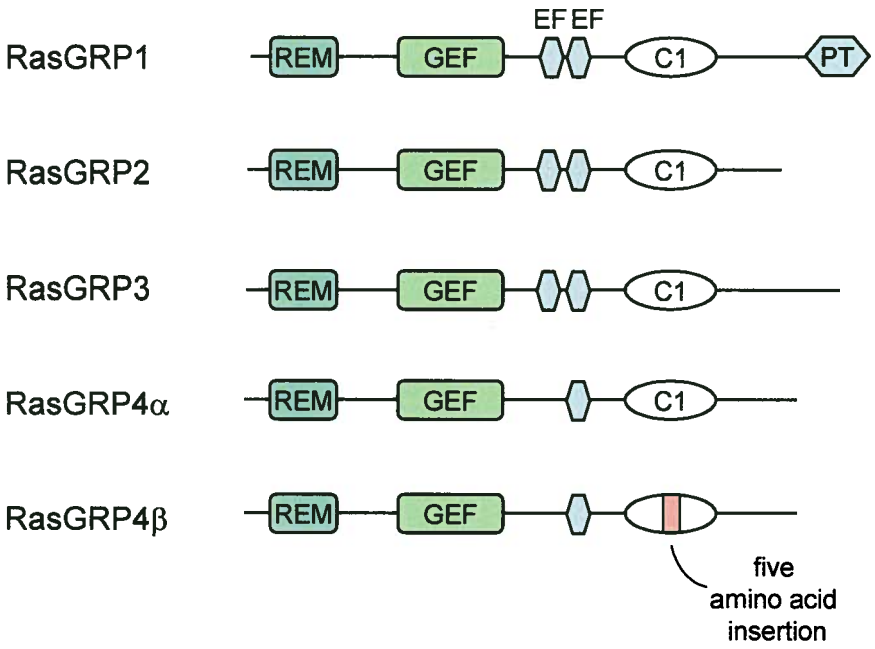

Figure 1.6 RasGRP protein structure

Domain structure of RasGRP proteins. REM, Ras exchange motif which is important for Ras activation. GEF, guanine nucleotide exchange domain that catalyzes GTP loading of Ras and Rap GTPases. EF, EF-hands. PT, plasma membrane targeting domain. RasGRP4β has a five amino acid insertion in the C1 domain.

1.6.2 RasGRP expression and function

RasGRPs are expressed predominantly in the hematopoietic system and brain with some degree of specificity (Clyde-Smith et al., 2000; Dupuy et al., 2001; Kawasaki et al., 1998a; Yamashita et al., 2000). In the hematopoietic system, there seems to be a certain level of restricted expression of RasGRP family members; RasGRP1 in T-cells (Ebinu et al., 1998), RasGRP2 in platelets (Crittenden et al., 2004), and T-cells (Ghandour et al., 2007), RasGRP1 (Coughlin et al., 2005) and 3 (Teixeira et al., 2003) in B-cells, and RasGRP1 (Liu et al., 2007) and 4 (Yang et al., 2002) in mast cells.

Knockout studies revealed that RasGRP1 is important for T-cell development (Dower et al., 2000; Priatel et al., 2002) and activation of both B-cells (Coughlin et al., 2005) and mast cells (Liu et al., 2007). RasGRP1 couples lymphocyte antigen receptors to Ras activation (Stone, 2006). Deregulated expression of RasGRP1 leads to lymphoma and leukemia (Dupuy et al., 2005; Klinger et al., 2005; Li et al., 1999; Mikkers et al., 2002; Suzuki et al., 2002), transformation of fibroblasts (Tognon et al., 1998) and keratinocytes (Oki-Idouchi and Lorenzo, 2007).

RasGRP2 has been shown to couple antigen receptor stimulation of T-cells with downstream Rap1 activation (Katagiri et al., 2004). Deletion of RasGRP2 in mice by Graybiel's group was shown to lead to defects in integrin dependent platelet aggregation (Crittenden et al., 2004) and leukocyte integrin mediated adhesion (Bergmeier et al., 2007). Wagner's group identified RasGRP2 deficient mice as a model for LAD-III (Bergmeier et al., 2007), and this was confirmed when two new cases of LAD-III, in which T lymphocytes, neutrophils and platelets have severe defects in integrin activation, were found to have defective RasGRP2 expression (Pasvolsky et al., 2007). Further work confirming the role of RasGRP2 and human T-cell adhesion showed an essential role for RasGRP2 in LFA-1 but not VLA-4 intergin mediated cell adhesion (Ghandour et al., 2007). Abberant expression of RasGRP2 leads to B lymphocyte (Mikkers et al., 2002; Suzuki et al., 2002) and myeloid transformation (Dupuy et al., 2001), which may be due to Rap1 activation of ERK1/2 via B-Raf. No reports have been made of B-cell specific adhesion defects in either LAD III patients or RasGRP2 knockout mice.

Less is known about the function of RasGRP3 and RasGRP4. RasGRP3 also couples antigen receptor signaling to Ras activation (Coughlin et al., 2005; Oh-hora et al., 2003) and RasGRP3 deficient DT40 B-cells have defects in B-cell activation (Oh-hora et al., 2003). RasGRP3 knockout mice exhibit hypogammaglobulinemia (disorder caused by a lack of B-lymphocytes) and with some evidence of splenomegaly (enlargement of the spleen) or autoimmunity (Coughlin et al., 2005). RasGRP4 was originally identified in a cDNA screen from acute myeloid leukemia (AML) patients (Reuther et al., 2002) and is thought to play a role in mast cell development (Stevens et al., 2005).

1.6.3 RasGRP GTPase specificity

RasGRPs have differential specificities for Ras versus Rap (Table 1.6.3). RasGRP1 has been shown to have robust activity towards Ras proteins including H-Ras, N-Ras, K-Ras, M-Ras, R-Ras and TC-21 (Ebinu et al., 2000; Kawasaki et al., 1998a; Mochizuki et al., 2000; Ohba et al., 2000a; Yamashita et al., 2000). RasGRP2 activates Rap1 and Rap2 (Dupuy et al., 2001; Eto et al., 2002; Katagiri et al., 2004; Kawasaki et al., 1998a; Ohba et al., 2000b; Ohba et al., 2000a) and has also been reported to activate Ras proteins, including R-Ras, N-Ras and TC-21 (Clyde-Smith et al., 2000; Ohba et al., 2000a; Yamashita et al., 2000). In addition, a myristoylatable, longer splice-variant of RasGRP2 (MyrRasGRP2) that is expressed in humans, has been shown to have moderate exchange activity towards N-Ras (Clyde-Smith et al., 2000). No further evidence of RasGRP2-mediated Ras activation has come to light. RasGRP3 has been shown to promote activation of H-Ras, N-Ras, R-Ras, M-Ras, TC-21 as well as Rap1 and Rap2 GTPases (Ohba et al., 2000b; Ohba et al., 2000a; Yamashita et al., 2000). RasGRP4 is reminiscent of RasGRP1 in its ability to activate H-Ras (Reuther et al., 2002; Yang et al., 2002).

	Ras/Rap GTPase specificity
RasGRP1	H-Ras, N-Ras, K-Ras, M-Ras, R-Ras, TC-21
RasGRP2	Rap1, Rap2, R-Ras, N-Ras, TC-21
RasGRP3	H-Ras, N-Ras, R-Ras, M-Ras, TC-21, Rap1, Rap2
RasGRP4	H-Ras

Table 1 GTPase specificities of RasGRPs
RasGRPs have different effector specificity. RasGRP1, 3 and 4 can activate Ras GTPases, although a human splice variant of RasGRP2 has been shown to activate R-Ras, N-Ras and TC-21. RasGRP2 can activate Rap GTPases and RasGRP3 has both Ras and Rap GTPase specificity.

1.7 REGULATION OF RASGRPS BY C1 DOMAINS

1.7.1 Membrane binding by C1 domains

Regulation of protein-membrane binding is governed by a variety of protein domains including C1, C2, PH and FYVE and PX domains, which have affinity for specific lipids at membranes [reviewed in (Cho and Stahelin, 2005)]. Protein kinase C (PKC) conserved 1 (C1) domains were first discovered in novel (PKCδ, ε, η, θ) and classical (PKCα, β, γ) isozymes, where they occur in tandem and are referred to as C1a and C1b. C1 domains are approximately 50 amino acids in length and are classified as typical or atypical depending on their ability to bind the ligand diacylglycerol (DAG). DAG is produced and maintained by several processes in the cell, including lipid metabolism which results in the synthesis of DAG de novo, and alternatively, external signals that activate membrane associated protein lipases and phosphatases which convert lipid precursors into DAG [reviewed in (Carrasco and Merida, 2006)]. One such canonical pathway involves the activation of phospholipase C (PLC) isozymes via receptor stimulation (e.g. T-cell receptor signaling), which cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into two products, DAG and inositol trisphosphate (IP3) (Figure 1.7). In addition, DAG is produced via the dephosphorylation of phosphatidic acid (PA) by phosphatidic-acid phosphatase (PAP). The

PA substrate for this reaction can also be generated during receptor tyrosine kinase stimulation through phospholipase D (PLD) cleavage of the phospholipid phosphatidyl serine. DAG that is generated and concentrated at membranes as a result of external signals acts as a ligand for C1 domain containing proteins. In this way this DAG facilitates the translocation of such proteins to these membrane locations. Most studies characterizing typical and atypical C1 domains have used the ligand phorbol ester (PE), a functional DAG analog which is not naturally present in cells, but for which many typical C1 domains have very high affinity. For the most part typical C1 domains have higher affinity for phorbol esters than for DAG, although in some instances they have greater affinity for DAG [reviewed in (Cho and Stahelin, 2005)].

Studies in the 1990s using PKC inhibitors that target the DAG/PE binding site of C1 domains (e.g. calphostin C), as well as treatment with exogenous PE, revealed a large number of pathways and cellular responses that at the time were thought to be solely dependent on novel and classical PKCs. This perspective dramatically changed with the discovery of five other protein families, which also contain typical DAG-responsive C1 domains: protein kinase D (PKD), DAG kinase (DGK), Munc13, chimaerins and RasGRPs [reviewed in (Brose et al., 2004)]. Pharmacological inhibitors of C1 domains and PE treatment can theoretically also negatively and positively regulate all proteins that contain a typical C1 domain; therefore their effects and specificities have been reanalysed in the context of a complex network of DAG responsive proteins [reviewed in (Kazanietz, 2002)]. Proteins have also been identified that have C1 domains that are not responsive to DAG, termed atypical C1 domains. These proteins include atypical PKCs, DGKs, Vav, Raf and kinase suppressor of Ras (KSR). In the case of KSR and Vav, while unresponsive to DAG/PE, the C1 domains within these proteins are required for their activity (see section 1.7.3).

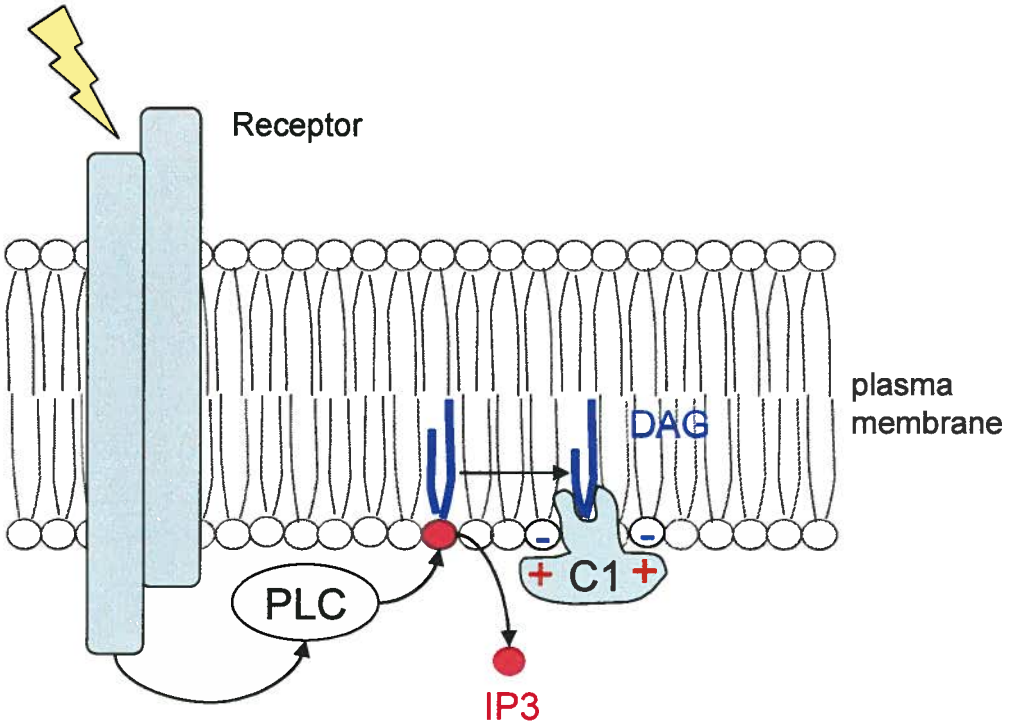

Figure 1.7 DAG production and C1 domain localization at membranes

Receptor stimulation leads to phospholipase C (PLC) activation which cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). DAG is the ligand for C1 domains, which bind the headgroup of DAG via the DAG binding pocket. Basic residues (+) on either side of the C1 domain DAG binding pocket are thought to interact with the anionic phospholipids (-) present in the membrane via electrostatic interactions, thereby stabilizing membrane binding.

1.7.2 PKC δ C1b: the archetypal typical C1 domain

NMR spectroscopy of the PKCδ C1b C1 domain determined that two zinc cation (Zn^{2+}) binding sites form during globular folding of the domain (Hommel et al., 1994). The structure of this C1 domain) bound to the phorbol ester, phorbol 13-acetate (PMA), was subsequently characterized by co-crystallization (Zhang et al., 1995). These studies together with validation by site-directed mutagenesis have helped to develop an understanding of how a typical C1 domain folds and interacts with membranes containing DAG/PE [reviewed in (Cho and Stahelin, 2005; Colon-Gonzalez and Kazanietz, 2006)]. All typical C1 domains

have the consensus sequence $Hx_{11-12}Cx_2Cx_{12-14}Cx_2Cx_4Hx_2Cx_{6-7}C$ (where H = histidine, C = cysteine, x = other amino acid) (Figure 1.8), which folds to form a compact structure featuring two small β sheets and a small C-terminal α helix. The two histidines and six cysteines in the consensus sequence create two cavities, that each coordinate a Zn^{2+} ion. The two β sheets form a narrow polar binding pocket with two loops (loop A and loop B), within which DAG/PE can insert lengthwise (Figure 1.9). In unbound form, the two β sheets are in a "zipped" conformation with hydrogen-bonded water molecules filling the binding pocket. Binding to phorbol ester leads to unzippering of the β sheets, allowing hydrogen bonds to form with positions C3, C4 and C20 of the phorbol ring with the "unzipped" groove. Basic residues (arginine, lysine or histidine) surrounding the binding pocket are involved in non-specific electrostatic interactions with anionic phospholipids, which accelerate initial membrane adsorption of the C1 domain and position the C1 domain at the surface of the membrane. Hydrophobic and aromatic residues at the tips or base of the loops A and B, which surround the binding pocket, are thought to be involved in insertion and penetration of the membrane, which in turn is thought to be required to stabilize binding to membrane-embedded DAG. Initial binding of the C1 to DAG or PE (in conjunction with these hydrophobic/aromatic residues) creates a contiguous hydrophobic binding surface which may facilitate subsequent insertion into the membrane. Residues in the PKC δ C1b sequence which were found to be essential for ligand binding including Phe3, Tyr8, Pro11, Leu20, Leu21, Trp22, Leu24, Gln27 and Val38 [reviewed in (Colon-Gonzalez and Kazanietz, 2006)].

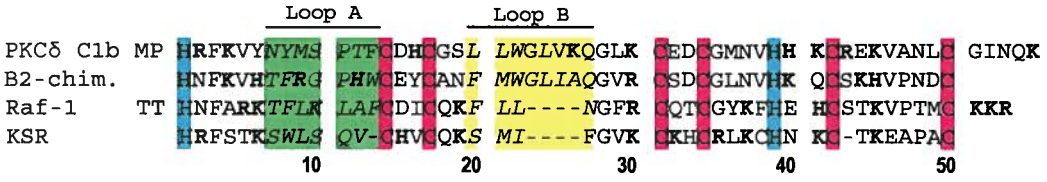

Figure 1.8 Typical and atypical C1 domain sequence comparison

Typcial C1 domains (PKCδ C1b and B2-chimaerin) compared to atypical C1 domains (Raf-1 and kinase suppressor of Ras (KSR). Loop A and loop B (italicized) form a DAG binding pocket. Cationic residues which have the potential to interact with anionic phospholipids within membranes are bolded. While Raf-1 and KSR, still have the consensus two histidines and six cysteines structure which coordinate zinc, they each have a deletion in loop B which precludes binding to DAG.

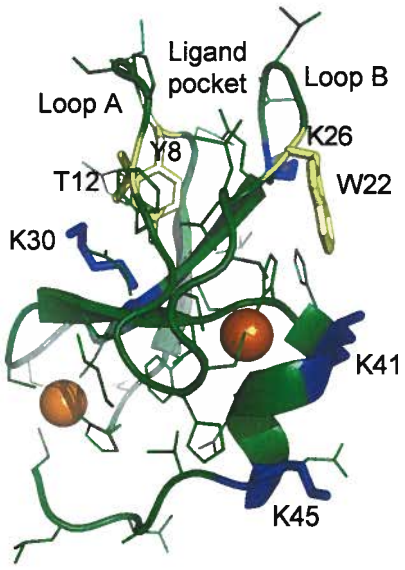

Figure 1.9 The C1b domain structure of PKCδ

The C1b domain of PKCδ (protein data bank code 1ptq) illustrates the generic structure of a C1 domain that binds DAG or phorbol ester. The zinc atoms are shown as orange balls. Y8, T12 and W22 are highlighted in yellow. Four of the cationic residues implicated in membrane interactions, K26, K30, K41 and K45, are highlighted in blue. The image was generated with PyMol and is also in Figure 2.1.

1.7.3 The function of atypical C1 domains

Atypical C1 domains have several sequence deviations in loops A and B that render them unable to bind DAG or PE. The structure of both C-Raf and KSR have been determined by spectroscopic NMR (Mott et al., 1996; Zhou et al., 2002). In both cases the C1 domains of these proteins lack a crucial proline residue (Pro11) in loop A and have a deletion at position 22-25 that structurally removes loop B [reviewed in (Colon-Gonzalez and Kazanietz, 2006)]. These changes predict an inflexible β sheet conformation that is unable to open to accommodate PE binding. In the case of the C-Raf C1 domain, the lack of this loop leads to a flat hydrophobic surface, which would lack penetrating capabilities. In addition, both C-Raf and KSR lack Gln27, which is important for maintenance of unzipped β sheet structure of typical C1 domains.

Atypical C1 domains do still retain some hydrophobic residues thought to be required for membrane insertion, and basic residues that may promote anionic phospholipid electrostatic interactions. It has been suggested that for C-Raf and KSR these interactions are responsible for membrane binding (Ghosh et al., 1996; Zhou et al., 2002). KSR translocates to the plasma membrane in response to growth factor treatment, in a C1 domain-dependent manner (Michaud et al., 1997). Other evidence for a functional role of atypical C1 domains comes from studies which showed that the C1 domain of Vav is required for its ability to induce oncogenic transformation (Booden et al., 2002; Coppola et al., 1991). Also, mutations in the C1 domain of C-Raf were shown to affect C-Raf binding to and activation of Ras (Drugan et al., 1996; Winkler et al., 1998).

1.7.4 Regulation of RasGRP1 localization by the C1 domain

The RasGRP family of guanine nucleotide exchange factors (RasGRP1-4) have a single C-terminally located C1 domain. Much information about the functional role of the C1 domains of this family of proteins has been gleaned from research with RasGRP1. The RasGRP1 C1 domain binds to DAG or PE (Ebinu et al., 1998; Irie et al., 2004; Lorenzo et al., 2000; Madani et al., 2004; Rong et al., 2002), [reviewed in (Carrasco and Merida, 2006)] and RasGRP1 translocates to membranes in response to DAG or PE treatment (Bivona et al., 2003; Caloca et al., 2003b; Ebinu et al., 1998; Rambaratsingh et al., 2003; Tognon et al., 1998). RasGRP1 also activates Ras in response to DAG/PE (Caloca et al., 2004; Ebinu et al., 1998; Kawasaki et al., 1998a; Priatel et al., 2002; Rambaratsingh et al., 2003; Tognon et al., 1998). The C1 domain is required for RasGRP1 translocation to endomembranes (endoplasmic reticulum (ER) and Golgi) in response to serum (Caloca et al., 2003b; Tognon et al., 1998) and is also required for RasGRP1 mediated NIH 3T3 transformation (Tognon et al., 1998). RasGRP1 translocates to the plasma membrane (Carrasco and Merida, 2006; Zugaza et al., 2004) or alternatively the Golgi (Bivona et al., 2003; Perez de Castro et al., 2004) in response to T-cell receptor (TCR) stimulation. Regulation of DAG levels by conversion to phosphatidic acid (PA) by DAG kinases has been proposed as a method for spatially restricting activation of RasGRP1 in selective membrane compartments (Zha et al., 2006). RasGRP1 is found at the plasma membrane in response to

antigen presenting cell (APC) interactions (Daniels et al., 2006), and at the Golgi when T-cells are anergized before APC engagement (Zha et al., 2006). Recently it was discovered that RasGRP1 could translocate to both membrane compartments in the same cell (Mor et al., 2007). Mor and colleagues found that TCR stimulation alone leads to RasGRP1 accumulation and subsequent Ras activation at the Golgi, whereas co-stimulation with ICAM-1 (to crosslink LFA-1) allowed RasGRP1 translocation to and Ras activation at the plasma membrane (Mor et al., 2007). Strikingly, this group also determined that RasGRP1 translocation to the plasma membrane was dependent on phospholipase D 2 (PLD2) mediated generation of PA at this membrane compartment, and subsequent dephosphorylation by PA phosphatase (PAP) to produce DAG. RasGRP1 translocation to DAG at the Golgi however, relies on PLCγ, which cleaves PIP2 to generate DAG and IP3, a regulator of intracellular calcium levels (Mor et al., 2007). In DT40 B-cells RasGRP1 has also been shown to translocate to the plasma membrane in response to B-cell receptor (BCR) ligation, and this translocation is enhanced by the C1 domain (Beaulieu et al., 2007). Other evidence has accumulated which points to a central role of the C1 domain in regulating RasGRP1. The C1 domain is required for TCR mediated activation of RasGRP1 (Roose et al., 2005) and TCR and BCR mediated RasGRP1-dependent Ras activation requires PLCγ (Beaulieu et al., 2007; Caloca et al., 2003b; Ebinu et al., 1998; Perez de Castro et al., 2004; Reynolds et al., 2004; Zugaza et al., 2004). RasGRP1-mediated Ras activation in Jurkat T-cells via TCR ligation is also inhibited by DAG kinases (Sanjuan et al., 2003), and ectopic DAG kinase expression in mature T-cells impairs RasGRP1 translocation to the plasma membrane during TCR stimulation (Zha et al., 2006). In addition, inhibitors of PLCγ1 inhibit accumulation of RasGRP1 in membranes (Ebinu et al., 2000; Reynolds et al., 2004) and translocation of RasGRP1 in response to BCR stimulation is greatly reduced in DT40 B-cells deficient in PLCγ2 (Beaulieu et al., 2007).

Clearly DAG and PLC activity are required for optimal translocation of RasGRP1, but RasGRP1 activation also requires phosphorylation by PKCs (Aiba et al., 2004; Roose et al., 2005; Zheng et al., 2005), which are also dependent on DAG. This raises the possibility that RasGRP1 translocation may not always be regulated by PLC and DAG directly. Also, recent evidence suggests that RasGRP1 translocation to the plasma membrane in DT40 cells

in response to BCR stimulation is primarily regulated by another domain, the plasma membrane targeter (PT) domain, and is secondarily assisted by the C1 domain (Beaulieu et al., 2007). However, the PT domain does not regulate RasGRP1 localization or transformation in NIH 3T3 cells, and therefore its importance may be cell type-specific (Beaulieu et al., 2007). While other domains and mechanisms of activation are required for RasGRP1 translocation and activation, a great deal of evidence supports the role of the C1 domain as a positive regulator of RasGRP1 via DAG binding at membranes.

1.7.5 The C1 domains of RasGRP2, 3 and 4

Comparative analysis of the RasGRP1 through 4 coding sequences with PKC δ C1b shows that all four C1 domains have the basic structure of two histidines and six cysteines that are required for Zn^{2+} binding (Figure 1.10). The RasGRP2 C1 domain diverges from the consensus PKCδ C1b sequence in a few key residues; Tyr8, Thr12 and Trp22 (Figure 1.7.5). These residues are thought to be important for the structure of the DAG/PE binding pocket (Colon-Gonzalez and Kazanietz, 2006). The RasGRP3 C1 domain has very similar sequence to that of RasGRP1 with minor differences in loop A and B. RasGRP4 has multiple splice variants although only two of these RasGRP4α and β, have intact C1 domains (Li et al., 2003; Yang et al., 2002). The RasGRP4α C1 domain retains all the key residues compared to PKCδ C1b, however RasGRP4β has a 5 amino acid insert in loop B (VSTGP).

Conflicting evidence has led to confusion concerning the role of the RasGRP2 C1 domain. RasGRP2 mediated Rap1 activation (Clyde-Smith et al., 2000; Kawasaki et al., 1998a) and adhesion to fibronectin (Dupuy et al., 2001) can be induced by PE. In addition, RasGRP2 deficient mouse platelets are unable to activate Rap1 or aggregate in response to PE (Crittenden et al., 2004). Also, *si*RNA knockdown of RasGRP2 in human T-cells also leads to abrogation of PE induced Rap1 activation and adhesion to ICAM-1 (Ghandour et al., 2007) and RasGRP2-mediated TCR-induced adhesion to ICAM-1 via Rap1 activation is dependent on PLC enzymes (Katagiri et al., 2004). However, it remains a possibility that these results reflect PE dependent PKC activation of RasGRP2. RasGRP1 and RasGRP3 have been shown to be phosphorylated by PKC isozymes. Interestingly, PMA-induced Rap1

activation in human T-cells was inhibited by the pan PKC inhibitor Gö 6850 and the classical PKC inhibitor Gö 6976, although this may be due to effects other than inhibition of PKC-dependent RasGRP2 activation (Ghandour et al., 2007). RasGRP2 was shown to translocate to membranes in response to PE treatment (Clyde-Smith et al., 2000), however, no other evidence has arisen which shows that translocation of RasGRP2 or the RasGRP2 C1 domain to membranes in response to DAG/PE. In fact, this C1 domain was shown to be incapable of binding PE in contrast to RasGRP1, 3 and 4α in a vesicle binding assay (Irie et al., 2004). Therefore it remains to conclusively be established whether the RasGRP2 C1 domain can respond to DAG.

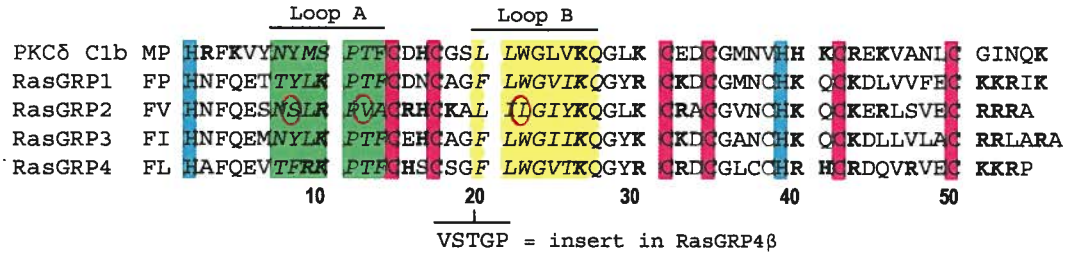

Figure 1.10 RasGRP C1 domains

Sequence comparison of RasGRP C1 domains compared to PKCδ C1b. Loop A and loop B (italicized) form a DAG binding pocket. Cationic residues which have the potential to interact with anionic phospholipids within membranes are bolded and residues that are divergent in RasGRP2; Tyr^{8}, Thr^{12} and Trp^{22} are circled. RasGRP4β C1 domain has a five amino acid insertion (VSTGP) at the base of loop B.

On the other hand, substantial evidence exists to support a positive regulatory role for the RasGRP3 C1 domain as a DAG receptor. RasGRP3 translocates to membranes and activates Ras in response to DAG or PE (Lorenzo et al., 2000) and RasGRP3 plasma membrane translocation in response to antigen receptor ligation requires both PLC activity and the C1 domain (Oh-hora et al., 2003). RasGRP4α has been shown to activate Ras (Katsoulotos et al., 2008) and to translocate to the plasma membrane (Katsoulotos et al., 2008; Shalom-Feuerstein et al., 2008) in response to PE treatment. Both RasGRP4α and β (splice variants of RasGRP4) promoted PE dependent transformation of NIH 3T3 fibroblasts (Li et al., 2003; Yang et al., 2002) and membrane translocation of the C1 domain of RasGRP4α can also be induced by PE treatment (Reuther et al., 2002). While the RasGRP4α

C1 domain can bind PE like the RasGRP1 and 3 C1 domains (Irie et al., 2004), no such evidence exists for RasGRP4β C1 binding directly to DAG/PE. RasGRP4β C1 domain has a 5 amino acid insertion exactly at the DAG binding pocket, which could preclude DAG binding.

Thus, the C1 domains of RasGRPs are not necessarily identical with respect to binding affinity for the second messenger DAG. Understanding the specific binding capability of the C1 domains with DAG (and PE) is crucial to determine the potential for these proteins to be regulated by upstream receptors that generate DAG.

1.8 THESIS OBJECTIVES AND APPROACH

My overall research aim was to determine which of the RasGRP C1 domains bind membranes, and whether this occurs by direct binding to DAG. Evidence has accumulated that supports the hypothesis that C1 domains of RasGRP1, 3 and 4α bind to DAG/PE. Most models of RasGRP2 regulation are based on the assumption that the C1 domain binds to DAG, but recent research points to the fact that the RasGRP2 C1 domain may be incapable of doing so. Therefore, my first hypothesis was that the RasGRP2 C1 domain would not bind DAG or PE in membranes. Although RasGRP4β can transform NIH 3T3 cells in response to PE, no evidence exists for DAG binding to the RasGRP4β C1 domain and moreover, sequence analysis of the binding pocket of this C1 domain reveals an insertion that could disrupt DAG binding. Thus, my second hypothesis was that RasGRP4β would also not bind to DAG or PE in membranes. The first objective of my thesis was to determine whether all RasGRP C1 domains have similar ability to bind to DAG or PE in membranes. The experimental approach I took was to look at the subcellular localization of GFP-tagged C1 domains in cells using fluorescence microscopy with the aim of establishing which RasGRP C1 domains localize to membranes and which RasGRP C1 domains can relocalize to membranes in response to DAG or PE.

The most important finding from my thesis research in Chapter 2 was that the RasGRP2 C1 domain was unable to recognize DAG as a ligand, although it was able to bind

to membranes enriched in anionic phospholipids. This result raised additional questions which were the starting points for my research described in Chapter 3:

What is the function of the C1 domain of RasGRP2? Does RasGRP2 localize to membranes and if so how is this achieved in the absence of a DAG-binding C1 domain? Therefore my second objective was to examine the role of the C1 domain in RasGRP2 membrane localization. To this end I used fluorescence microscopy to establish the localization pattern of RasGRP2 in a number of different cell lines, and to determine whether the C1 domain was required for the observed pattern.

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CHAPTER 2: DIFFERENTIAL MEMBRANE BINDING AND DIACYLGLYCEROL RECOGNITION BY C1 DOMAINS OF RASGRPS

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2.1 INTRODUCTION

Diacylglycerol (DAG) is a lipid second messenger that is involved in the transduction of signals from most cell surface receptors (Brose et al., 2004; Carrasco and Merida, 2006; Gomez-Fernandez et al., 2004). Signal transduction by DAG is mediated by its binding to C1 domains, which are found on a wide variety of kinases, exchange factors and other proteins (Colon-Gonzalez and Kazanietz, 2006; Hurley et al., 1997). The binding of DAG to their C1 domains serves to translocate these proteins to membranes, where they can interact with signaling complexes and find their substrates and regulators (Cho and Stahelin, 2005; Hurley, 2006). In some cases, the binding of C1 domains to DAG within membranes has the potential to release other domains of the signaling proteins and thus contributes to enzymatic activation as well as translocation to membranes (Colon-Gonzalez and Kazanietz, 2006; Newton, 2001). In the case of PKCδ, when the C1B domain binds to DAG in membranes, in coordination with binding of the C2 domain to membranes, this enables the dislocation of the N-terminal pseudosubstrate domain from the C-terminal catalytic kinase domain, allowing the latter to bind substrate and catalyze downstream target activation (Newton, 2001). Conversely, conformational changes could be mitigated by other domains and lead to the liberation of the C1 domain. For example, β2-chimaerin translocates to the membrane by an unknown mechanism, after which interactions with acidic phospholipids lead to a conformational change of the protein that in turn releases the C1 domain from N-terminal auto-inhibition, allowing it to bind DAG in membranes and also to bind and activate RapGAP (Hall et al., 2005).

C1 domains are functionally heterogeneous in terms of their ligand binding capabilities (Cho and Stahelin, 2005; Colon-Gonzalez and Kazanietz, 2006). Most protein kinase Cs (PKCs) have a pair of C1 domains, but each of these can differ considerably in their affinities for DAG (Cho and Stahelin, 2005). PKC C1 domains can also differ in their relative affinities for DAG versus the phorbol esters which are commonly used as artificial ligands for C1 domains (Cho and Stahelin, 2005). In general, these C1 domains have higher affinity for phorbol esters, but in some cases DAG is the more effective ligand (Ananthanarayanan et al., 2003; Stahelin et al., 2004). Some C1 domains have no detectable affinity for either DAG or phorbol esters, and thus may have functions other than serving as

DAG receptors (Colon-Gonzalez and Kazanietz, 2006) or may be C1 domains, which are no longer functional in terms of providing membrane localization.

C1 domains are readily identified by sequence analysis due to the characteristic spacing of two histidines and six cysteines (Figure 2.1A). These amino acids lock the domain into a compact structure by coordinating two zinc atoms (Figure 2.1B). In C1 domains capable of binding DAG or phorbol esters and with known structures, such as the second C1 domain (C1b) of PKCδ (Zhang et al., 1995) or the C1 domain of β2-chimaerin (Canagarajah et al., 2004), two projecting loops insert into membranes and form a ligand binding pocket (Figure 2.1B) that can accommodate either the head group of diacylglycerol or an analogous segment of phorbol ester. While the atomic interactions between the C1b domain of PKCδ and phorbol ester have been identified, the precise structural basis for the diacylglycerol-C1 domain interaction is unknown. The C1 domains of Raf-1 and KSR, which do not bind DAG or phorbol ester, have radical alterations in loops A and B (Mott et al., 1996; Zhou et al., 2002). In particular, they lack the proline that dictates the structure of loop A and are missing half of loop B (Figure 2.1A). As a consequence, these two C1 domains lack a ligand binding pocket.

In addition to specific ligand binding via the pocket, C1 domains interact with the membrane surface (Cho and Stahelin, 2005). Hydrophobic residues projecting from or at the base of loops A and B facilitate membrane insertion (Cho and Stahelin, 2005; Wang et al., 2001; Zhang et al., 1995). Basic residues distributed on the periphery of the C1 domain surface and at the C-terminus (Figure 2.1A) provide electrostatic interactions with anionic phospholipids and thus enhance DAG-mediated binding to membranes (Irie et al., 2004; Johnson et al., 2000; Medkova and Cho, 1999; Zhang et al., 1995). For KSR and Raf-1 these hydrophobic and electrostatic interactions may enable DAG-independent membrane binding (Bondeva et al., 2002; Zhou et al., 2002).

RasGRPs are exchange factors for membrane-bound Ras or Rap GTPases (Stone, 2006). All RasGRP family members contain C1 domains. This suggested that the activity of all RasGRP family members could be regulated by membrane translocation mediated by

binding of their C1 domains to DAG . Evidence for this concept has been obtained for RasGRP1 and to a lesser extent for RasGRP3. Activation of RasGRP1 and RasGRP3 by antigen receptors requires DAG-generating phospholipase Cγ enzymes (Caloca et al., 2003b; Oh-hora et al., 2003; Zugaza et al., 2004) and is inhibited by DAG kinases (Jones et al., 2002; Sanjuan et al., 2003; Topham and Prescott, 2001; Zha et al., 2006). This does not necessarily reflect involvement of a DAG-C1 domain interaction, because activation of both RasGRP1 and 3 requires their phosphorylation by DAG-dependent PKCs (Aiba et al., 2004; Brodie et al., 2004; Roose et al., 2005; Teixeira et al., 2003; Zheng et al., 2005). However, for RasGRP1 it is clear that a direct DAG-C1 domain interaction contributes to the membrane localization step of its activation process. Phospholipase Cγ1 is required for, and DAG kinase is inhibitory to, T cell antigen receptor-induced translocation of RasGRP1 to membranes (Bivona et al., 2003; Reynolds et al., 2004; Sanjuan et al., 2003), while deletion of the C1 domain eliminates binding of RasGRP1 to membranes (Caloca et al., 2003a; Ebinu et al., 1998; Tognon et al., 1998). The isolated C1 domain of RasGRP1 translocates in vivo in response to DAG (Carrasco and Merida, 2004; Tognon et al., 1998) and phorbol esters (Ebinu et al., 1998; Tognon et al., 1998) and binds directly to DAG (Carrasco and Merida, 2004) and phorbol esters (Ebinu et al., 1998; Irie et al., 2004; Lorenzo et al., 2000).

While direct binding of the C1 domain of RasGRP3 to DAG has not yet been demonstrated, it does bind phorbol ester (Irie et al., 2004). The large differences in affinities for DAG versus phorbol esters of several PKC C1 domains (Cho and Stahelin, 2005) shows that caution is needed in assuming that phorbol ester binding equates with DAG binding, although it hasn't been possible to predict from the sequence of a C1 domain whether it would have higher affinity for DAG versus PE, or vice versa. Antigen receptor-induced membrane translocation of RasGRP3 also requires its C1 domain and phospholipase C activity (Oh-hora et al., 2003), and is induced by either DAG or phorbol ester (Lorenzo et al., 2001). Therefore, the available evidence supports the hypothesis that RasGRP3, like RasGRP1, is regulated by membrane translocation via a DAG-C1 domain interaction.

RasGRP4 has several splice variants (Li et al., 2003; Yang et al., 2002). Two of these, which we will refer to as RasGRP4α and RasGRP4β, have intact C1 domains, although they

differ by the insertion of five amino acids within the RasGRP4β C1 domain (Figure 2.1A). Phorbol ester treatment induces membrane translocation and activation of RasGRP4α (Reuther et al., 2002). Both RasGRP4α and β synergize with phorbol esters to induce transformation of fibroblasts, while another RasGRP4 splice variant with a truncated C1 domain is unable to do this (Li et al., 2003; Yang et al., 2002).

The isolated C1 domain of RasGRP4α binds directly to phorbol esters within phospholipid vesicles (Irie et al., 2004). Even though phorbol ester binding by the RasGRP4β C1 domain, as well as DAG binding by either of the RasGRP4 C1 domains, have not been examined, the combination of results so far is compatible with the hypothesis that both RasGRP4α and RasGRP4β are activated via membrane translocation driven by binding of DAG to their C1 domains. However, the evidence for this is not well established, particularly for RasGRP4β.

The evidence for regulation of the Rap-specific exchange factor RasGRP2 via a DAG-C1 domain interaction is somewhat contradictory. RasGRP2 is activated by phorbol esters in several cell types (Clyde-Smith et al., 2000; Crittenden et al., 2004; Dupuy et al., 2001; Kawasaki et al., 1998) and the ability of RasGRP2 to enhance T cell antigen receptor-induced adhesion via Rap GTPase activation is dependent on TCR-coupled phospholipase C enzymes (Katagiri et al., 2004). Because translocation of RasGRP2 to membranes and its activation of membrane-associated Rap GTPases is induced by PMA (Clyde-Smith et al., 2000) and a variety of receptors coupling to phospholipase C enzymes (Crittenden et al., 2004; Eto et al., 2002; Guo et al., 2001; Katagiri et al., 2004), a frequently stated assumption is that RasGRP2 is equivalent to RasGRP1 and 3 in being regulated by binding of its C1 domain to DAG-enriched membranes. However, a synthetic RasGRP2 C1 domain had no detectable affinity for phorbol esters in a vesicle binding assay (Irie et al., 2004), in contrast to the C1 domains of RasGRP1, 3 and 4α. This result has to be interpreted with some caution, because the C1 domains of munc-13 and unc-13, which bind phorbol ester when expressed in cells (Betz et al., 1998; Kazanietz et al., 1995a), failed to bind phorbol ester when synthesized and analysed in the same way as the RasGRP2 C1 domain (Irie et al., 2004). Therefore, it is possible that the lack of phorbol ester binding reflected misfolding of the synthetic RasGRP2 C1 domain. Binding to DAG was not examined with the synthetic

RasGRP2 C1 domain. Moreover, the localizations of full length RasGRP2 versus RasGRP1 are different in several cell types (Bivona et al., 2003; Caloca et al., 2003a). This is not what is expected if the localization of both of these RasGRPs is entirely determined by their C1 domains seeking out membranes enriched in DAG. However, other domains of both RasGRP1 (Carrasco and Merida, 2004) and RasGRP2 (Caloca et al., 2003b; Caloca et al., 2004) can have dominant effects on their localizations, and thus could obscure equivalent contributions of their C1 domains to localization. Thus, it remains an open question whether or not the C1 domain of RasGRP2 contributes to its localization to membranes, and if so, whether it does this by binding DAG.

The objective of our current study was to determine the capabilities of the C1 domains of RasGRP2, 4α and 4β to mediate membrane binding via DAG. Our *in vivo* and *in vitro* experiments demonstrated that the C1 domain of RasGRP4α binds DAG within membranes, although with moderately reduced affinity relative to the RasGRP1 C1 domain. In contrast, the C1 domains of RasGRP2 and 4β did not bind DAG, although they were equivalent to the other C1 domains in being able to bind membrane vesicles enriched in anionic phospholipids. From these results, we conclude that RasGRP2 and RasGRP4β cannot be directly regulated by DAG binding to their C1 domains, thus disproving the common assumption that all RasGRPs are regulated by membrane translocation driven by a DAG-C1 domain interaction (Bunney and Katan, 2006; Clyde-Smith et al., 2000; Crittenden et al., 2004; Eto et al., 2002; Guo et al., 2001; Mitin et al., 2005). Instead, our finding that all of the RasGRP C1 domains can interact with anionic phospholipids suggests that this mechanism could contribute to membrane localization of RasGRPs.

2.2 MATERIALS AND METHODS

2.2.1 Construction and expression of GFP/C1 domain fusion proteins

cDNAs encoding the C1 domains were generated by PCR from plasmids encoding full length proteins and fused with the enhanced green fluorescence protein (GFP) cDNA from pEGFP-C1 (Clontech). The sequences of the encoded C1 domain peptides are shown in Figure 2.1A. All C1 domains are murine, with the exception of human Raf-1. The sequence of the C-terminal portion of the GFP peptide in these constructs is: ...DELYKSGLRSLKST, with this sequence being fused to the N-termini of the C1 domain sequences in Figure 2.1A. The fusion cDNAs encoding the GFP-C1 domain constructs were cloned into the retroviral vector pCTV211 (Guilbault and Kay, 2004), converted to retroviral particles by transfection into the BOSC 23 packaging cell line (Pear et al., 1993) and transduced into the NIH 3T3 (American Type Culture Collection, ATCC) WEHI-231 (ATCC) or DO11.10 (Haskins et al., 1983) cell lines. Polyclonal populations of transduced cells were obtained by puromycin selection.

2.2.2 Fluorescence microscopy

Transduced NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose (DMEM) plus 10% calf serum on cover slips for 24 hours, then transferred to the same medium containing 10% or 0% calf serum for an additional 16 hours. The cells were treated with 2 μM PMA (Sigma-Aldrich, Oakville, ON) or 100 μM dioctanoylglycerol (a short chain DAG) (Sigma-Aldrich) from stocks in DMSO, or with DMSO alone, for 5 minutes. DO11.10 T cells and WEHI-231 B cells were adhered to poly-L-lysine coated coverslips and cultured in DMEM 10% fetal bovine serum for 24 hours. After fixation with 4% formaldehyde in PBS for 2 minutes, the cells were permeabilized with 0.2% Triton X-100 in PBS and stained with Alexa Fluor 488-conjugated anti-GFP antibody (Invitrogen, Carlsbad, CA). This increases sensitivity of detection of GFP, compared to GFP fluorescence on its own. To mark Golgi membranes, cells were co-stained with anti-GFP as above, along with mouse anti-GM130 (BD Biosciences, Mississauga, ON) followed by

Alexa Fluor 647-conjugated anti-mouse IgG (Invitrogen). Endoplasmic reticulum was marked by treating unfixed cells with glibenclamide BODIPY-texas red (ER Tracker, Invitrogen), followed by fixation with formaldehyde in PBS and staining with anti-GFP as described above. Images were photographed with an Axioplan 2 imaging microscope (Carl Zeiss, Toronto, ON), using OpenLab imaging software (Improvision, Coventry, England).

2.2.3 Preparation of cellular membrane and cytosol fractions

Transduced NIH 3T3 cells at 50% confluency were serum-starved for 3.5 hours by culture in DMEM plus 1 mg/ml BSA, and then treated for the indicated time with DMEM, plus 1 mg/ml BSA containing 300 μM dioctanoylglycerol. On ice, cells were washed 3 times with cold PBS, scraped into 0.25 ml lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM PMSF, 2.5 μg/ml leupeptin, 2 μg/ml chymostatin, 1 μg/ml antipain, 2 μg/ml pepstatin, 10 μg/ml benzamidine, 10 μg/ml p-aminobenzadine) and lysed by sonication. The NaCl concentration was adjusted to 0.15 M and the lysate was centrifuged at 100,000 x g to separate cytosol from particulate. The resulting pellet was resuspended by sonication in lysis buffer with 0.15 M NaCl and 1% (w/v) Triton X-100, and centrifuged at 100,000 x g to separate detergent-soluble membranes from insoluble particulate. Equivalent volumes of each fraction were added to Laemmli sample buffer without β-mercaptoethanol or boiling (boiling would destroy GFP fluorescence) and were electrophoresed on 10% SDS-PAGE. Gels were immediately scanned using a Typhoon 9410 (488nm Blue2 laser, 520nm BP filter) to visualize the fluorescence of GFP-tagged proteins. The fluorescence of GFP-C1 domain bands were quantified using ImageQuant (GE Healthcare, Piscataway, NJ). The percent in the membrane fraction was calculated as the density of the band in the detergent-soluble microsome fraction relative to the sum of the densities of the protein bands in the three sub-cellular fractions. The recovery in the three subcellular fractions, expressed as a percent of the cell lysate density averaged 99 ± 9% (S.D.) with a range of 87% - 116%.

2.2.4 Construction of K-Ras/C1 fusions, RasGRP1Δ/C1 fusions and RasGRP4α and β

The K-Ras Q61N Δ173-188 mutant has the C-terminal amino acid sequence ...KEKMSKDG*STEA* (residues not naturally present in K-Ras are italicized). Fusions of K-Ras Q61N Δ173-188 to C1 domains have the sequence ...KEKMSKDG*ST,* with this sequence being fused to the N-termini of the C1 domain sequences in Figure 2.1. For the K-Ras/pren construct, the basic cluster and prenylation signal of K-Ras, along with an HA epitope tag, was re-attached to the K-Ras Q61N Δ173-188 mutant, resulting in the C-terminal sequence ...KEKMSKDG*STEAYPYDYASGS*RKHKEKMSKDGKKKKKKSKTKCVIM. The N-terminus of K-Ras Q61N Δ173-188 is fused to GFP, with the junction sequence being ...DELYKSGLRS*FLLK*MTEYKLVVV...

The N-terminally GFP-tagged and C-terminally deleted form of murine RasGRP1 (RasGRP1Δ) has GFP fused to amino acid 2 of RasGRP1 (GenBank accession NP035376). The sequence at this N-terminal fusion site is ...delyksglrssaqseGTLGKAR..., with natural sequence of RasGRP1 in upper case. The sequence at the C-terminal fusion junction is ...YSKLGst[C1 domain], with the C1 domain sequences shown in Figure 2.1.

Murine RasGRP4α and β with N-terminal GFP tags have the sequence ...delyksglrslksNRKDIKRKS.... at the GFP-RasGRP4 junction, with the natural sequence of RasGRP4 (starting at amino acid 2) in upper case. The encoded RasGRP4α is identical to Genbank accession AF331457, while the encoded RasGRP4β is identical except for the five amino acid insertion in the C1 domain.

2.2.5 NIH 3T3 transformation assays

cDNAs encoding the specified proteins were inserted into the retroviral vector pCTV211, which were converted to retroviruses, were transduced into NIH 3T3 cells and selected as described above. Transduced cells were plated at 10% confluence. For assessment of transformation in 10% serum, the cells were cultured continually until 3 days (For K-Ras) or 5-7 days (for RasGRPs) after the cells had formed a monolayer, then photographed using a Nikon Elipse TS100 microscope (Nikon, Mississauga, ON). For low

serum transformation assays, the cells were transferred to DMEM / 0.5% fetal calf serum for 1 day, followed by another 2 days in DMEM/ 0.5% fetal calf serum with or without 0.1 μM PMA. The cells were then photographed. The photographed regions were representative of the appearance of the cells across the entire culture dish. To quantify transformation efficiency, NIH 3T3 cultures transduced with the indicated constructs were seeded at low density. After colonies had grown up from individual cells, they were scored as transformed or non-transformed. Transformation efficiency = # of transformed colonies/total # of colonies. >30 colonies were scored.

2.2.6 Construction, expression and purification of GST/C1 fusion proteins

cDNAs encoding C1 domains were inserted into the glutathione S-transferase (GST) fusion vector pGEX-2T (Amersham, Piscataway, NJ), resulting in N-terminal GST fusions having the junction sequence ...LVPRGSLKST, with this sequence being fused to the N-termini of the C1 domain sequences in Figure 2.1A. The plasmids were transformed into *E. coli* strain AD202 and cultures in LB medium were grown overnight at 37° to saturation, then diluted 40-fold and grown at 37° to an OD_{600} of 0.8. After 10 min on ice, cells were induced with 0.5 mM IPTG for 5 hours at 28° C. The bacterial cell pellet was resuspended in cold PBS containing 1% (w/v) Triton X100, 2 mM PMSF, and cells lysed by two passes through a French press. Insoluble material was removed by centrifugation at 15,000 x g. GST-C1 domains in the soluble fraction were bound to glutathione-Sepharose (Amersham; 2 ml of 50% slurry per litre of cell culture, equilibrated in PBS/1% Triton X-100) during a 2-hour incubation at 4°. Beads were collected on a mini-column and washed with PBS /1% Triton X100, PBS, and finally 50 mM Tris, pH 7.5, 0.15 M NaCl before elution of GST fusion protein in 50 mM Tris pH 7.5, 0.15 M NaCl, 5 mM glutathione. Protein was quantitated by Bradford assay using ovalbumin as a standard. The GST fusion proteins were aliquotted and stored at -80° for use in the *in vitro* vesicle-binding assay. On average, 40 mg of the GST-C1 fusion protein, at 2 mg/ml was obtained per liter of bacterial culture.

2.2.7 Binding of GST-C1 domain fusion proteins to sucrose-loaded phospholipid vesicles

Binding of GST-C1 domain fusion proteins to sucrose-loaded large unilamellar vesicles (SLVs) was measured using a protocol based on that described previously (Buser and McLaughlin, 1998; Johnson et al., 2000). 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC), 1,2-dioleoyl-phosphatidylserine (PS), 1,2 dioleoyl-phosphatidylglycerol (PG), 1,2-dioleoylglycerol (DAG), 1-palmitoyl, 2-oleoyl-phosphatidic acid (PA), 1-palmitoyl, 2-hydroxy-glycerophosphate (lysoPA), and N-palmitoyl, D-erythro-sphingosine (ceramide) were obtained from Avanti Polar Lipids (Alabaster, AL) or Northern Lipids (Burnaby, BC), while oleic acid and arachidonic acid were from Sigma-Aldrich. Lipid vesicles were prepared by drying mixtures of lipids (including trace amounts of [^{3}H] di-palmitoyl PC for quantification of recovery) in chloroform on a rotary-evaporator, resuspending in 20 mM Hepes, pH 7.5, 170 mM sucrose by vigorous vortexing to a final lipid concentration of 2 mM, followed by five freeze-thaw cycles in liquid nitrogen. These multilamellar vesicles were stored at -20° C until use. On the day of the experiment, SLVs were prepared by extrusion at room temperature through a 100 nm membrane using a Lipofast Microextruder (Avestin, Mannheim, Germany). Extruded vesicles were diluted 5-fold in 100 mM NaCl, 20 mM Hepes pH 7.5 and centrifuged 100,000 x g for 30 min at 25° C to dilute the free sucrose. 80% of the supernatant was removed and the resulting pellet was resuspended by vigorous vortexing in the residual volume. PMA, from a DMSO stock, was subsequently added with vigorous vortexing such that the end concentration of DMSO was < 2 % (v/v). GST-C1 domain fusion proteins (0.38-0.48 μM) were incubated with SLVs (200 μM, except 125 μM for Fig. 2.6A) for 10 min in the presence of 20 mM Hepes, pH 7.5, 100 mM NaCl, 0.05 mg/ml ovalbumin (non-specific adsorption blocker) in a volume of 120 μL. Vesicle-bound protein was separated from unbound by centrifugation at 100,000 x g for 30 min at 25 °C. Greater than 90% of the [^{3}H] di-palmitoyl PC label was in the pellet fraction. Separate samples of fusion proteins were treated in the same way in the absence of added SLVs to quantify vesicle-independent sedimentation. Protein in each fraction was determined by analysis of supernatant and pellet fractions, and resolved by SDS-PAGE on a Tricine gel system (Schagger and von Jagow, 1987). Gels were stained with Sypro-Orange stain (Molecular Probes, Invitrogen) according to the manufacturer's instructions, and imaged on a

Typhoon 9410 (488nm Blue2 laser, 580nm BP filter). Fluorescence intensities of the GST-C1 bands were quantitated using ImageQuant. Percent bound= 100% x P/(S + P) where P is intensity of fluorescence band from the pellet fraction, and S is intensity of fluorescence band from the supernatant fraction. The pellet intensities were corrected for contaminating supernatant, as well as protein that sedimented in the absence of vesicles. The latter correction resulted in some % bound values being less than zero. The apparent association constant, K_a = (B/F) x 1/L where B is fraction of protein bound (in pellet), F is fraction of protein free (in supernatant), and L is the molar concentration of accessible lipid (0.5 x total lipid concentration, since the C1 domain only binds the outer leaflet) was calculated.

2.3 RESULTS

2.3.1 Sequence comparison of RasGRP C1 domains

Modeling of the RasGRP1 C1 domain indicated that its A and B loops can form a ligand binding pocket that is very similar to that of the C1b domain of PKCδ (Rong et al., 2002). The RasGRP3 C1 domain is identical to that of RasGRP1 in loop A except for asparagine versus threonine at the base of the loop, and has only an isoleucine/valine difference in loop B (Figure 2.1A). To assess the DAG- or phorbol ester-binding potentials of the C1 domains of RasGRP2 and the two splice variants of RasGRP4, we compared their ligand binding loops to those of RasGRP1 and 3, and to the C1b domain of PKCδ and the C1 domain of β2-chimaerin (Figure 2.1A).

There are two atypical residues in loop A of the RasGRP2 C1 domain. Position 8 is serine, versus tyrosine or phenylalanine in the DAG/phorbol ester-binding C1 domains. Mutation of tyrosine 8 to glycine in the PKCδ C1b domain reduces but does not eliminate phorbol ester binding (Kazanietz et al., 1995b). The other atypical residue in loop A is valine at position 12, which is threonine or histidine in the DAG and phorbol ester-binding C1 domains shown in Figure 2.1A. Mutation of threonine 12 to valine in PKCδ C1b caused a minor reduction in phorbol ester binding (Kazanietz et al., 1995b), while changing position 12 in the DAG kinase β C1a domain from alanine to threonine moderately increased phorbol ester binding (Shindo et al., 2003). The significant discrepancy in loop B of the RasGRP2 C1 domain is leucine 22, which is tryptophan in the DAG and phorbol ester-binding C1 domains shown in Figure 2.1. In the C1b domain of PKCβ, switching this residue from tyrosine to tryptophan increases affinity for DAG and enhances colocalization with perinuclear membranes (Dries et al., 2007). Tryptophan versus tyrosine occupancy at this position has the potential to affect DAG affinity by causing major perturbations in the shape of the ligand binding pocket (Dries et al., 2007). The effect of leucine at this position is unknown. While the variations at positions 8, 12 and 22 serve as warning flags that the DAG or phorbol ester binding capabilities of the RasGRP2 C1 domain should not be taken for granted, none of these changes by themselves are predicted to eliminate binding by either of these ligands.

A

```
                  Loop A          Loop B
PKCδ C1b   MPHRFKVYNYMSPTFCDHCGSLLWGLVKQGLKCEDCGMNVHHKCREKVANLCGINQKptea
B2-chim.     HNFKVHTFRGPHWCEYCANFMWGLIAQGVRCSDCGLNVHKQCSKHVPNDC
             1         10        20        30        40        50
RasGRP1    FPHNFQETTYLKPTFCDNCAGFLWGVIKQGYRCKDCGMNCHKQCKDLVVFECKKRIKptea
RasGRP2    FVHNFQESNSLRPVACRHCKALILGIYKQGLKCRACGVNCHKQCKERLSVECRRRAQptea
RasGRP3    FIHNFQEMNYLKPTFCEHCAGFLWGIIKQGYKCKDCGANCHKQCKDLLVLACRRLARAPSLSSNPtea
RasGRP4    FLHAFQEVTFRKPTFCHSCSGFLWGVTKQGYRCRDCGLCCHRHCRDQVRVECKKRPtea
                          VSTGP = insert in RasGRP4β
Raf-1      TTHNFARKTFLKLAFCDICQKFLL----NGFRCQTCGYKFHEHCSTKVPTMCkkrikptea
KSR          HRFSTKSWLSQV-CHVCQKSMI----FGVKCKHCRLKCHNKC-TKEAPAC
```

B

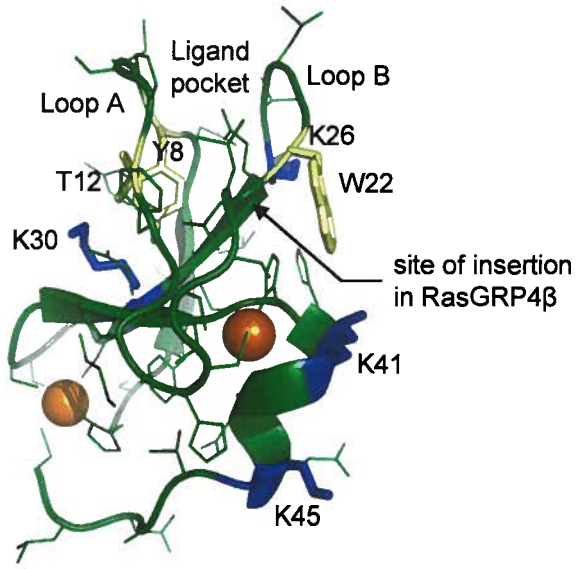

Figure 2.1: C1 domain sequences

A. The C1 domains of the RasGRPs are compared to four C1 domains of known structure. The C1b domain of PKCδ and the C1 domain of β2-chimaerin bind DAG and phorbol esters while the C1 domains of Raf-1 and KSR do not. The histidine and cysteine residues which coordinate zinc are indicated by the shaded bars. Loop A and B residues are italicized. Cationic residues which have the potential to interact with anionic phospholipids within membranes are bolded. For the C1 domains used in this study, the sequences extending to the C-termini of the expressed proteins are shown, with non-natural amino acids in lower case. The N-termini indicate where the C1 domains were fused to GFP, GST or K-Ras. Numbering of the residues within the C1 domains is shown above the RasGRP1 C1 domain sequence.

B. The C1b domain of PKCδ (protein data bank code 1ptq) illustrates the generic structure of a C1 domain that binds DAG or phorbol ester. The zinc atoms are shown as orange balls. Y8, T12 and W22 are highlighted in yellow. Four of the cationic residues implicated in membrane interactions, K26, K30, K41 and K45, are highlighted in blue. The image was generated with PyMol.

The RasGRP4α C1 domain has no atypical residues in loop A or B relative to the DAG/phorbol ester-binding C1 domains shown in Figure 2.1A. Therefore, it is expected to bind both DAG and phorbol esters. In RasGRP4β there is an insertion of five amino acids at the N-terminus of loop B. While this insertion could radically disrupt the ligand binding pocket, it is possible that the inserted amino acids project outward, with most of loop B remaining in position to form one side of the ligand-binding pocket. In the latter case, the RasGRP4β C1 domain could retain binding of DAG and/or phorbol ester, or could have altered ligand specificity relative to the C1 domain of RasGRP4α.

All of the RasGRP C1 domains have cationic residues within and C-terminal to their C1 domains (Figure 2.1A) which are positioned to interact electrostatically with anionic phospholipids (Hurley, 2006). In conjunction with the hydrophobic residues projecting from loops A and B, these have the potential to either cooperate with DAG to enhance membrane binding affinity or to mediate DAG-independent interactions with membranes.

To summarize, the C1 domain of RasGRP4α is expected to bind both DAG and phorbol ester based on its similarities in loop A and B to other C1 domains, but the capabilities of the C1 domains of RasGRP2 and 4β to bind either of these ligands cannot be predicted with confidence from their sequences. All of the RasGRP C1 domains have the potential to bind membranes independently of DAG or phorbol ester, via electrostatic and hydrophobic interactions. A combination of *in vivo* and *in vitro* experiments are needed to resolve the question of whether all RasGRP C1 domains can mediate the membrane binding required for juxtaposing these exchange factors with their Ras or Rap GTPase substrates, and to assess whether the C1 domains achieve this by binding directly to DAG.

2.3.2 Only the C1 domains of RasGRP 1, 3 and 4α co-localize with membranes and translocate in response to DAG or phorbol ester

Fusions of C1 domains with GFP were expressed in NIH 3T3 fibroblasts to compare the localization of the five RasGRP C1 domains to the DAG-binding PKCδ C1b domain versus the Raf-1 C1 domain, which does not bind DAG. The GFP/C1 domain fusions for the RasGRPs and PKCδ included the clusters of cationic amino acids which are naturally found

immediately C-terminal to the C1 domains, as these have been shown to contribute to membrane binding in vivo and in vitro (Irie et al., 2004; Tognon et al., 1998). For the Raf-1 C1 domain, the cationic cluster from RasGRP1 was attached to ensure that any deficiencies in membrane binding by the Raf-1 C1 domain were not simply due to its lack of a cluster of cationic amino acid residues.

In NIH 3T3 cells cultured in 10% serum, the Raf-1 C1 domain, as well as GFP alone, was distributed throughout the nucleus and cytoplasm (Figure 2.2A). In contrast, the PKCδ C1b domain and the RasGRP1 C1 domain were excluded from the nucleus and concentrated in the perinuclear cytoplasm where they co-localized with internal membranes, as shown by staining GFP-RasGRP1 C1 domain-expressing cells with ER Tracker [glibenclamide, which binds to sulfonylurea receptors in the endoplasmic reticulum (Hambrock et al., 2002)] or with antibody to the Golgi-associated protein GM130 (Nakamura et al., 1995) (Figure 2.2B). The C1 domain of RasGRP3 was also largely co-localized with internal membranes, while the C1 domain of RasGRP4α was similar but with more accumulation away from internal membranes, particularly evident as partial localization in the nucleus. The C1 domains of RasGRP2 and RasGRP4β were radically different, being distributed throughout the cytoplasm and nucleus, as was seen for the Raf-1 C1 domain and GFP alone. A similar pattern of C1 domain localization was seen in the T cell line DO11.10 and the B cell line WEHI-231, with the C1 domains of RasGRP1 and 3 strongly co-localizing with internal membranes, the C1 domains of RasGRP2 and 4β being dispersed throughout the cells, and the C1 domain of RasGRP4α being partially excluded from the nucleus but only weakly co-localizating with internal membranes (Figure 2.3).

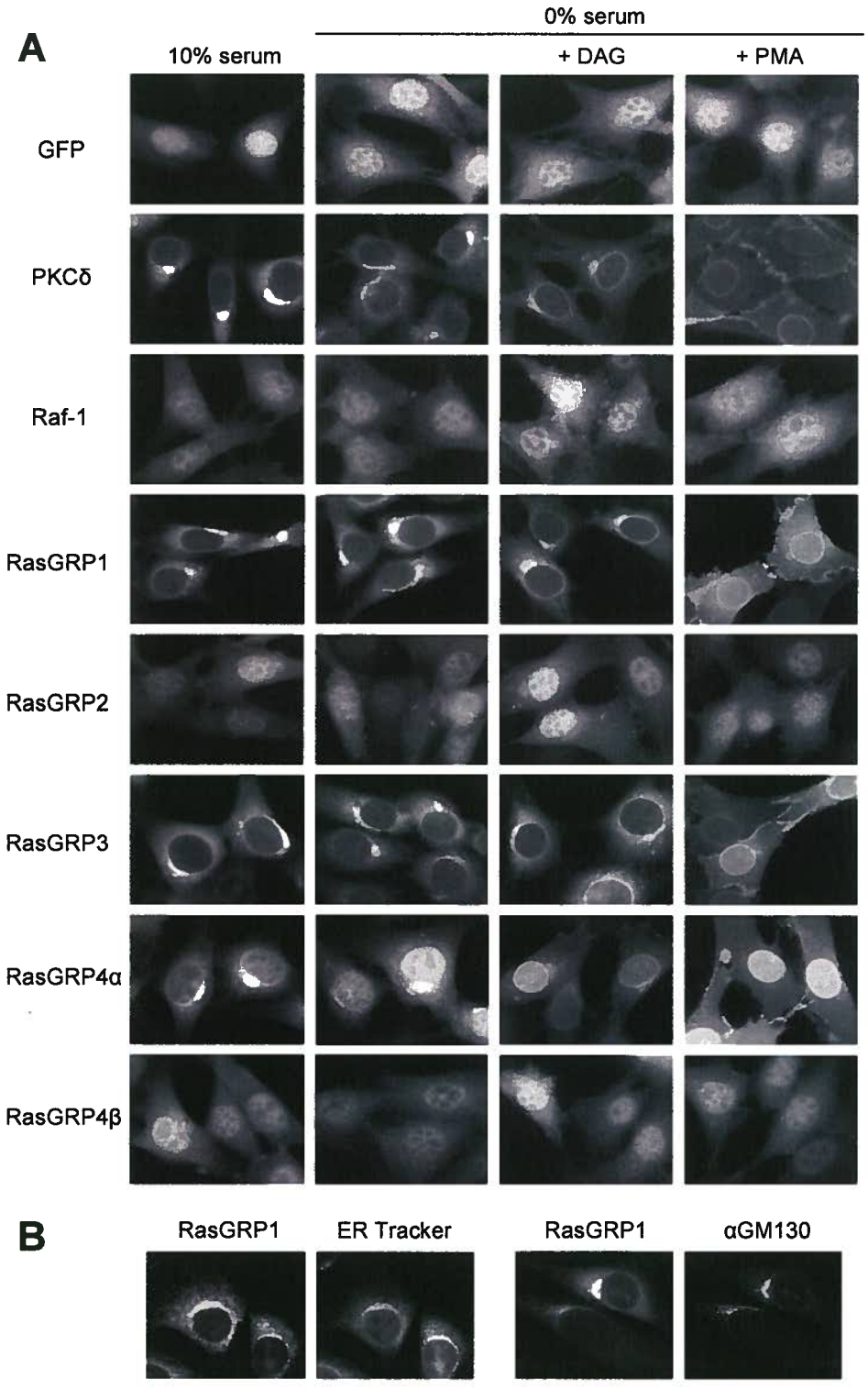

Figure 2.2 Only the C1 domains of RasGRP1, 3 and 4α co-localize with membranes and translocate in response to DAG or phorbol ester

Figure 2.2. Only the C1 domains of RasGRP1, 3 and 4α co-localize with membranes and translocate in response to DAG or phorbol ester (page 58)
A. GFP fusions of the indicated C1 domains, or GFP alone, were expressed in NIH 3T3 cells. After culture in DMEM containing 10% or 0% calf serum +/- 100 μM dioctanoylglycerol (DAG) or 2 μM PMA as indicated, the cells were fixed and photographed by fluorescence microscopy.
B. NIH 3T3 cells expressing the GFP-RasGRP1 C1 domain fusion were stained with either ER Tracker to mark endoplasmic reticulum or anti-GM130 to mark Golgi membranes, as described in Materials and Methods. Individual cells showing fluorescence from the GFP-tagged RasGRP1 C1 domain and either ER Tracker or GM130 staining are shown.

Culturing NIH 3T3 cells in medium lacking serum caused a considerable shift in the distribution of the RasGRP4α C1 domain away from perinuclear membranes and into the nucleus (Figure 2.2A). Serum starvation had no noticeable effect on the other C1 domains. Relative to the RasGRP1 and 3 C1 domains, the RasGRP4α C1 domain appears to be less co-localized with internal membranes and more dependent on serum stimulation to maintain its localization at those membranes. Nonetheless, it is clearly distinguished from the C1 domains of RasGRP2 and RasGRP4β which lack co-localization with membranes.

Treatment of the serum-starved NIH 3T3 cells with dioctanoylglycerol, a short chain DAG, induced accumulation of the C1 domains of PKCδ and RasGRP1, 3 and 4α in the nuclear envelope, while retaining their localization in the perinuclear region (Figure 2.2A). This membrane-selective relocalization presumably reflects accumulation of exogenous DAG at the nuclear envelope. Exogenous DAG could also have accumulated at the ER and Golgi, but with no discernible effect on the C1 domains because they were already located at these sites, potentially by endogenous DAG. PMA also caused relocalization of the C1 domains of RasGRP1, 3 and 4α, and PKCδ. PMA had no observable effect on the localizations of the C1 domains of Raf-1, RasGRP2 or RasGRP4β (Fig. 2.2A), nor did DAG when used at either 100 μM for 5 minutes (Fig. 2.2A) or 300 μM for either 5 or 15 minutes (data not shown).

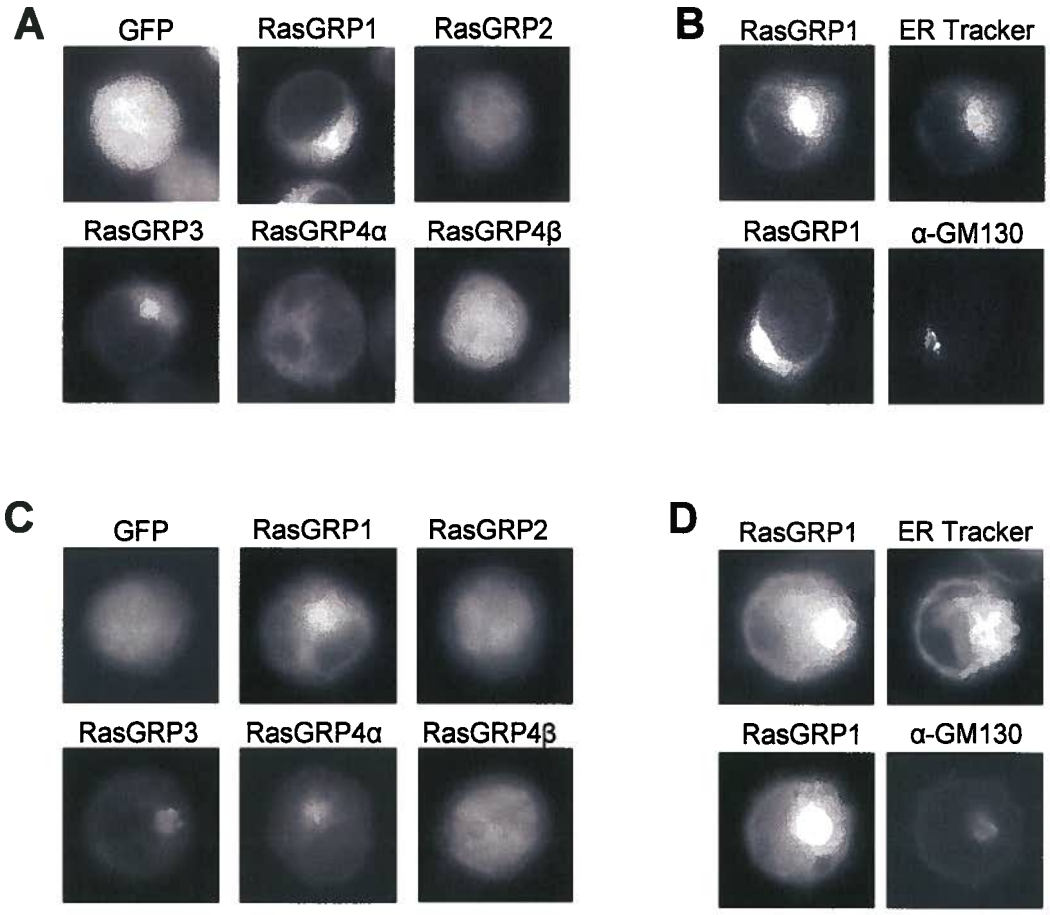

Figure 2.3 Distributions of C1 domains in DO11.10 T cells and WEHI-231 B cells
A and C. GFP fusions of the indicated C1 domains, or GFP alone, were expressed in DO11.10 T cells or WEHI-231 B cells. After culture in DMEM containing 10% fetal calf serum the cells were fixed and photographed.
B and D. DO11.10 or WEHI-231 cells expressing the GFP-RasGRP1 C1 domain fusion were stained with either ER Tracker to mark endoplasmic reticulum or anti-GM130 to mark Golgi membranes, as described in Materials and Methods.

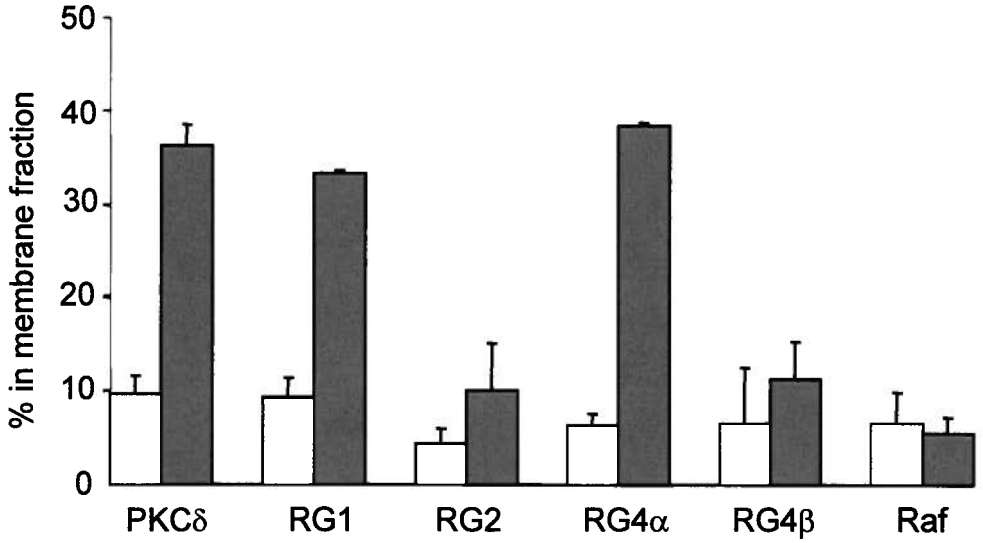

Figure 2.4 DAG induces translocation of the C1 domains of RasGRP1, 3 and 4α to membranes

NIH 3T3 cells expressing the indicated GFP-tagged C1 domains were cultured in serum-free medium for 3.5 h, then treated for 15 min with DMSO (white bars) or 300 μM dioctanoylglycerol (DAG, grey bars). Cells were fractionated and the GFP-C1 proteins in the membrane versus non-membrane fractions were quantified as described in Materials and Methods. Data are means +/- standard errors from two independent experiments.

Differential responses of the C1 domains of RasGRP1 and RasGRP4α versus RasGRP2 and RasGRP4β were also observed when DAG-induced membrane binding was assessed by fractionation of NIH 3T3 cells. GFP-tagged C1 domains were quantitated in cell lysates, cytosol, a detergent-solubilized particulate fraction (regarded as the membrane fraction), and a detergent-insoluble particulate fraction. The percent of each C1 domain in the membrane fraction is shown in Figure 2.4. In serum-starved cells, less than 10% of each C1 domain was in the membrane fraction. After DAG treatment 35-40% of the C1 domains of PKC, RasGRP1 and RasGRP4α were membrane-associated, whereas the RasGRP2 and RasGRP4β C1 domains, like the Raf-1 C1 domain, did not show significant DAG-induced changes in membrane binding.

2.3.3 The C1 domains of RasGRP1 and 4α bind directly to DAG within phospholipid membranes, while the C1 domains of RasGRP2 and 4β do not

To determine if the differences in membrane localization of the RasGRP C1 domains reflected differences in their abilities to directly bind DAG or phorbol ester within phospholipid bilayers, we compared their binding to unilamellar phospholipid vesicles *in vitro*. C1 domains were expressed in *E. coli* as GST fusion proteins, and purified by affinity chromatography on glutathione-agarose. The GST-C1 fusion proteins ran as the expected single 34 kDa species on SDS-PAGE, with the exception of the GST-RasGRP3 C1 construct which had a significant amount of a smaller 26 kDa GST species present, indicating partial premature termination of translation near the GST/C1 junction (data not shown).

Binding of the C1 domains to phospholipid bilayers was assessed by their cosedimentation with sucrose-loaded large unilamellar vesicles (SLVs) composed of the neutral phospholipid phosphatidylcholine (PC) supplemented with 5 mol% of the anionic phospholipid phosphatidylserine (PS), either alone or in combination with 5 mol% long-chain DAG (1,2-dioleoylglycerol), or 1 mol% PMA. The PKCδ C1b and Raf-1 C1 domains were used as positive and negative controls to test this experimental system (Figure 2.5A). The PKCδ C1b domain bound poorly to the PC/5% PS vesicles, and very well to vesicles containing DAG or PMA. In contrast, the Raf-1 C1 domain had no significant vesicle binding in the absence or presence of DAG or PMA.

The RasGRP1 C1 domain was very similar to the PKCδ C1b domain, with the addition of either DAG or PMA inducing nearly complete binding to the vesicles (Figure 2.5A). The RasGRP3 and 4α C1 domains were equivalent to the C1 domain of RasGRP1 in having nearly complete binding to the PMA-containing vesicles, but bound less effectively to DAG-containing vesicles (Figure 2.5A). The C1 domains of RasGRP2 and RasGRP4β had minimal vesicle binding, and this was not altered by the addition of DAG or PMA.

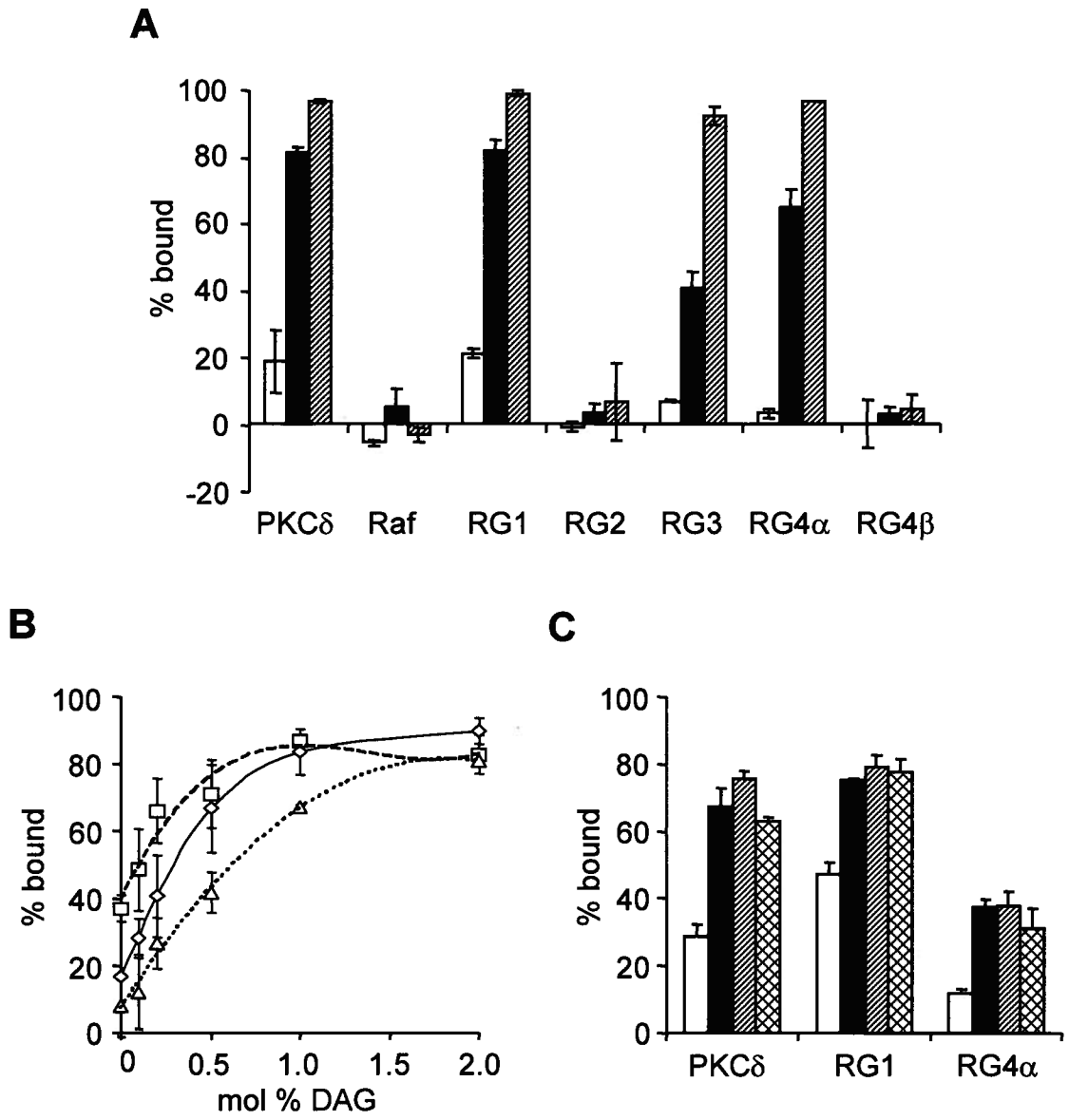

Figure 2.5 Binding of the C1 domains of RasGRP1, 3, and 4α to DAG or PMA within membrane vesicles

A. GST fusions of the indicated C1 domains were mixed with SLVs composed of PC and 5 mol% PS (open bars) or 5 mol% PS and 5 mol% DAG (black bars) or 5 mol% PS and 1 mol% PMA (striped bars). Binding was assessed by co-sedimentation as described in Materials and Methods. Some values are less than 0% because of correction for the amount of protein which sedimented in the absence of vesicles. Data are means +/- range of 2 independent experiments. **B.** Binding of GST fusions of the RasGRP1 (□), RasGRP4α (Δ), and PKCδ C1 (◊) domains to PC SLVs containing 10 mol% PS with various mol% DAG. Data are means +/- range of 2 independent experiments.
C. Binding of GST fusions of the RasGRP1, RasGRP4α and PKCδ C1 domains to PC SLVs containing 2 mol% DAG and either no anionic lipid (open bars), 5 mol% PS (black bars), 2.5 mol% PS and 2.5 mol% PG (striped bars), or 5 mol% PG (hatched bars). Data are means +/- range of 2 independent experiments.

These vesicle binding properties mirror the results we obtained with the in vivo experiments, and confirm that direct binding to DAG and PMA is restricted to the C1 domains of RasGRP1, 3 and 4α. This initial vesicle binding experiment also indicated that there could be quantitative heterogeneity among these three DAG-binding C1 domains. However, the lower DAG binding of the RasGRP3 C1 domain seen in Figure 2.5A may be artifactual, because the protein was produced as a mixture of full-length and truncated GST/C1 fusion proteins. Because GST dimerizes, this would result in some GST-GST/C1 heterodimers, which would have reduced avidity for membranes relative to GST/C1-GST/C1 homodimers, due to the presence of one versus two DAG-binding sites within the dimer. Considering this anomaly, the RasGRP3 C1 domain was not used in subsequent experiments aimed at quantifying differences in DAG-dependent and DAG-independent membrane binding.

2.3.4 The C1 domains of RasGRP1 and 4α have different affinities for DAG

Figure 2.5B compares the binding of the RasGRP1 versus 4α C1 domains to PC/10 mol% PS vesicles with DAG concentrations ranging from 0 to 2 mol%. Binding of the RasGRP4α C1 domain was less sensitive to DAG at low concentrations, and reached saturation at 2 mol% DAG, versus 1 mol% DAG for the RasGRP1 C1 domain. The binding curve for the PKCδ C1b domain was intermediate between those of RasGRP1 and 4α at low DAG concentrations, and like the RasGRP1 C1 domain it reached saturation at a lower DAG concentration than was required for the RasGRP4α C1 domain. From these binding curves we calculated apparent affinity constants for membranes containing 10 mol% PS and 0.2 mol% DAG. The values are 21,000, 7500, and 3800 M^{-1} for the RasGRP1, PKCδ, and RasGRP4α C1 domains, respectively. Figure 2.5B also indicates that the RasGRP1 C1 domain binds more strongly to 10 mol% PS vesicles containing no DAG, in comparison to the RasGRP4α or PKCδ C1b domains.

The role of anionic phospholipid in the response to DAG is shown in Figure 2.5C. Binding of the C1 domains of PKCδ, RasGRP1 or RasGRP4α to vesicles containing 2 mol% DAG was enhanced by either of the anionic phospholipids PS or phosphatidylglycerol (PG),

included at 5 mol%. Of the three, the RasGRP4α C1 domain showed the weakest binding and was the most dependent on anionic phospholipid. Membrane binding of the RasGRP 1 and 4α C1 domains does not specifically require the phosphatidylserine head group. Instead, they appear to respond to the negative charge on the membrane surface, as is the case for the PKCδ C1b domain as well as other PKC C1 domains (Johnson et al., 2000).

2.3.5 Phosphatidic acid, lysophosphatidic acid, ceramide, fatty acids and sphingosine-1-phosphate are not alternate ligands for RasGRP2 or RasGRP4β

The RasGRP2 and 4β C1 domains do not bind to DAG, but their sequences are compatible with their having a modified pocket structure that could bind an alternative ligand. To address the hypothesis that the C1 domains of RasGRP2 and 4β are specialized to recognize lipid second messengers other than DAG, we tested candidate lipid ligands which have small head groups suitable for occupying the pocket and which act as signaling molecules.

Phosphatidic acid was of particular interest as a ligand, because it can be generated from DAG via DAG kinases which have been functionally coupled to RasGRPs (Regier et al., 2005; Sanjuan et al., 2003; Topham and Prescott, 2001; Zha et al., 2006). However, phosphatidic acid at 5 mol% did not promote vesicle binding of any of the C1 domains, under conditions in which 5 mol% DAG promoted nearly complete binding of the C1 domains of RasGRP1, RasGRP4α and PKCδ (Figure 2.6A). Other signaling lipids with larger headgroups that we examined, lyso-phosphatidic acid (lysoPA) and sphingosine 1-phosphate, also failed to promote binding of the C1 domains of RasGRP1, 2 or 4β (Figure 2.6B). Ceramide is structurally similar to DAG and has been postulated as a potential ligand for DAG binding-incompetent C1 domains of Raf-1, PKCs and DAG kinases, although a direct interaction with these or any other C1 domain has not been demonstrated (Kashiwagi et al., 2002; van Blitterswijk, 1998; van Blitterswijk et al., 2003).

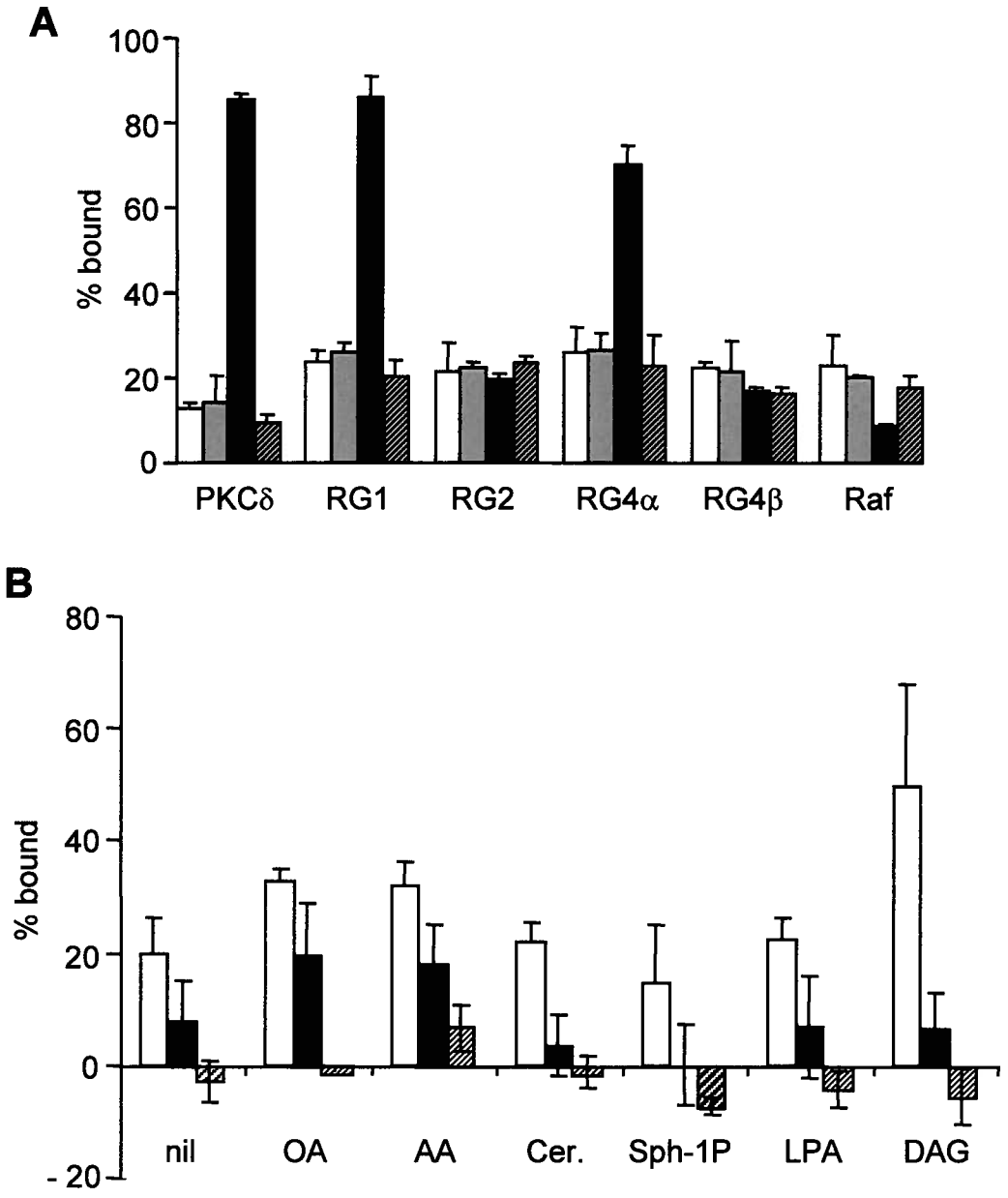

Figure 2.6 Lack of binding of RasGRP C1 domains to alternative lipid ligands
A. GST fusions of the indicated RasGRP C1 domains were assayed for binding to PC SLVs (open), or PC SLVs containing 10 mol% PS (grey), 10 mol% PS and 5 mol% DAG (black), or 10 mol% PS and 5 mol% PA (striped). The percent bound values were not corrected for sedimentation in the absence of lipid. Data are means +/- range of 2 independent experiments.
B. GST fusions of the C1 domains of RasGRP1 (open bars), RasGRP2 (black bars), and RasGRP4β (striped bars) were mixed with PC SLVs containing 5 mol% PS, and 5 mol% of oleic acid (OA), arachidonic acid (AA), ceramide, sphingosine-1-phosphate (sph-1-P), lysophosphatidic acid (lysoPA), or 1 mol% DAG. Data are means +/- range of 2 independent experiments.

Ceramide at 5 mol% was also ineffective as a ligand for the C1 domains of the RasGRP1, 2 or 4β (Figure 2.6B). Fatty acids, particularly polyunsaturated species such as arachidonic acid, have been reported to influence the activity of some PKC isoforms (Khan et al., 1995). Arachidonic acid causes a C1b-dependent redistribution of PKCε in CHO cells (Kashiwagi et al., 2002), suggesting that it could be a ligand for this and other C1 domains. Arachidonic acid and another fatty acid, oleic acid, induced minor increase in membrane binding of the C1 domains of RasGRP1 and 2 (Figure 2.6B). However, this is likely due to the increased membrane negative charge provided by these anionic lipids (see below).

2.3.6 High concentrations of anionic phospholipids enable membrane binding by RasGRP C1 domains in the absence of DAG

Electrostatic interactions with anionic phospholipids have the potential to provide a DAG-independent mechnism for membrane binding by C1 domains. The anionic lipid dependence for membrane binding in the absence of DAG was tested in Figure 2.7A. All RasGRP C1 domains bound vesicles in an anionic lipid-dependent manner. GST alone did not bind these vesicles, regardless of the anionic lipid composition (data not shown). All C1 domains were >60% bound to vesicles containing 40 mol% PS, but sensitivities to lower PS concentrations were variable. The RasGRP1 C1 domain was the most PS-sensitive of all the C1 domains tested, with considerable binding to vesicles containing just 10 mol% PS and only minor increases in binding at higher PS concentrations. Along with the PKCδ C1b domain, the RasGRP4β C1 domain had the weakest binding response, requiring greater than 20 mol% PS for appreciable binding. The C1 domains of RasGRP2, 4α and Raf-1 had intermediate dependencies on PS, with transitions from low to high binding occurring between 10% and 20% PS.

In addition to their charges, the specific structures of anionic phospholipid headgroups could influence C1 domain binding to membranes. This is of considerable biological interest, because the C1b domain of PKCδ has selectivity for PS which contributes

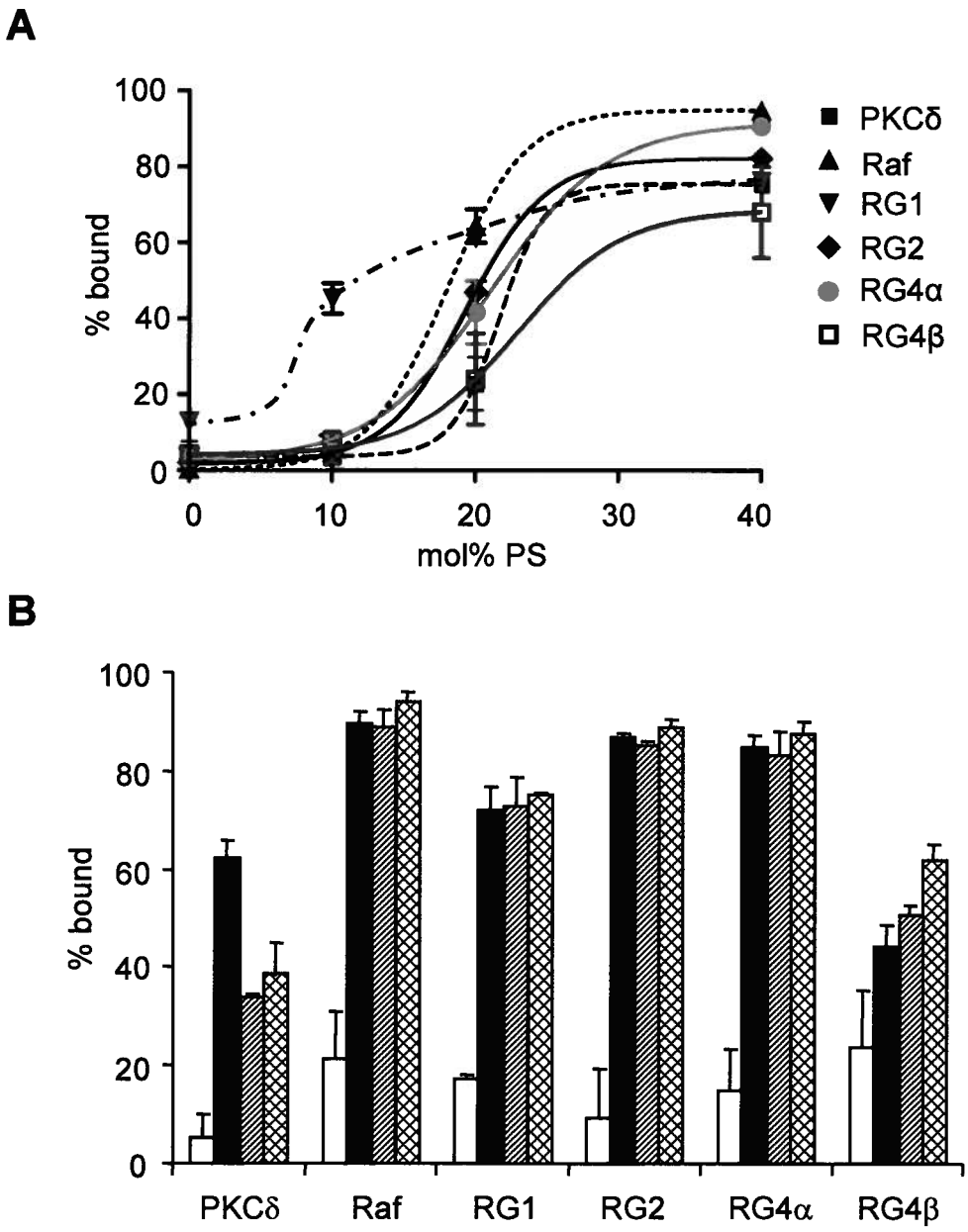

Figure 2.7 RasGRP C1 domains bind to vesicles enriched in anionic phospholipids
A. Dependence of C1 domain binding on the mol% PS. GST fusions of the indicated C1 domains were mixed with SLVs composed of PC and the indicated amounts of PS. Data are means +/- range of 2 independent experiments. The curves were generated by the sigmoidal dose dependence variable slope option of Prism Graph Pad, with the exception of the curve for the RasGRP1 C1 domain which was drawn by hand to give a sigmoidal curve fitting the known % bound value of 20% at 5 mol% PS (Fig. 5A).
B. C1 domain binding to anionic phospholipids is not head group-specific. GST fusions of the indicated C1 domains were assayed for binding to SLVs containing PC alone (open bars), or 70 mol% PC and 30 mol% PS (solid black bars), 30 mol% PG (striped bars), or 30 mol% PA (hatched bars). Data are means +/- range of 2 independent experiments.

to its preferential localization at the PS-enriched plasma membrane (Stahelin et al., 2005; Stahelin et al., 2004). We observed a selectivity for PS only for the C1b domain of PKCδ (Figure 2.7B). The RasGRP C1 domains, like the Raf-1 C1 domain, showed no significant preference for PS versus PG versus PA, and therefore are likely to interact with anionic phospholipids strictly through an electrostatic interaction rather than specific headgroup recognition. The data in Figure 2.7B also show weaker binding of RasGRP4β and PKCδ C1 domains to anionic lipids compared to the others.

These experiments demonstrate that all RasGRP C1 domains can bind to membranes in the absence of DAG, if anionic phospholipids are present at sufficient concentrations. Because anionic phospholipid concentrations range between 10% and 20% of total lipid content in cellular membranes (Daum, 1985), this property of RasGRP C1 domains may be of biological significance, and in particular could contribute to the differential targeting of RasGRPs to specific membranes within cells. In the following experiments, we tested the hypothesis that the C1 domains of RasGRP2 and RasGRP4β could contribute to membrane binding *in vivo* despite being unable to bind to DAG.

2.3.7 Only the DAG-binding C1 domains of RasGRPs can complement a membrane binding deficiency mutation in K-Ras

Complementation of a membrane localization-defective mutant of K-Ras has been used previously to demonstrate the ability of the RasGRP1 C1 domain to confer membrane binding (Tognon et al., 1998). We used this approach to test the abilities of the other RasGRP C1 domains to bind membranes *in vivo*, for the purpose of determining if membrane binding could occur even when the C1 domain was unable to bind specifically to DAG.

In NIH 3T3 cells, constitutive signalling from the mutationally-activated Q61N form of K-Ras induces oncogenic transformation, detectable by cell contraction from the substratum, high refractility and loss of contact inhibition. Signal transduction by K-Ras is

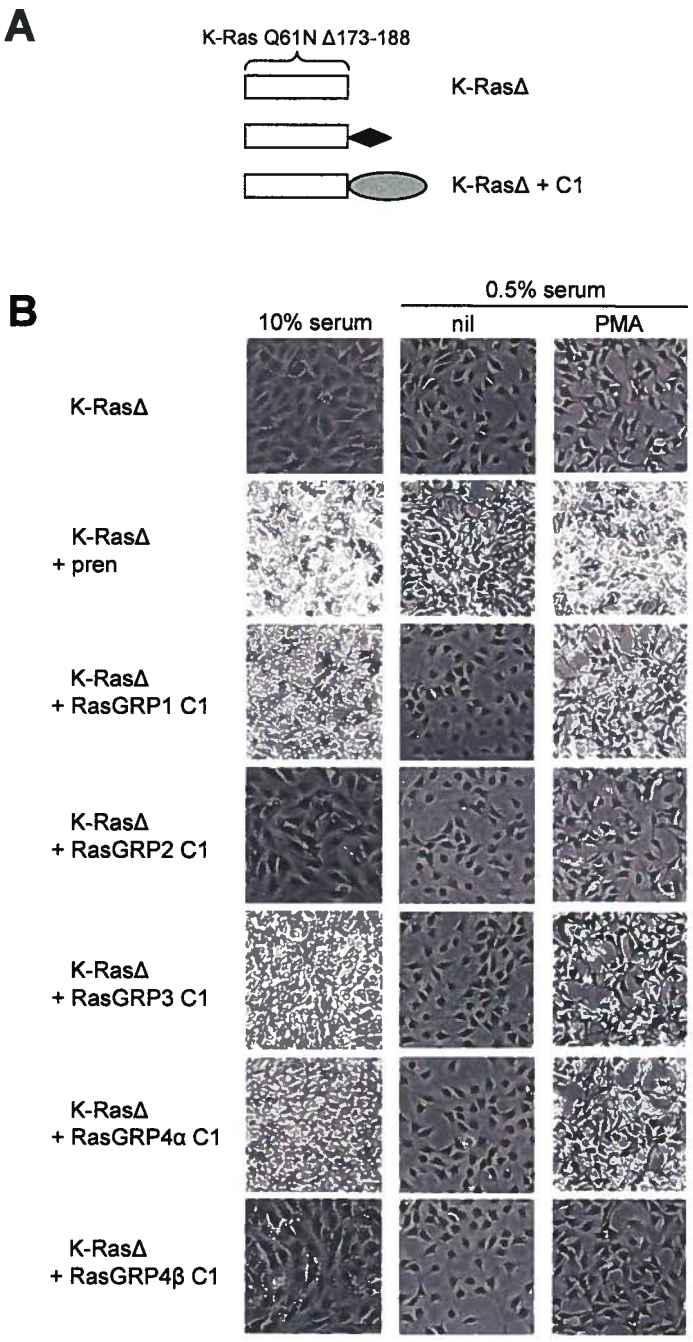

Figure 2.8 Only the C1 domains of RasGRP1, 3 and 4α provide serum- or phorbol ester-dependent complementation of a membrane binding-deficient K-Ras mutant

A. Structures of K-Ras proteins. K-RasΔ is shorthand for K-Ras Q61N Δ173-188. K-RasΔ + pren is K-Ras Q61N Δ173-188 with the K-Ras basic cluster + prenylation signal re-attached. K-RasΔ + C1 is fusion of a C1 domain to the C-terminus of K-Ras Q61N Δ173-188.

B. Transformation assays. The indicated constructs were expressed in NIH 3T3 cells. After culture in medium containing 10% calf serum, or 0.5% serum +/- PMA, the cell cultures were photographed to distinguish those forming a non-refractile contact-inhibited monolayer (non-transformed) from those exhibiting contraction from the substratum, high refractility and loss of contact inhibition (transformed via K-Ras activation).

entirely dependent on its membrane localization, which is naturally provided by C-terminal prenylation, in combination with a polybasic cluster of amino acids (Figure 2.8A). As a result, the Q61N Δ173-188 K-Ras double mutant (K-RasΔ), which has a deletion of the C-terminal basic cluster and prenylation signal, is non-transforming. Reattachment of the basic cluster and prenylation signal to K-RasΔ resulted in restoration of transformation, which was evident even when the cells were cultured in only 0.5% serum (Figure 2.8B).

Attachment of the RasGRP1 C1 domain to the C-terminus of K-RasΔ resulted in transformation when the cells were cultured in 10% serum, but not when they were cultured in 0.5% serum. The serum-independent transformation by the prenylated K-Ras versus the serum-dependent transformation by the K-Ras utilizing the RasGRP1 C1 domain may reflect depletion of a serum-induced C1 ligand, presumably DAG. This interpretation is supported by the observation that transformation via the K-Ras/RasGRP1 C1 fusion protein did occur when the low serum medium was supplemented with a C1 domain ligand, PMA. Equivalent experiments using DAG supplementation were impractical because DAG is rapidly metabolized.

The C1 domains of RasGRP 3 and 4α were equivalent to the RasGRP1 C1 domain in conferring serum- or PMA-dependent complementation of the membrane binding mutation in K-RasΔ (Figure 2.8B). In contrast, the fusions of K-RasΔ to the C1 domains of RasGRP2 or 4β were non-transforming under any conditions. This experiment confirms that stable membrane binding and responsiveness to PMA are restricted to the C1 domains of RasGRP1, 3 and 4α, and demonstrates that the C1 domains of RasGRP2 and 4β are incapable of conferring membrane binding to K-Ras at the level and/or correct subcellular location required to trigger NIH 3T3 cell transformation.

2.3.8 Despite lacking discernible membrane localization in vivo, the C1 domain of RasGRP2 can functionally replace the C1 domain within RasGRP1

Expression of RasGRP1 also induces transformation of NIH 3T3 cells, via its stimulation of GTP loading of Ras GTPases (Tognon et al., 1998). Because all Ras GTPases are membrane-localized, RasGRP1 presumably has to interact with membranes to act on its

substrate Ras, although this interaction could be weak and transient. Deletion of its C-terminal region including the C1 domain completely eliminates membrane localization and transforming activity of RasGRP1, while re-attachment of just the C1 domain fully restores membrane localization and transforming activity (Tognon et al., 1998). Along with the observation that the C1 domain can be functionally replaced by a membrane-localization signal, this demonstrates that transformation by RasGRP1 is dependent on the ability of its C1 domain to confer membrane localization (Tognon et al., 1998). This enables functional replacement of the C1 domain of RasGRP1 to serve as a test for the ability of another C1 domain to confer membrane binding sufficient to support Ras activation by RasGRP1.

Similarly to their effects on K-Ras, the C1 domains of RasGRP3 and 4α were also able to restore transformation via the deleted form of RasGRP1 (RasGRP1Δ), while the C1 domain of RasGRP4β did not (Figure 2.9A, B). Unexpectedly, the C1 domain of RasGRP2 was as effective as the DAG-binding C1 domains of RasGRP1, 3 or 4α in restoring the transforming activity of RasGRP1Δ. The localization of RasGRP1Δ fused to the RasGRP1 or 3 C1 domains was very similar to the localization of the isolated C1 domains, being concentrated in the regions occupied by ER and golgi (Figure 2.9B). In contrast, the RasGRP1Δ fusions to the C1 domains of RasGRP2, 4α and 4β were much more diffusely distributed, such that they were not readily distinguishable from GFP alone. Therefore, the abilities of the RasGRP2 and 4α C1 domains to functionally replace the C1 domain within RasGRP1 occurs despite their inabilities to mimic the observable membrane localization properties of the RasGRP1 C1 domain. It is evident from this experiment that although membrane localization of RasGRPs is essential for accessing membrane-bound Ras and Rap, this can occur at a low and transient level which is not evident by microscopy. Membrane binding by the RasGRP4α C1

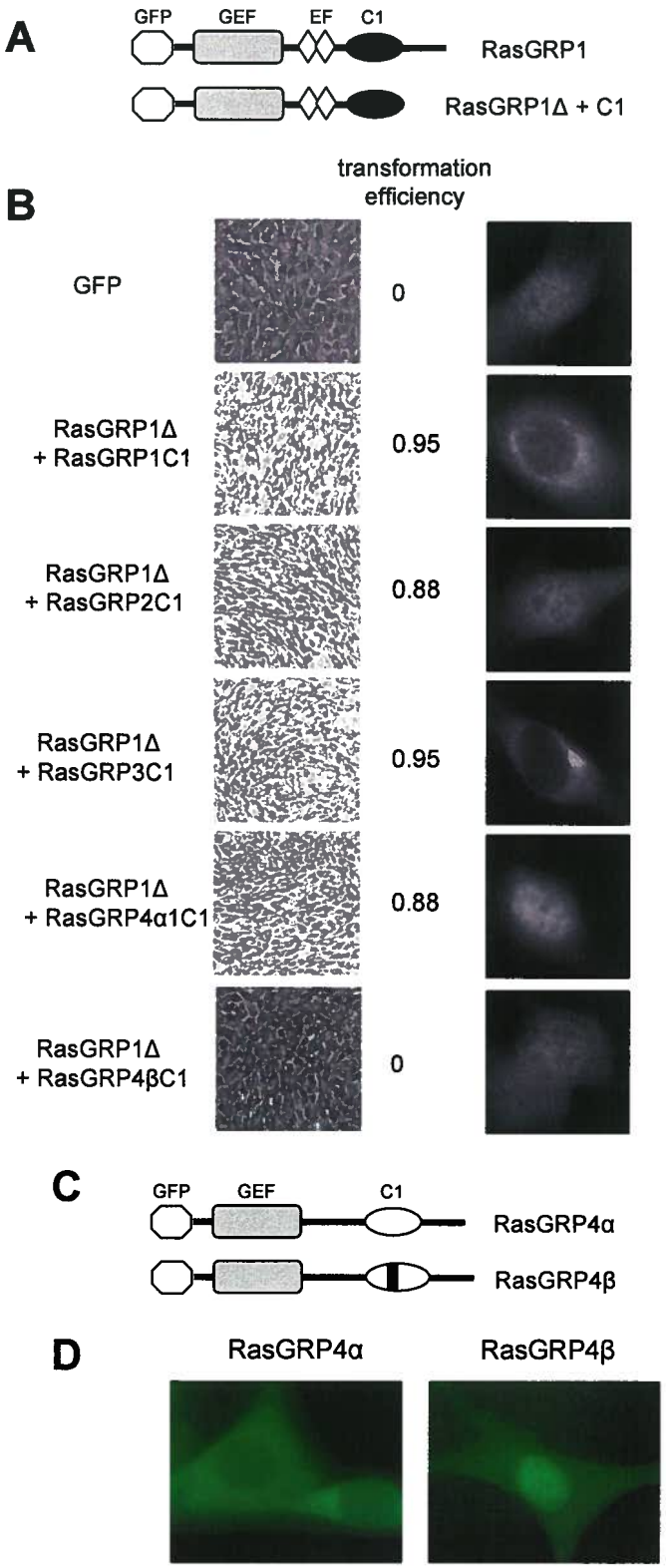

Figure 2.9 The C1 domains of RasGRP1, 2, 3 and 4α are functional within RasGRPs, while the C1 domain of RasGRP4β is not

Figure 2.9 The C1 domains of RasGRP1, 2, 3 and 4α are functional within RasGRPs, while the C1 domain of RasGRP4β is not (page 73).
A. Structure of RasGRP1 compared to the deleted form of RasGRP1 used to test functionality of attached C1 domains. The guanine nucleotide exchange domains (GEF), EF hands (EF) and C1 domains are shown, along with the N-terminal GFP tag.
B. NIH 3T3 cells expressing the indicated RasGRP1Δ + C1 fusion proteins, or expressing GFP alone as a control, were assessed for oncogenic transformation (low magnification pictures of cell cultures at left, with high refractility and loss of contact inhibition indicating transformation), and for localization of the GFP-tagged proteins (higher magnification fluorescence microscopy of typical individual cells at right). The efficiency of transformation of each construct was determined as described in Materials and Methods.
C. Structures of GFP-tagged RasGRP4α and β. The bar represents the five amino acid insertion in the C1 domain.
D. NIH 3T3 cells were transduced with retroviral vectors expressing the GFP-tagged RasGRP4 constructs and photographed by fluorescence microscopy.

domain appears to be partially destabilized by attachment of the RasGRP1Δ protein, but a weak interaction with DAG in membranes may explain the ability of this C1 domain to restore transforming activity to RasGRP1Δ. However, the experiments demonstrating a lack of DAG binding by the RasGRP2 C1 domain imply that the RasGRP1Δ + RasGRP2 C1 domain fusion protein must be activated by another mechanism, possibly reflecting the ability of the RasGRP2 C1 domain to bind weakly to membranes via anionic phospholipids.

The C1 domain of RasGRP4β was unable to activate RasGRP1Δ (Figure 2.9B), thus distinguishing it from the RasGRP2 C1 domain. It is possible that the RasGRP4β C1 domain makes a functionally significant contribution to membrane localization, but that this is effective only in cooperation with other domains of RasGRP4. To test this, we compared the localizations of RasGRP4α versus RasGRP4β in NIH 3T3 cells, as these two proteins differ only by the five amino acid insertion in the C1 domain of RasGRP4β (Figure 2.9C). RasGRP4α was nuclear excluded and partially concentrated in the perinuclear region occupied by ER (Figure 2.9D), which was similar to the distribution of the isolated C1 domain of RasGRP4α (Figure 2.2A). In contrast, RasGRP4β was distributed throughout the cells (Figure 2.9D), equivalent to the distribution of GFP alone or the isolated C1 domain of RasGRP4β (Figure 2.2A). These results indicate that the C1 domain is the primary determinant of RasGRP4α membrane localization in NIH 3T3 cells, and that the five amino acid insertion in the RasGRP4β C1 domain renders it non-functional as a membrane localizer.

2.4 DISCUSSION

The occurence of C1 domains in all RasGRP proteins, followed by the convincing demonstration that RasGRP1 is regulated by DAG binding directly to its C1 domain, has fostered the assumption that all RasGRPs are regulated in the same way as RasGRP1 (Bunney and Katan, 2006; Crittenden et al., 2004; Eto et al., 2002; Guo et al., 2001; Mitin et al., 2005). We have directly addressed the question of whether all RasGRP C1 domains are functionally equivalent or distinct by several independent experimental approaches – fluorescence microscopy and cell fractionation of the distribution of GFP tagged C1 domains, direct lipid vesicle binding assays, and PMA- and serum-dependent complementation by C1 domains of membrane binding defective K-Ras. The results were consistent in showing that the RasGRP C1 domains divide into two distinct classes. The translocation of the C1 domains of RasGRP 1, 3, and 4α to membranes can be driven by their binding to DAG or its functional analog PMA, although the RasGRP4α C1 domain has reduced affinity for DAG *in vitro* and shows less intense co-localization with membranes *in vivo*. In contrast, the C1 domains of RasGRP2 and 4β were unable to bind DAG or PMA and did not detectably co-localize with membranes *in vivo*. Previous reports of phorbol ester-induced or PLC-dependent activation of RasGRP2 or 4β, which were interpreted as evidence for direct binding of their C1 domains to phorbol ester or DAG (Clyde-Smith et al., 2000; Crittenden et al., 2004; Dupuy et al., 2001; Katagiri et al., 2004; Kawasaki et al., 1998; Yang et al., 2002), may have instead reflected the involvement of DAG or phorbol ester-dependent PKCs in the activation of these two RasGRPs.

In the RasGRP2 C1 domain, the combined effects of the alterations at positions 8, 12 and 22 may alter the structure of the ligand binding pocket sufficiently to prevent DAG or phorbol ester binding. The position 8 alteration is of particular interest, because mutation of this residue from tyrosine to serine (the residue in RasGRP2) eliminates localization of RasGRP1 to internal membranes (Caloca et al., 2003a), which could reflect loss of DAG binding. Our data demonstrate that the five amino acid insertion at the base of the B loop of the ligand binding pocket of the RasGRP4β C1 domain is sufficient to eliminate DAG as well as phorbol ester binding, and also eliminates membrane binding as detected by microscopy or complementation of the membrane binding-defective mutants of K-Ras or

RasGRP1. The alternative splicing event affecting this C1 domain apparently provides a mechanism for generating two functionally distinct forms of RasGRP4, one with and one without the capability of being activated by DAG-mediated translocation to membranes.

RasGRP 2 and 4β presumably underwent opportunistic evolution away from DAG-mediated regulation following the expansion of the RasGRP family via gene duplication and acquisition of alternative splicing. Conservation of the basic structure of these two C1 domains, and their retention of membrane binding via anionic phospholipids, suggests that they still make significant contributions to the interactions of RasGRP2 and 4β with membranes. We tested the hypothesis that these C1 domains have evolved to recognize different lipid signal transducers, but neither these nor the other C1 domains had specific binding to PA, lysoPA, ceramide, sphingosine 1-phosphate, oleic acid or arachidonic acid in the vesicle binding assay. An alternative hypothesis is that the C1 domains of RasGRP2 and 4β, as well as those of the other RasGRPs, provide a weak membrane binding site via their electrostatic interactions with anionic phospholipids. The surface of the PKCδ C1b domain contains basic residues which are positioned to interact with an anionic membrane surface (Rong et al., 2002; Zhang et al., 1995). The RasGRP C1 domains contain these and additional basic residues positioned appropriately for interaction with anionic surfaces, particularly at positions 10 and 32 (Figure 2.1). The RasGRP C1 domains are also bordered at their C-termini by a three or four residue basic patch (Figure 2.1A), which enhances binding of the RasGRP1 and 3 C1 domains to PMA/PS micelles (Irie et al., 2004). Our analyses have demonstrated that all C1 domains bind phospholipid vesicles in proportion to the anionic lipid content, and that this binding is insensitive to the specific headgroup and can occur in the absence of DAG. In the case of a DAG-binding C1 domain, the weak electrostatic interaction with negatively charged phospholipds may facilitate a 2-dimensional search for DAG on the membrane surface, as well as reinforcing the membrane binding strength of the ligated C1 domain. For the C1 domains of RasGRP2 and 4β, a DAG-independent electrostatic interaction with membranes is evidently insufficient to dictate strong membrane binding *in vivo*, since RasGRP2 and 4β did not noticeably co-localize with membranes or complement the membrane binding-defective K-Ras mutant. However, the RasGRP2 C1 domain can functionally replace the C1 domain within RasGRP1. Because the

C1 domain is essential and sufficient for RasGRP1 membrane localization leading to Ras GTP loading (Tognon et al., 1998), this implies that the RasGRP2 C1 domain can provide membrane binding to a physiologically significant extent despite its lack of detectable DAG binding and lack of membrane localization observable by microscopy. It appears that the association of RasGRP1 with membranes can be quite weak and still be sufficient for Ras activation, as detected by transformation of NIH 3T3 cells. In contrast, K-Ras may have to be more stably bound to membranes in order to transduce signals sufficient to maintain transformation of NIH 3T3 cells.

In addition to the RasGRP2 C1 domain, there are other examples of C1 domains that do not bind DAG and do not provide microscopy-detectable binding to membranes on their own, but which nonetheless are required for efficient localization to membranes. The Raf-1 C1 domain is required to stabilize membrane binding via the adjacent Ras-binding domain (Bondeva et al., 2002), while the C1 domain of KSR makes an essential contribution to constitutive localization to internal membranes and is needed for cytokine-induced translocation to the plasma membrane (Zhou et al., 2002). The inability of the RasGRP4β C1 domain to confer transforming activity on either RasGRP1 or RasGRP4 may be due to its lower binding to anionic phospholipids, relative to the C1 domain of RasGRP2, as seen in Figure 2.7. It is possible that under some circumstances (although not in NIH 3T3 cells) this C1 domain can also make physiologically significant contributions to membrane binding by RasGRP4. However, it could be that the alternative splicing event which converts RasGRP4α into RasGRP4α has the purpose of eliminating any contribution of the C1 domain to RasGRP4 activation.

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CHAPTER 3: LOCALIZATION OF RASGRP2 IS SPECIFIED BY THE C1 DOMAIN AND A POTENTIAL BINDING SITE FOR PDZ PROTEINS

A version of this chapter will be submitted for publication: Goulding RE and Kay RJ. Localization of RasGRP2 is specified by the C1 domain and a potential binding site for PDZ proteins.

3.1 INTRODUCTION

RasGRP2 is a guanine nucleotide exchange factor with specificity for the Rap1, Rap2, R-Ras and TC-21 GTPases (Dupuy et al., 2001; Eto et al., 2002; Katagiri et al., 2004; Kawasaki et al., 1998; Ohba et al., 2000b; Ohba et al., 2000a). RasGRP2 is selectively expressed in the brain (Kawasaki et al., 1998) platelets, megakaryocytes (Crittenden et al., 2004) and T-cells (Ghandour et al., 2007). Proviral insertions in the RasGRP2 locus have been shown to result in B-cell lymphoma (Mikkers et al., 2002; Suzuki et al., 2002) and myeloid leukemia (Dupuy et al., 2001), which is presumed to reflect deregulated expression of RasGRP2. The normal pattern of expression in B-cells and myeloid cells has not been investigated. Targeted disruption of RasGRP2 leads to severe defects in integrin-mediated platelet aggregation, adhesion, spreading and thrombus formation (Bernardi et al., 2006; Crittenden et al., 2004), and abolishes LFA-1 mediated adhesion of primary human T-cells to ICAM-1, in response to the chemokine SDF-1α (Ghandour et al., 2007).

In order to be active, RasGRP2 must be localized to cell membranes where Rap1 GTPases are constitutively bound. All members of the RasGRP family (RasGRP1, 2, 3 and 4) have a single C1 domain. Some C1 domains bind diacylglycerol (DAG), which is generated at membranes by a number of signal transduction mechanisms including phospholipase C (PLC)-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and inositol 1,4,5-triphosphate (Carrasco and Merida, 2006). There is evidence that membrane localization of RasGRP1, 3 and 4 is regulated by binding of their C1 domains to DAG. RasGRP1 (Bivona et al., 2003; Caloca et al., 2004; Ebinu et al., 1998; Rambaratsingh et al., 2003; Tognon et al., 1998), RasGRP3 (Lorenzo et al., 2001; Shao et al., 2001) and RasGRP4 (Katsoulotos et al., 2008) translocate to membranes in response to treatment with DAG or phorbol ester (PE), which acts as a DAG mimetic. RasGRP1 (Caloca et al., 2004; Ebinu et al., 1998; Kawasaki et al., 1998; Priatel et al., 2002; Rambaratsingh et al., 2003), RasGRP3 (Lorenzo et al., 2001) and RasGRP4 (Katsoulotos et al., 2008) have been shown to activate their target GTPases, as a consequence of DAG or PE treatment. A combination of microscopy, membrane fractionation, vesicle binding and direct binding assays has shown that the C1 domain of RasGRP1 (Ebinu et al., 1998; Irie et al., 2004;

Johnson et al., 2007; Lorenzo et al., 2000; Madani et al., 2004; Rong et al., 2002), RasGRP3 (Irie et al., 2004; Johnson et al., 2007) and RasGRP4 (Irie et al., 2004; Johnson et al., 2007; Reuther et al., 2002) all bind DAG or PE. The C1 domain of RasGRP1 is required for serum-stimulated fibroblast transformation (Tognon et al., 1998) and its translocation to endoplasmic reticulum and Golgi membranes in fibroblasts and COS cells (Caloca et al., 2003; Toki et al., 2001). Antigen receptor stimulation of lymphocytes, which leads to DAG production at membranes via PLCγ cleavage of PIP2, also leads to RasGRP1 translocation to the plasma membrane or Golgi (Beaulieu et al., 2007; Bivona et al., 2003; Caloca et al., 2004; Perez de Castro et al., 2004; Zugaza et al., 2004). B-cell receptor (BCR) mediated translocation of RasGRP1 (Beaulieu et al., 2007) or RasGRP3 (Oh-hora et al., 2003) to the plasma membrane requires the C1 domain. In addition, the C1 domain is required for T-cell receptor (TCR) (Roose et al., 2007) or BCR (Beaulieu et al., 2007) mediated activation of RasGRP1. Thus it has been established that the C1 domain is required for membrane localization of RasGRP1 and RasGRP3, and also for RasGRP1 activation.

Compared to other RasGRPs, relatively little is known about RasGRP2 localization. In BHK cells RasGRP2 was found to localize in the cytosol, with no evidence of plasma membrane localization (Clyde-Smith et al., 2000), while in COS cells RasGRP2 displays a dispersed localization in the cytoplasm with some detection in plasma membrane ruffles (Caloca et al., 2004). It is not clear what role the C1 domain plays in regulating the localization and activation of RasGRP2. Most evidence implicating the C1 domain in the regulation of RasGRP2 comes from studies using PE treatment. In response to prolonged PE exposure RasGRP2 accumulates in the subcellular fraction of COS cells which includes membranes (Clyde-Smith et al., 2000), however no PE-induced change in localization of RasGRP2 is observable in these cells using standard microscopy techniques (Caloca et al., 2004). Both RasGRP2-mediated Rap1 activation in COS or 293T cells (Clyde-Smith et al., 2000; Kawasaki et al., 1998) and RasGRP2-mediated adhesion of 32D cells to fibronectin (Dupuy et al., 2001) can be induced by PE. In addition, RasGRP2-deficient mouse platelets are unable to activate Rap1 or aggregate in response to PE (Crittenden et al., 2004). shRNA mediated silencing of RasGRP2 in primary human T-cells also leads to abrogation of PE-induced Rap1 activation and adhesion to ICAM-1 (Ghandour et al., 2007). Also, RasGRP2-

mediated TCR-induced adhesion of Jurkat T-cells to ICAM-1 via Rap1 activation is dependent on PLCs , which has been interpreted as reflecting direct binding of the C1 domain of RasGRP2 to DAG generated by PLCs (Katagiri et al., 2004). However, when interpreting these results it is important to note that RasGRP1 and RasGRP3 have been shown to be phosphorylated and activated by protein kinase Cs (PKCs) (Teixeira et al., 2003; Zheng et al., 2005), which also contain DAG-binding C1 domains and are themselves activated by DAG or PE (Colon-Gonzalez and Kazanietz, 2006). Thus, it remains a possibility that activation of RasGRP2 by DAG or PE occurs via PKCs rather than the C1 domain of RasGRP2. In support of this hypothesis, PE-induced Rap1 activation in human T-cells was shown to be inhibited by the PKC inhibitor Go6850 (Ghandour et al., 2007).

While RasGRP1, 3 and 4 C1 domains directly bind PE (Irie et al., 2004) or DAG (Johnson et al., 2007), the RasGRP2 C1 domain does not (Irie et al., 2004; Johnson et al., 2007). Sequence analysis of the RasGRP2 C1 domain shows that although the basic structure of two histidines and six cysteines that are required for Zn^{2+} binding is intact, this C1 domain diverges from the C1 domains of RasGRP1, 3 and 4 at three key residues (Irie et al., 2004; Johnson et al., 2007), which are predicted to be important for forming the DAG/PE binding pocket (Colon-Gonzalez and Kazanietz, 2006). Although unable to bind DAG with high affinity, the C1 domain of RasGRP2 can bind to membranes enriched in anionic phospholipids (Johnson et al., 2007) and thus has the potential to mediate localization to membranes by this mechanism.

In this study we examined the role of the C1 domain as a determinant of RasGRP2 localization in fibroblasts and T-cells. We found that RasGRP2 is targeted to two membrane locations: the plasma membrane and the Golgi. The C1 domain is responsible for plasma membrane localization and is required for SDF-1α induced translocation of RasGRP2 to the plasma membrane. In contrast, localization of RasGRP2 to the Golgi occurs independently of the C1 domain and instead requires a PDZ-binding motif.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and reagents

NIH 3T3 cells from American Type Culture Collection (ATCC) (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM, StemCell Technologies Vancouver, BC) containing 10% bovine calf serum (Hyclone Laboratories, Logan, UT). Jurkat cells from ATCC were cultured in RPMI 1640 (StemCell Technologies) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). DO11.10 cells (Morgan *et al.*, 1999) were obtained from Barbara Osborne (University of Massachusetts), and were cultured in DMEM containing 10% fetal bovine serum. DT40 cells were obtained from Mike Gold (University of British Columbia, Vancouver, BC), and were originally from T. Kurosaki (RIKEN Research Center, Yokohama, Japan). DT40 cells were cultured in RPMI 1640 medium (StemCell Technologies) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 2% chicken serum (Invitrogen, Carlsbad, CA), and 50 μM 2-mercaptoethanol. All Jurkat and DT40 cells used in this study were transfected with an expression plasmid expressing the ecotropic retroviral receptor, to make them permissible for infection with murine retroviral vectors. Anti-human CD3ε (OKT3) and anti-CD28 were from eBiosciences (San Diego, CA), and anti-chicken IgM polyclonal antibody was from Bethyl Laboratories (Montgomery, TX). SDF-1α was from the Biomedical Research Centre (University of British Columbia). Anti-Rap1 was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-GFP was from Roche (Laval, QC). HRP-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-GM130 was from BD Biosciences (Mississauga, ON) and Alexa fluor 647-conjugated secondary antibody (AF647) and ER Tracker was from Invitrogen.

3.2.2 Construction of modified forms of RasGRP2

The N-terminally GFP-tagged form of full length murine RasGRP2 (RG2) has the green fluorescent protein (GFP) coding sequences from pEGFP-C1 (Clontech, Mountain View, CA) fused to amino acid 1 of RasGRP2. The sequence at the GFP-RasGRP2 fusion junction in RG2 is DELYK*SGLRSLKSAPAA*MTSTLDL, with italics indicating the linker

between the C-terminus of eGFP and the N-terminus of RasGRP2. The remainder of the RasGRP2 sequence is identical to GenBank 131888394. RG2ΔC1 has a deletion of amino acids 497 to 552 in RasGRP2. The sequence at the deletion junction is GGRMG*ST*QSVSL, with italics indicating amino acids that are not naturally in RasGRP2. RG2-PBμ has a mutation which replaces the last residue at position 0 (leucine) with three residues: alanine, serine and glutamine. The sequence at the C-terminus of RG2-PBμ is VFDIH*ASG*, with italics indicating amino acids that are not naturally in RasGRP2. The GFP-tagged C1 domain of RasGRP2 (RG2C1) is described in (Johnson et al., 2007). RG2C1x2 was derived from this by joining two copies of the RasGRP2 C1 domain via a linker containing an HA epitope tag to serve as a hydrophilic spacer between the two C1 domains. The sequence at the junction of the two C1 domains is VECRRAQ*TEAYPYDVPDYASGST*FVHNF with italics indicating the linker, and underlining indicating the C-terminal zinc-binding cysteine of the first C1 domain and the N-terminal zinc-binding histidine of the second C1 domain. The RG2-C1+C-term construct is comprised of amino acids 496 to 608 (C-terminus) of RasGRP2, with an N-terminal GFP tag. The sequence around the GFP fusion is DELYK*SGLRSLKST*FVHNFQE, with italics indicating the linker between GFP and RasGRP2 sequence. The RG2-C-term construct is comprised of amino acids 553 to 608 of RasGRP2, with an N-terminal GFP tag. The sequence around the GFP fusion is DELYK*SGLRSLKST*QSVSLEG, with italics indicating the linker between GFP and RasGRP2 sequence. RG2-pr has the C1 domain and C-terminal region (amino acids 497 to 608) replaced by the basic cluster and prenylation signal of K-Ras4B. The sequence at the junction between RasGRP2 and K-Ras is GGRMG*STEAYPYDYASGS*RKHKEKMSKDGKKKKKKSKTKCVIM* with italics representing the linker and the asterisk indicating the C-terminus of KRas4B. RG2-1C1 has the C1 domain and C-terminal region (amino acids 497 to 608) of RasGRP2 replaced by the C1 domain of RasGRP1. The sequence at the junction between RasGRP2 and the RasGRP1 C1 domain is GGRMG*ST*FPHNF with italics representing the short linker sequence not naturally found in either RasGRP2 or RasGRP1. The remainder of the C1 domain of RasGRP1 is as described (Johnson et al., 2007).

3.2.3 Retroviral transduction of cell lines

Transfection of BOSC23 ecotropic packaging cells with retroviral vector plasmid DNA was performed as described (Pear et al., 1993). Virus-containing medium was supplemented with polybrene to 20 μg/ml and then added to an equal volume of Jurkat, DO11.10 or DT40 cells (2 x 10^6/ml) in appropriate medium. After 5 to 10 hours of culture, 2-3 volumes of medium were added. Transduced cells were selected by addition of puromycin (all RasGRP2 constructs) 30 to 48 hours following infection. GFP-positive cells were then sorted by flow cytometry. Adherent NIH 3T3 cells were transduced in the same way, except with complete changes of medium. Following antibiotic selection, cells expressing high levels of GFP-tagged forms of RasGRP2 were sorted by flow cytometry using the FACSVantage SE cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ); these cells represented about 40-80% of the total population prior to sorting, depending on transduction efficiency. For certain experiments, populations of RasGRP2 expressing cells were sorted to acquire different levels of expression (e.g. high, medium or low). After sorting, cells were kept on ice, spun down and resuspended in NIH 3T3, Jurkat, DO11.10 or DT40 medium with 10% serum at a density of 5 x 10^5 cells/ml or lower. Post sorting, expression levels of the different constructs were assessed by FACS analysis for all cell lines after 24-48 hours.

3.2.4 Fluorescence microscopy

NIH 3T3 cells expressing different mutant forms of RasGRP2 were grown on glass coverslips overnight and then fixed with 4% formaldehyde. Jurkat cells were plated on 0.1 mg/ml poly-$_L$-lysine-coated glass coverslips. Prior to stimulation with SDF-1α, Jurkat cells were cultured in serum-free medium for 8 hours. Jurkat cells were then stimulated in Hank's buffer (StemCell Technologies) with 10 μg/ml anti-CD3 and anti-CD28, 5 μg/ml anti-IgM or 10 ng/ml SDF-1α, and fixed with 4% formaldehyde in PBS. To mark Golgi membranes, fixed NIH 3T3 and Jurkat cells were stained with mouse anti-GM130 followed by staining with AF647-conjugated secondary antibody. ER was marked by treating unfixed NIH 3T3 cells with glibenclamide BODIPY-Texas Red (ER Tracker; Invitrogen), followed by fixation with formaldehyde in PBS for 2 minutes at 37° C. Fluorescence microscopy images were photographed with an Axioplan 2 imaging microscope (Carl Zeiss, Toronto, ON), using a

450-485 nm excitation filter and a 500-545 nm emission filter for analysing GFP-tagged constructs, and a 530-585 nm excitation filter and 615 nm (long-pass) emission filter for analysis of secondary antibody AF647 staining or ER Tracker. Images were captured using OpenLab (Improvision, Coventry, England) imaging software. The individual cells displayed in the figures were chosen to be representative of the majority of the population of cells. Changes in the original images in the following figures were made: in Figure 3.1C all images were reduced in brightness, in Figure 3.1E the image for RG2C1x2 was brightened to match background levels of the other two images, in Figure 3.5A RG2ΔC1 and RG2-PBμ panels were increased in brightness and contrast to match images from GFP and RG2 panels, in Figure 3.7A, B, C and D all images from each experiment were equally increased in brightness and contrast, and in Figure 3.10 contrast was increased equally in all images. For two colour fluorescent images that were subsequently merged, contrast and brightness were adjusted to a common background and saturation level in images with the same fluorescent marker [e.g. GFP, GM130 (AF647), ER Tracker]. Images were merged using OpenLab imaging software.

3.2.5 Cell colony boundary and scratch test assays

NIH 3T3 cells were seeded at a low density and allowed to grow into colonies of between 3 and 5 mm in diameter before being counted and scored as either normal or as having a tight boundary phenotype by microscopy. A minimum of 50 colonies were counted for each mutant. Images were acquired using OpenLab imaging software with a light microscope. For scratch test assays, NIH 3T3 cells were seeded at 1 x 10^6/ml in 10 cm dishes. After 24 hrs scratches were made with a cell scraper, scratch areas were marked and microscopy images were photographed with a Nikon Elipse TS100 microscope (Nikon, Mississauga, ON) and acquired using OpenLab imaging software. After 16 hours, images of the same areas were captured.

3.2.6 Cell stimulation, lysis and affinity purification of activated Rap1 GTPase by RalGDS-RBD chromatography

1 x 10^7 NIH 3T3 cells were lysed with 1 ml of ice cold lysis buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM $NaMoO_4$) containing 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mM PMSF and 1 mM activated Na_3VO_4. 1.5 x 10^7 Jurkat cells or 2.5 x 10^7 DT40 cells were incubated in activation buffer (25 mM HEPES, pH 7.2, 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1% glucose, 0.1% bovine serum albumin (BSA), and 50 μM 2- mercaptoethanol) (Saxton *et al.*, 1994) for 10 min at 37°C before stimulation. In the case of Jurkat cells anti-CD3/CD28 or SDF-1α was then added to a concentration of 10 μg/ml or 10 ng/ml respectively, for the indicated times. For DT40 cell stimulation anti-chicken IgM was then added to a concentration of 5 μg/ml to DT40 cells, for the indicated times. Cells were immediately lysed with 2.5 volumes of ice-cold lysis buffer and lysed as for NIH 3T3 cells. Lysates were incubated for 30 min with GST-Rap binding domain (GST-RapBD) fusion protein which had been prepared and pre-bound to glutathione-agarose beads as described (Taylor et al., 2001). The GST-RapBD was expressed from pGEX4T3-RalGDS-RBD as described in (Spaargaren and Bischoff, 1994). After washing, samples were eluted in Bio-Rad XT sample buffer and electrophoresed, levels of Rap1 were detected by western blot as described below.

3.2.7 Western blot analysis

Equal volumes of lysates were denatured using Bio-Rad XT sample buffer, separated by gel electrophoresis on 12% XT-Criterion acrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) by electroblotting. After blocking the membranes for one hour at room temperature in TBST (25 mM Tris-HCl pH 7.4, 3 mM KCl, 150 mM NaCl, 0.05% Tween-20) containing 5% bovine serum albumin (BSA), primary antibodies were applied to the membrane for 90 min at room temperature in 2% BSA TBST, followed by 3 x 10 min washes with TBST. Horseradish peroxidase-conjugated secondary antibodies were then applied for 45 min at

room temperature in TBST containing 1% BSA, followed by 3 x 10 min washes with TBST. Membranes were exposed to substrate/ECL (Santa Cruz Biotechnology) and chemiluminescence was detected using the VersaDoc 5000 imaging system (Bio-Rad Laboratories). Levels of Rap1-GTP and total Rap1 were quantified by band volume analysis using Quantity One software (Bio-Rad Laboratories).

3.3 RESULTS

3.3.1 The C1 domain of RasGRP2 specifies plasma membrane localization

Our initial goal was to identify the domains of RasGRP2 that regulate membrane localization. Given the importance of the C1 domain in regulating the localization of other RasGRPs, we hypothesized that the C1 domain of RasGRP2 would be involved in specifying its intracellular localization. In subconfluent cultures, GFP-tagged RasGRP2 (Figure 3.1A) was mostly dispersed throughout the cytoplasm, but a minor portion was concentrated at the Golgi membrane, as detected by its co-localization with the Golgi-associated protein GM130 (Figure 3.1B). When the NIH 3T3 cells reached confluence, RasGRP2 was also detected at the plasma membrane at sites of cell-cell contact (Figure 3.1C). To test whether the C1 domain was determining localization of RasGRP2 in NIH 3T3 fibroblasts, we expressed a form of RasGRP2 that had a deletion of the C1 domain (RG2ΔC1) (Figure 3.1A). GFP-tagged RG2ΔC1 localized in the cytoplasm and at the Golgi. However, plasma membrane localization in confluent cultures was not detectable (Figure 3.1C). Therefore, the RasGRP2 C1 domain specifies plasma membrane targeting of RasGRP2 but is not needed for Golgi targeting.

We expressed a GFP fusion construct of the isolated C1 domain of RasGRP2 (RG2C1, Figure 3.1D) to determine if the C1 domain was sufficient as well as necessary for plasma membrane targeting. Unlike full length RasGRP2, the isolated C1 domain did not detectably localize to cell-cell contacts in confluent NIH 3T3 cells (Figure 1E), indicating that other parts of RasGRP2 must act in conjunction to achieve stable localization at the plasma membrane. This may be due to the C1 domain having only a weak interaction with the plasma membrane, or the C1 being required for the function of another domain of RasGRP2 which serves as the sole site of contact with the plasma membrane.

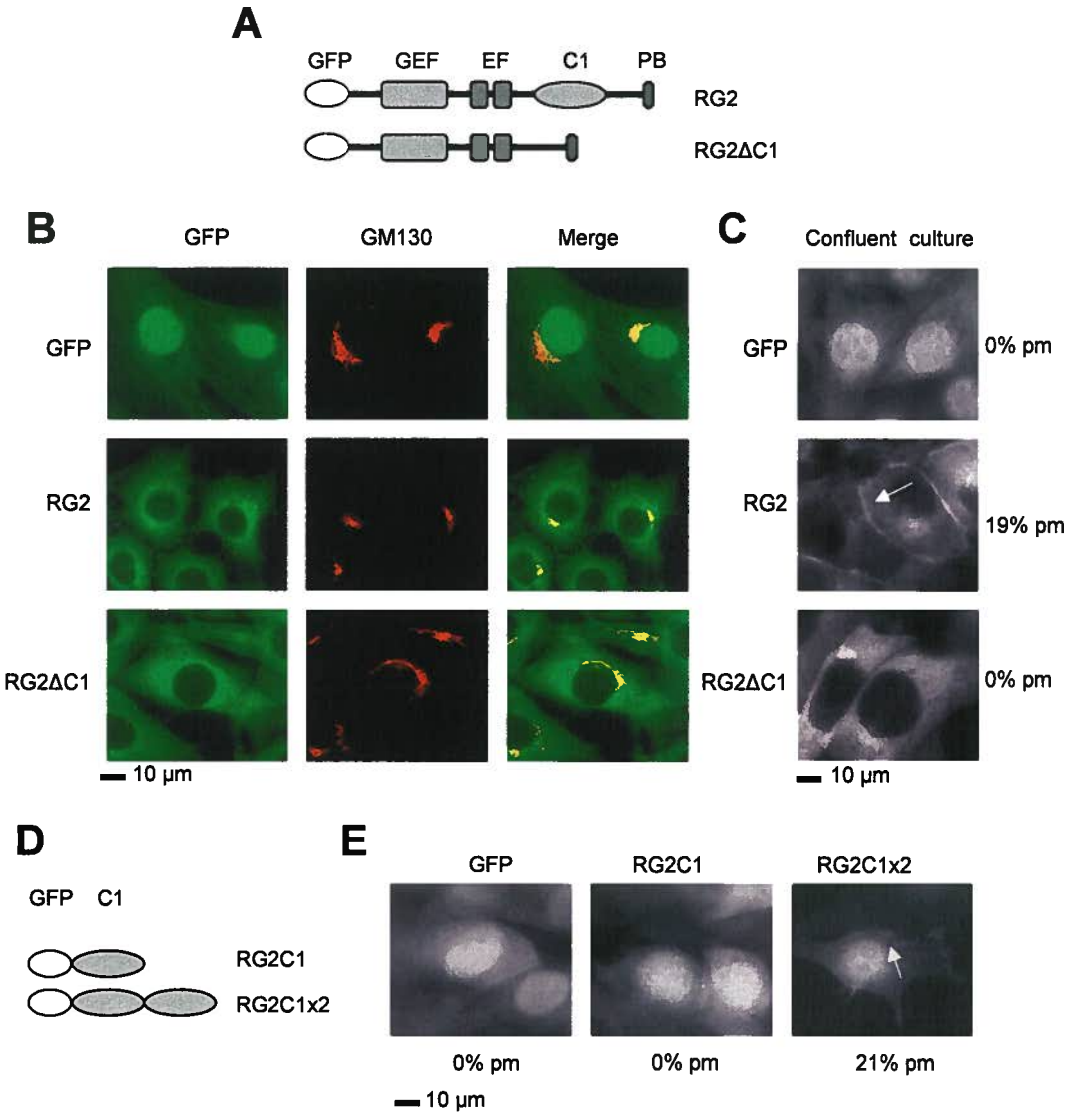

Figure 3.1 RasGRP2 plasma membrane localization is specified by the C1 domain

Figure 3.1 RasGRP2 plasma membrane localization is specified by the C1 domain
A. Domain structures of GFP-tagged RasGRP2 proteins used in this experiment. GEF, guanine nucleotide exchange domain that catalyzes GTP loading of Rap1 GTPase. EF, EF-hands. PB, PDZ-binding motif is the Golgi targeting regulatory domain identified in this study.
B. Subconfluent NIH 3T3 cells, expressing GFP alone or the GFP-tagged RG2 or RG2ΔC1 constructs, were fixed, stained with anti-GM130 (secondary antibody conjugated to AF647) to mark Golgi membranes and photographed by fluorescence microscopy as described in Materials and Methods. Representative cells are shown.
C. Confluent cultures of NIH 3T3 cells expressing GFP alone, or the GFP-tagged RG2 or RG2ΔC1. Cells were fixed and photographed by fluorescence microscopy. Arrow indicates GFP-tagged RG2 localization at the plasma membrane at sites of cell-cell contact. The results shown are representative of 3 independent experiments. Percentages to the right of images are numbers of cells that show plasma membrane localization in one experiment (a total of at least 100 cells were counted), which also correlates with expression level, in that bright cells typically display plasma membrane localization whereas dim cells typically do not.
D. Domain structures of GFP-tagged RG2C1 domain and RG2C1x2 constructs used in this experiment.
E. Localization of the GFP-tagged isolated C1 (RG2C1) and tandem C1 domain (RG2C1x2) of RasGRP2 in NIH 3T3 cells. Subconfluent cells were fixed and photographed by fluorescence microscopy. Arrow indicates GFP-tagged RG2C1x2 localization at the plasma membrane. Representative cells are shown.

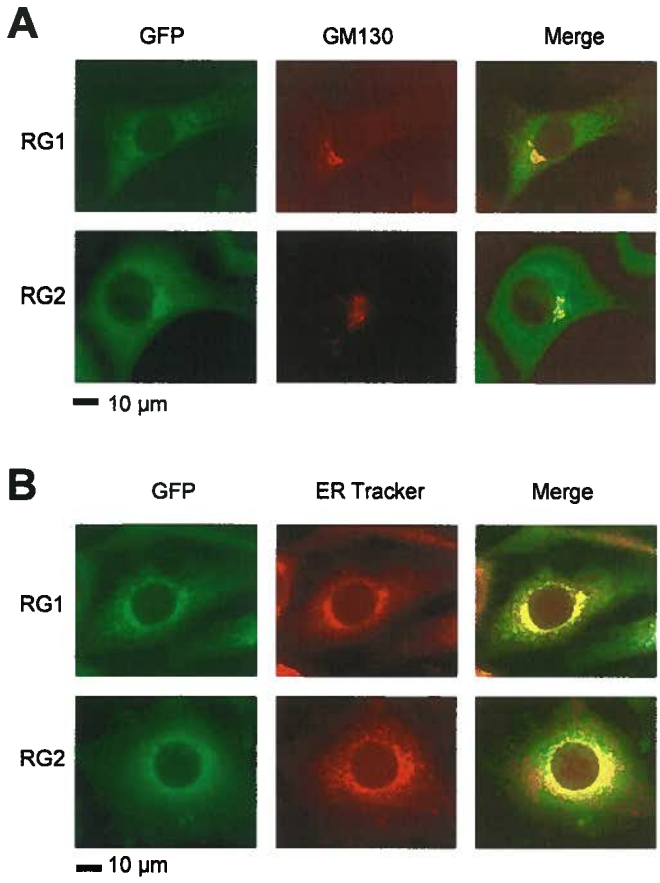

Figure 3.2 RasGRP1 localization at the Golgi and ER compared to RasGRP2 in NIH 3T3 cells

Subconfluent NIH 3T3 cells, expressing GFP-tagged RasGRP1 or GFP-tagged RasGRP2, were fixed, stained with anti-GM130 (secondary antibody conjugated to AF647) to mark Golgi membranes (A) or ER Tracker to mark endoplasmic reticulum (B), and photographed by fluorescence microscopy as described in Materials and Methods. Representative cells are shown.

To determine if the C1 domain of RasGRP2 is capable of directly but weakly mediating plasma membrane localization, we generated two tandem C1 domains (RG2C1x2, Figure 3.1D) to increase its potential avidity for membranes. The tandem pair of RasGRP2 C1 domains was detectable at the plasma membrane at areas of cell-cell contact (Figure 3.1E) demonstrating that the C1 domain of RasGRP2 can directly and independently mediates plasma membrane localization, although in a monomeric form it does this with insufficient affinity to confer stable membrane binding. In combination, these results indicate that the C1 domain of RasGRP2 specifies its localization to the plasma membrane, but must cooperate with other domains in RasGRP2 to attain detectable binding at this membrane compartment.

RasGRP1 also localizes at the Golgi (Figure 3.2A), however RasGRP1 localization at this site is totally dependent on the C1 domain (Beaulieu et al., 2007; Tognon et al., 1998). RasGRP1 localization is also different to that of RasGRP2 in that RasGRP1 localizes more distinctly at the endoplasmic reticulum (ER) (Figure 3.2B), a pattern of localization which also requires the C1 domain (Beaulieu et al., 2007; Tognon et al., 1998). In contrast RasGRP2 appears to be more dispersed through the cytoplasm rather than ER-localized in comparison to RasGRP1 (Figure 3.2B). These results show that these two RasGRPs have similar Golgi and divergent ER patterns of endomembrane localization, but from previous studies we know that RasGRP1 has a different mode of localization compared to RasGRP2; the C1 domain of RasGRP1 targets the Golgi/ER while the our results show that the C1 domain of RasGRP2 targets the plasma membrane.

3.3.2 A potential binding site for PDZ proteins specifies Golgi localization of RasGRP2

The C-terminus of RasGRP2 has the sequence features of a class II PDZ-binding motif. To bind cognate PDZ proteins, a class II PDZ-binding motif needs to be located at the C-terminus of a protein and must have hydrophobic residues at the C-terminal and -2 positions (Nourry et al., 2003), as is the case for the PDZ-binding motif of RasGRP2 (Figure 3.3A). By binding to PDZ proteins at the plasma membrane, the PDZ-binding motif of

RasGRP2 could cooperate with the C1 domain to confer stable plasma membrane localization. To test this hypothesis, we made one mutant in which we mutated the PDZ-binding motif by attaching an additional two amino acids, thus moving the motif away from its required position at the C-terminus and we changed the hydrophobic leucine formerly at position 0 to an alanine. This mutant (RG2-PBμ, Figure 3.3B) had notably reduced Golgi localization (Figure 3.3C), however still localized to the plasma membrane in confluent NIH 3T3 cells (Figure 3.3D). The RG2-PBμ mutant was found in the nucleus as well as the cytoplasm, which could reflect dispersal of RasGRP2 once Golgi tethering via the PDZ-binding motif is lost. Therefore the PDZ-binding motif at the C-terminus of RasGRP2 is not what cooperates with the C1 domain to confer plasma membrane localization, but instead has the distinct task of localizing a portion of RasGRP2 to the Golgi.

To confirm that the C-terminal region of RasGRP2 specified localization at the Golgi, we expressed a form of RasGRP2 that included only the part of RasGRP2 C-terminal to the C1 domain. This form of RasGRP2 (RG2-C-term) localized to the Golgi (Figure 3.3C), but did not localize to the plasma membrane (Figure 3.3D). A form of RasGRP2 that contained both this C-terminal region and the C1 domain (RG2-C1+C-term), localized both at the Golgi (Figure 3.3C) and at the plasma membrane (Figure 3.3D), indicating that the C1 domain and the C-terminus were solely responsible for the Golgi and plasma membrane pattern of RasGRP2 localization. Since the C1 domain of RasGRP2 in isolation is unable to localize to the plasma membrane, unless it is expressed as a tandem pair of C1 domains (Figure 3.1D), we hypothesize that a region in between the C1 domain and the PDZ-binding motif may be responsible for stabilizing RasGRP2 C1-mediated plasma membrane localization. Also because the C-terminal region on its own is incapable of localizing at the plasma membrane, this indicates that the C-terminal region is interdependent with the C1 domain in terms of providing plasma membrane localization.

3.3.3 Both the C1 domain and the PDZ-binding motif contribute to RasGRP2 activity as an exchange factor for Rap1

Expression of RasGRP2 in NIH 3T3 fibroblasts led to a 2 to 4 fold increase in levels of GTP bound Rap1 even when RasGRP2 expression levels were low (Figure 3.4A). This demonstrates that RasGRP2 is constitutively active in these cells as a Rap-specific exchange factor. To determine whether membrane localization was a requirement for RasGRP2 activity we analysed Rap1-GTP levels in NIH 3T3 cells expressing mutated forms of RasGRP2, deficient in either plasma membrane or Golgi targeting. Cells expressing RG2ΔC1, which has no plasma membrane localization, had reduced Rap1-GTP levels compared to cells expressing RasGRP2, but levels were still significantly greater than those in control cells (Figure 3.4A, 3.4B). Mutation of the PDZ-binding motif, which abrogates Golgi, but not plasma membrane, localization, also reduced but did not eliminate RasGRP2-mediated GTP loading of Rap1 (Figure 3.4A, 3.4B). Therefore while the C1 and the PDZ-binding motif specify RasGRP2 localization to distinctly different sites in NIH 3T3 fibroblasts, they both contribute to RasGRP2 activity within these cells.

3.3.4 Changes in cell morphology and colony edge phenotype induced by RasGRP2 require plasma membrane localization via the C1 domain

Expression of RasGRP2 in NIH 3T3 fibroblasts caused a distinct change in cell shape (Figure 3.5A). When approaching confluence, NIH 3T3 fibroblasts have a disordered morphology, with cell projections growing on top of other cells, and with irregular spaces in between cells. In comparison to control cells, confluent RasGRP2 expressing cells were more regular in shape and grew as tessellated (cobblestone-like) monolayers with a reduction in the numbers of spaces in between cells and without overgrowth of neighbouring cell projections. In addition, colonies of RasGRP2 expressing cells had very tight discrete boundaries, lacking dispersed single cells at the edge as seen in colonies of control cells (Figure 3.5A, 3.5C). This could be due to reduced cell migration, a phenotype that is induced in MDCK epithelial cells by expression of the Rap-specific GEF Epac1 (Lyle et al., 2008). Using a scratch test assay we found that RasGRP2-expressing NIH 3T3s cells could move into the cleared area, indicating that they did not have a complete migration defect

(Figure 3.5B). However, the front of migrating cells was very tight, lacking single cells migrating beyond the advancing boundary of cells (Figure 3.5B).

Deletion of the C1 domain eliminated the ability of RasGRP2 to induce changes in cell and colony morphology, such that cells expressing RG2ΔC1 had the disordered monolayers and dispersed colony boundaries typical of control NIH 3T3 cells (Figure 3.5A, 3.5C). In contrast, mutation of the PDZ-binding motif had no effect on the ability of RasGRP2 to induce morphological changes in NIH 3T3 cells (Figure 3.5A, 3.5C). This indicated that the morphological changes are induced by RasGRP2 when it is localized to the plasma membrane via the C1 domain, but not when it is localized to Golgi by the PDZ-binding motif. To further test how localization determines cellular responses to RasGRP2, we constructed variants of RasGRP2 with mechanistically distinct modes of targeting to either the plasma membrane or Golgi membranes. RasGRP2 was targeted to the plasma membrane by replacing its C1 domain and C-terminus with the basic cluster and prenylation signal of K-Ras4B (RG2-pr, Figure 3.6A). For Golgi membrane targeting, we replaced the C1 and C-terminus of RasGRP2 with the C1 domain of RasGRP1 (RG2-1C1, Figure 3.6A). In contrast to the selectivity of the RasGRP2 C1 domain for the plasma membrane, the C1 domain of RasGRP1 is selectively localized at the Golgi of NIH 3T3 cells (Johnson et al., 2007) apparently due to its recognition of high concentrations of DAG at this site. As intended, RG2-pr localized predominantly to the plasma membrane while RG2-1C1 localized predominantly to the Golgi (Figure 3.6B). Expression of either RG2-pr or RG2-1C1 increased GTP loading of Rap1 relative to control cells (Figure 3.6C, 3.6D). However, only the cells expressing the plasma membrane-localizing RG2-pr construct showed the tesselated monolayers and tight colony boundaries that typify RasGRP2-expressing NIH 3T3 cells (Figure 3.6E, 3.6F). Therefore, it is the plasma membrane-localized form of RasGRP2 that is capable of modifying the morphology of NIH 3T3 cells.

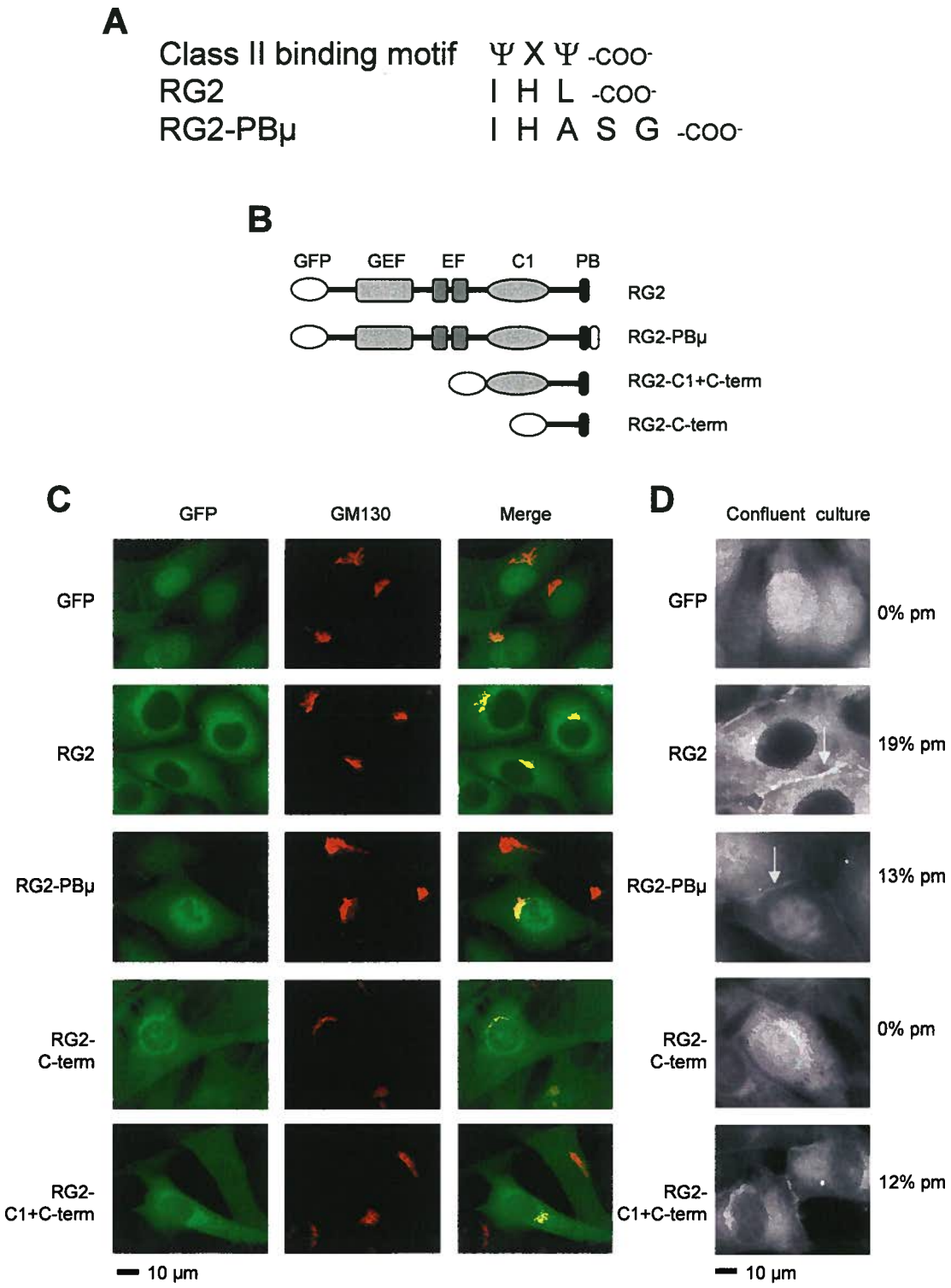

Figure 3.3 RasGRP2 localization at the Golgi requires a PDZ-binding motif

Figure 3.3 RasGRP2 localization at the Golgi requires a PDZ-binding motif
A. RasGRP2 C-terminus has sequence features of a class II PDZ-binding motif with hydrophobic (Ψ) amino acids at position 0 (leucine) and at position -2 (isoleucine). Disruption of this motif was facilitated by attaching two amino acids, thus moving the motif away from the C-terminus, and by changing the leucine formerly at position 0, to an alanine.
B. Domain structures of GFP-tagged RasGRP2 proteins used in this experiment.
C. Subconfluent NIH 3T3 cells, expressing GFP alone, or the GFP-tagged RG2, RG2-PBμ, RG2-C-term or RG2-C1+C-term constructs, were fixed, stained with anti-GM130 to mark Golgi membranes and photographed by fluorescence microscopy as described in Materials and Methods. Representative cells are shown.
D. Confluent cultures of NIH 3T3 cells expressing the GFP alone, or the GFP-tagged RG2, RG2-PBμ, RG2-C-term or RG2-C1+C-term constructs. Cells were fixed and photographed by fluorescence microscopy. Arrow indicates GFP-tagged RG2 and RG2-PBμ localization at the plasma membrane. The results shown are representative of 3 independent experiments. Percentages to the right of images are numbers of cells that show plasma membrane localization in one experiment (a total of at least 100 cells were counted). Percentages may be an underestimate because bright cells typically display plasma membrane localization whereas dim cells typically do not.

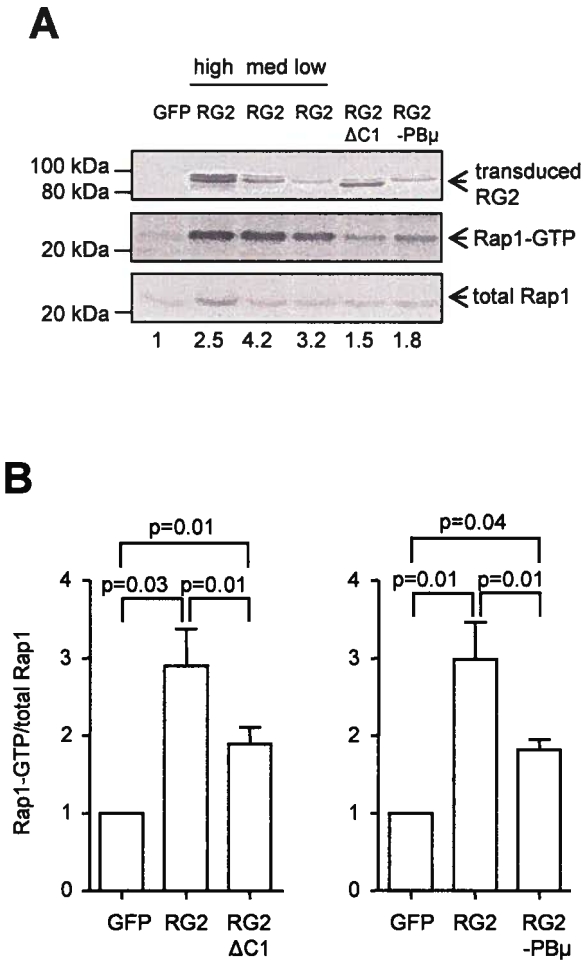

Figure 3.4 The C1 domain and the potential PDZ-binding motif are required for full activation of RasGRP2

A. NIH 3T3 cells, expressing GFP alone or GFP-tagged RG2, RG2ΔC1 or RG2-PBμ constructs, were lysed and assayed for levels of GTP bound Rap1 by Raf-RBD chromatography and detected by Western blot with anti-Rap1. Levels of Rap1-GTP and total Rap1 were quantified by band volume analysis using Quantity One software (Bio-Rad Laboratories). The numbers below the Rap1 Total blot are the ratios of Rap1-GTP over total Rap1 in RG2, RG2ΔC1 and RG2-PBμ expressing cells, relative to Rap1-GTP/total Rap1 in cells expressing GFP alone. The expression levels of transduced GFP-tagged RG2 protein in each sample determined by Western blot with an anti-GFP antibody, are shown in the upper blot.

B. Rap1 activation by RG2, RG2ΔC1 and RG2-PBμ in serum grown cells, relative to cells expressing GFP alone. Rap1-GTP/total Rap1 levels were measured in NIH 3T3 cells expressing RG2, RG2ΔC1 and RG2-PBμ and are displayed relative to GFP alone. Cells expressing GFP tagged-mutant RasGRP2 were compared with cells expressing a similar level of GFP-tagged wild type RasGRP2. Bars show SEM. The two-tailed p values are for comparison to a hypothetical mean of 1, or in the case of RG2 compared to mutant, the hypothetical mean was that of RG2. Results are from seven separate experiments with RG2ΔC1 and five separate experiments with RG2-PBμ.

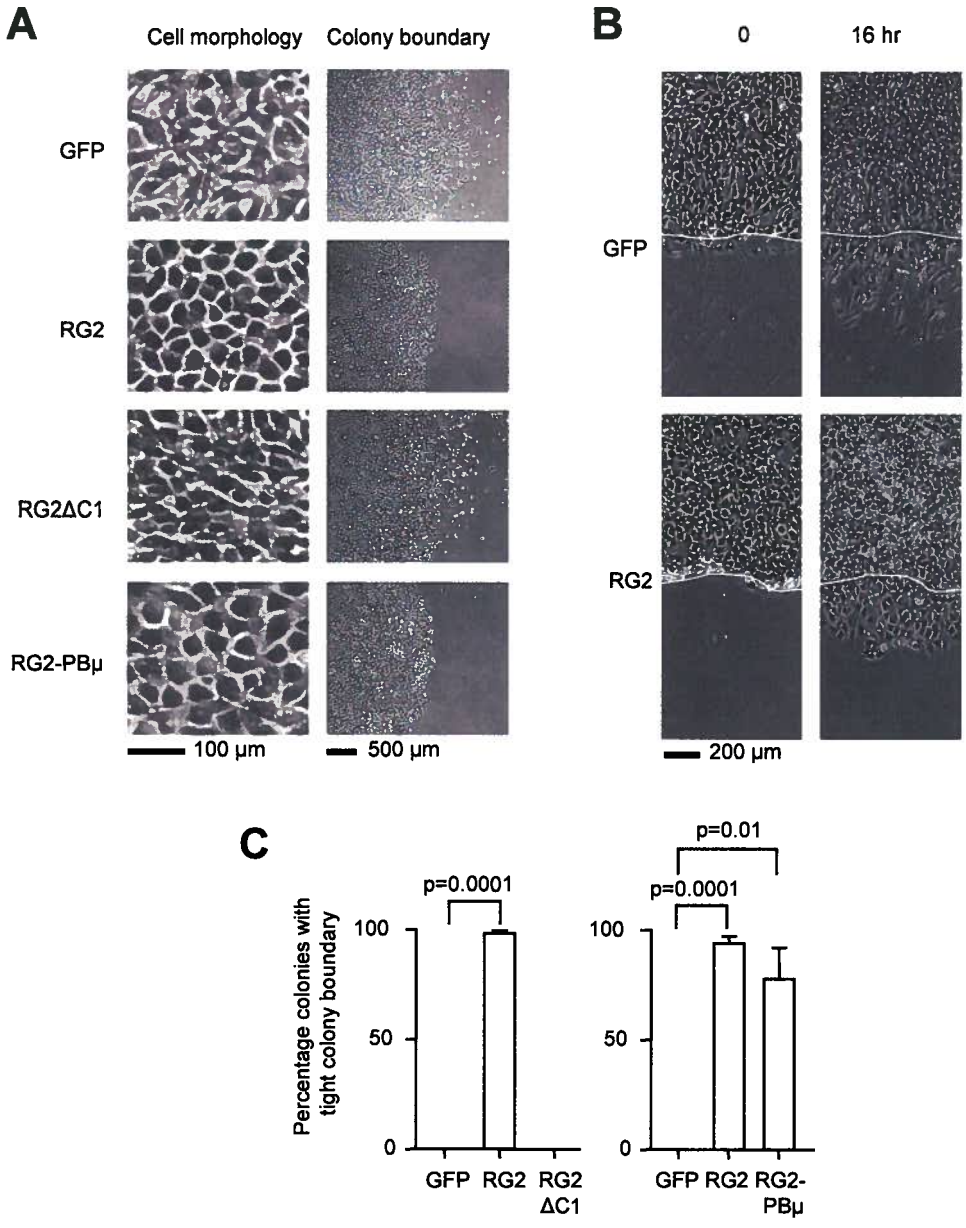

Figure 3.5 The C1 domain is required for RasGRP2 induced changes in morphology

Figure 3.5 The C1 domain is required for RasGRP2 induced changes in morphology (page 99)

A. NIH 3T3 cells expressing GFP alone, GFP-tagged RG2, RG2ΔC1 or RG2-PBμ were seeded at low density, grown into colonies and photographed as described in Materials and Methods to show cell morphology (left panels) and colony boundary (middle panels). GFP alone, GFP-tagged RG2, RG2ΔC1 or RG2-PBμ expressing cells were seeded on glass coverslips, and when confluent were fixed, stained with Phalloidin TRITC and photographed (right panels) as described in Materials and Methods.

B. NIH 3T3 cells expressing either GFP (left panels) or GFP-tagged RG2, (right panels) showing cell migration, were seeded and photographed 16 hr post scratch test as described in Materials and Methods.

C. Percentage of colonies with RG2-induced colony boundary morphology phenotype. A total of at least 70 colonies from six separate (GFP, RG2, RG2ΔC1) or four separate (GFP, RG2, RG2-PBμ) experiments were counted. Bars show SEM. The two-tailed p values are for comparison to hypothetical mean of 0.

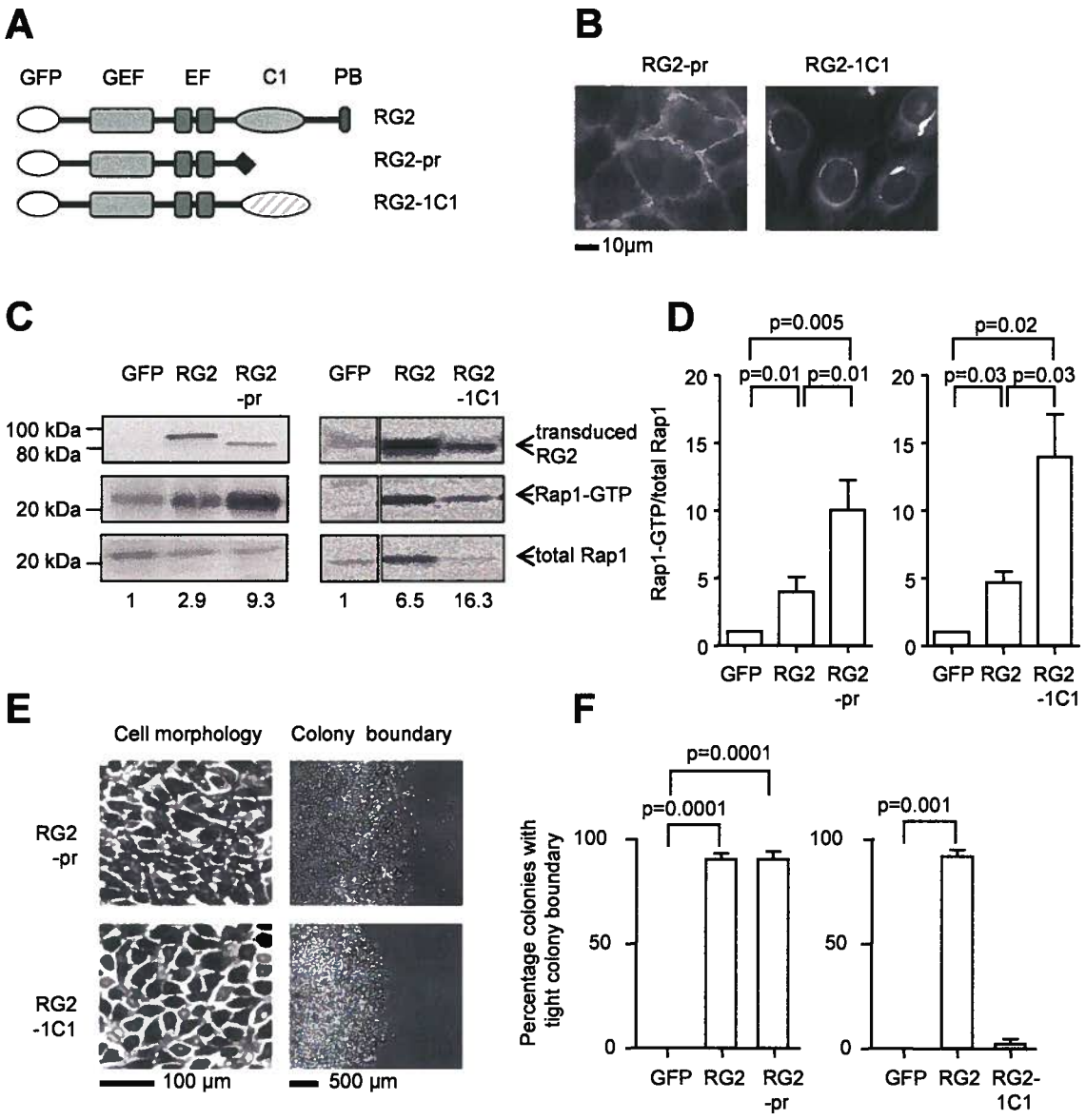

Figure 3.6 Targeting RasGRP2 to the plasma membrane induces colony morphology

Figure 3.6 Targeting RasGRP2 to the plasma membrane induces colony morphology phenotype (page 101)

A. Domain structures of GFP-tagged RasGRP2 proteins used in this experiment. To a truncated form of RasGRP2 the plasma membrane targeting K-Ras basic cluster and C-terminal prenylation signal (RG2-pr), or the C1 domain of RasGRP1 (RG2-1C1) were attached.

B. NIH 3T3 cells expressing RG2-pr or RG2-1C1 were grown on cover slips, fixed and photographed as described in Materials and Methods.

C. NIH 3T3 cells expressing GFP alone or GFP-tagged RG2, RG2-pr or RG2-1C1 were lysed and assayed for levels of GTP-bound Rap1 by Raf-RBD chromatography and detected by Western blot with anti-Rap1. Levels of Rap1-GTP and total Rap1 were quantified by band volume analysis using Quantity One software (Bio-Rad). The numbers below the total Rap1 blot are the ratios of Rap1-GTP over total Rap1 in RG2, RG2-pr and RG2-1C1 expressing cells, relative to Rap1-GTP/total Rap1 in cells expressing GFP alone. The expression levels of transduced RasGRP2 protein in each sample, determined by Western blot with an anti-GFP antibody, are shown in the upper blot.

D. Rap1 activation by RG2, RG2-pr and RG2-1C1 in serum grown cells, relative to cells expressing GFP alone. The numbers below the total Rap1 blot are the ratios of Rap1-GTP over total Rap1 in RG2, RG2-pr and RG2-1C1 expressing cells, relative to Rap1-GTP/total Rap1 in cells expressing GFP alone. The expression levels of transduced GFP-tagged RG2 protein in each sample determined by Western blot with an anti-GFP antibody, are shown in the Figure 3.5C. Cells expressing GFP tagged-mutant RasGRP2 were compared with cells expressing a similar level of GFP- tagged wild type RasGRP2. Rap1-GTP/total Rap1 levels were measured in NIH 3T3 cells expressing RG2, RG2-pr and RG2-1C1 and are displayed relative to GFP alone. Bars show SEM. The two-tailed p values are for comparison to hypothetical mean of 1, or in the case of RG2 compared to mutant, the hypothetical mean was that of RG2. Results are from nine separate experiments with RG2-pr and four separate with RG2-1C1.

E. NIH 3T3 cells expressing RG2-pr (upper panels) or RG2-1C1 (lower panels) grown to confluence and photographed by fluorescence microscopy (left panels) or seeded at low density, grown into colonies and photographed by light microsope (right panels) as described in Materials and Methods.

F. Percentage of colonies with RG2 induced colony boundary morphology phenotype. A total of at least 70 colonies from four separate (GFP, RG2, RG2-pr) or three separate (GFP, RG2, RG2-1C1) experiments were counted. Bars show SEM. The two-tailed p values are for comparison to hypothetical mean of 0.

3.3.5 The C1 domain and PDZ-binding motif specify plasma membrane versus Golgi localization of RasGRP2 in Jurkat T-cells

The experiments with NIH 3T3 cells showed that RasGRP2 localization at the plasma membrane is determined by the C1 domain, while Golgi localization is regulated by the PDZ-binding motif. RasGRP2 is expressed in primary human T-cells (Pasvolsky et al, 2007; Ghandour et al, 2007), where it is thought to play a role in integrin-mediated T-cell adhesion (Pasvolsky et al, 2007; Ghandour et al, 2007). RasGRP2 is also expressed in the Jurkat T-cell line, which has has been used as an experimental model for studying the role of RasGRP2 in Rap1 activation (Katagiri et al., 2004). Jurkat T-cells were used to address the question of whether the C1 and PDZ-binding motif would determine RasGRP2 localization in a cell line which normally expresses RasGRP2. In Jurkat cells, we found that GFP-tagged RasGRP2 was localized predominantly in the cytoplasm, with some localization at the plasma membrane, which was most readily detected at regions of cell-cell contact (Figure 3.7A), and was also found to be localized at the Golgi (Figure 3.8). RG2ΔC1 could not be detected at the plasma membrane, not even at areas of cell-cell contact, while localization in the cytoplasm (Figure 3.7A) and at the Golgi was unaffected by deletion of the C1 domain (Figure 3.8). The RG2-PBμ, mutant was detected at the plasma membrane at regions of cell-cell contact (Figure 3.7A), but Golgi localization was reduced to that seen for GFP alone (Figure 3.8). RG2-C1+C-term localized at both the plasma membrane, at sites of cell-cell contact, in the cytoplasm and at the Golgi, whereas RG2-C-term did not localize at the plasma membrane, but was in the cytoplasm and at the Golgi (Figure 3.7B, 3.8). These results indicate that in Jurkat cells, as in NIH 3T3 fibroblasts, the C1 domain of RasGRP2 specifies plasma membrane localization whereas the PDZ-binding motif specifies Golgi localization.

We also looked at the localization pattern of GFP-tagged RasGRP2 localization in the DO11.10 murine T-cell line and found that GFP-tagged RG2 and RG2ΔC1, RG-PBμ, RG2-C1+C-term and RG2-C-term localization was identical to that observed in Jurkat cells (Figure 3.7C, D).

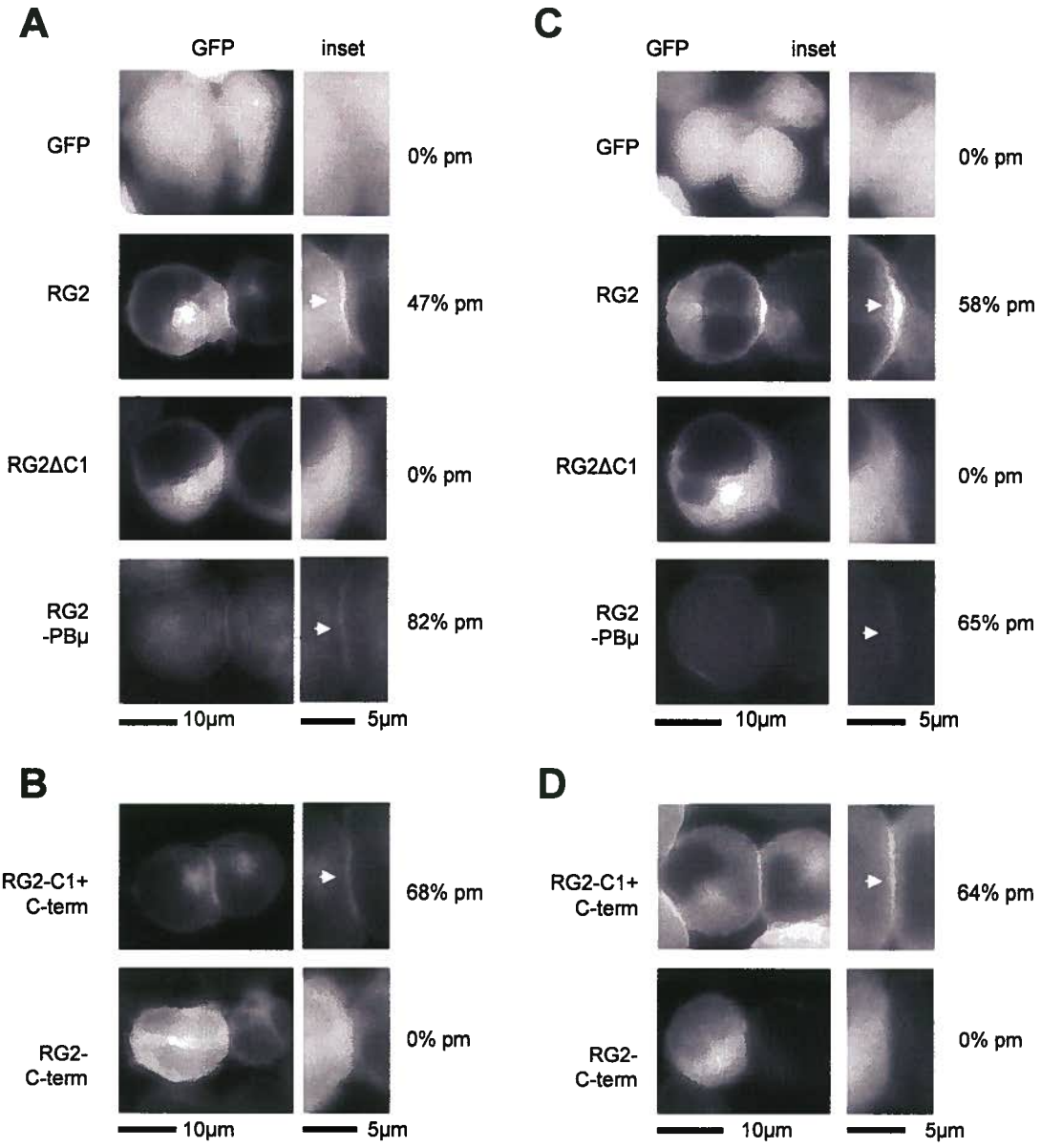

Figure 3.7 The C1 domain specifies plasma membrane localization of RasGRP2 in Jurkat and DO11.10 T-cells

Jurkat (A, B) and DO11.10 (C, D) T-cells expressing GFP alone or the GFP-tagged RG2, RG2ΔC1,RG2-PBμ, RG2-C1+C-term and RG2-C-term constructs were fixed, and photographed as described in Materials and Methods. Representative cells are shown. Percentages to the right of images are numbers of cells that show plasma membrane localization in one experiment (a total of at least 50 cells that demonstrated cell-cell contact were counted).

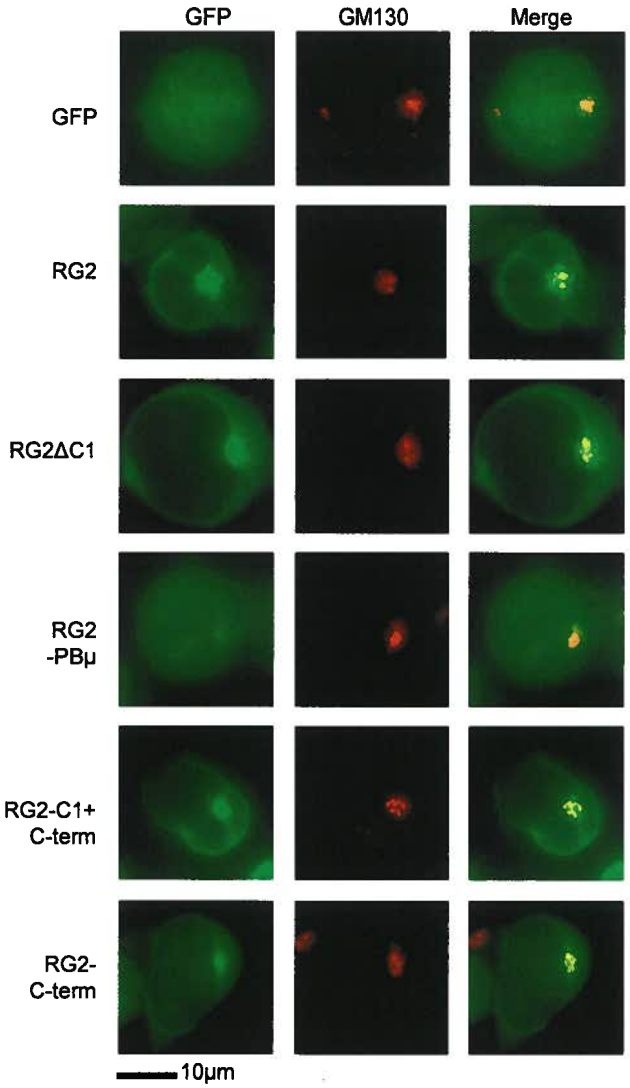

Figure 3.8 The PDZ-binding motif specifies RasGRP2 localization at the Golgi in Jurkat T-cells

Jurkat T-cells expressing GFP alone or the GFP-tagged RG2, RG2ΔC1, RG2-PBμ, RG2-C1+C-term and RG2-C-term constructs were fixed, stained with anti-GM130 to mark Golgi membranes and photographed as described in Materials and Methods. Representative cells are shown.

3.3.6 RasGRP2 does not respond to antigen receptor stimulation

RasGRP2 has been previously been shown to be activated by T-cell receptor (TCR) stimulation in Jurkat cells (Katagiri et al, 2004). Since RasGRP2 is able to localize at the plasma membrane in Jurkat cells, and the plasma membrane is the main site of activation of Rap1 in T-cells (Bivona et al, 2004), we expected that RasGRP2 might translocate to the plasma membrane in response to TCR stimulation. However TCR ligation via anti-CD3 in combination with co-stimulation via anti-CD28, did not cause any detectable redistribution of RasGRP2 (Figure 3.9A). Treatment with anti-CD3 and anti-CD28 increases Rap1-GTP levels in Jurkat cells (Figure 3.9B), but this was not increased by transfection of the cells with RasGRP2 (Figure 3.9B). While this may reflect saturation of Rap1 activation via endogenous RasGRP2, these experiments indicate that RasGRP2 is not activated by translocation in response to TCR stimulation.

RasGRP1 translocates to the plasma membrane in response to B-cell receptor stimulation of DT40 B-cells (Beaulieu et al, 2007). Rap1 is also activated in these cells in reponse to BCR stimulation (McLeod et al, 2002). In contrast to RasGRP1, we found that RasGRP2 did not translocate to the plasma membrane post BCR stimulation of DT40 cells, and we did not see an increase in Rap1 activation in RasGRP2-expressing cells compared to non-transduced cells under the same conditions. These results indicate that RasGRP2 also does not translocate to the plasma membrane in response to BCR stimulation.

3.3.7 The C1 domain is required for concentration of RasGRP2 at the plasma membrane in response to the chemokine SDF-1α

Treatment of Jurkat cells with the chemokine SDF-1α leads to a rapid increase in Rap1 activation, which is reduced when the level of endogenous RasGRP2 is lowered by RNA silencing (Ghandour et al., 2007). SDF-1α stimulation induced rapid and transient accumulation of RasGRP2 at the plasma membrane with concurrent reductions in cytosolic and Golgi-localized RasGRP2 (Figure 3.10A). However, we did not observe an increase in Rap1 activation in RasGRP2-expressing cells compared to non-transduced cells after

stimulation with SDF-1α (Figure 3.10B). This could either be the result of a lack of catalytic activation of RasGRP2 despite translocation, or alternatively due to saturation of Rap1 activation in response to SDF-1α by endogenous levels of RasGRP2.

Given that the C1 domain is required for plasma membrane localization in unstimulated cells, we postulated that this domain would also be required for SDF-1α induced recruitment of RasGRP2 to the plasma membrane. As predicted, RG2ΔC1 did not accumulate at the plasma membrane in SDF-1α treated cells (Figure 3.10A). These results further demonstrate the essential role of the C1 domain in regulating RasGRP2 localization to the plasma membrane.

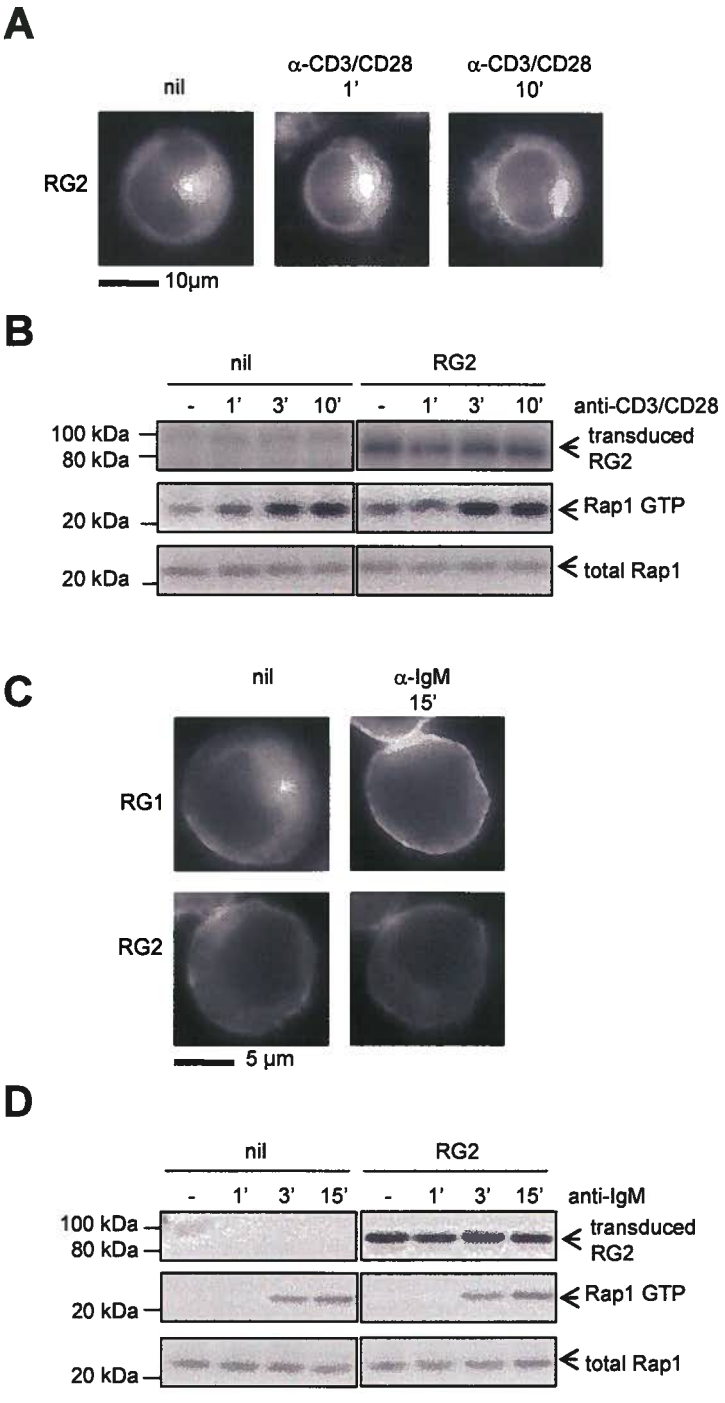

Figure 3.9 RasGRP2 does not respond to TCR or BCR stimulation

Figure 3.9 RasGRP2 does not respond to TCR or BCR stimulation
A. Jurkat T-cells expressing GFP-tagged RG2 were starved for 4 hr in serum free media, stimulated with 10 µg/ml anti-CD3 and anti-CD28 for the 1 or 10 minutes, fixed photographed by fluorescence microscopy as described in Materials and Methods. Representative cells are shown.
B. Jurkat T-cells expressing GFP alone or GFP-tagged RG2 were starved for 4 hr in serum free media, stimulated with 10 µg/ml anti-CD3 and anti-CD28 for 1, 3 or 10 minutes, and then were lysed and assayed for levels of GTP-bound Rap1 by Raf-RBD chromatography and detected by Western blot with anti-Rap1. The expression levels of transduced RasGRP2 protein in each sample, determined by Western blot with an anti-GFP antibody, are shown in the upper blot. Results are representative of three experiments.
C. DT40 B-cells expressing GFP-tagged RG1 or the GFP-tagged RG2 were starved for 4 hr in serum free media, stimulated with 5 µg/ml anti-IgM for 15 minutes, fixed photographed by fluorescence microscopy as described in Materials and Methods. Representative cells are shown.
D. DT40 B-cells expressing GFP alone or GFP-tagged RG2 were starved for 4 hr in serum free media, stimulated with 5 µg/ml anti-IgM for 1, 3 or 15 minutes, and then were lysed and assayed for levels of GTP-bound Rap1 by Raf-RBD chromatography and detected by Western blot with anti-Rap1. The expression levels of transduced RasGRP2 protein in each sample, determined by Western blot with an anti-GFP antibody, are shown in the upper blot. Results are representative of three experiments.

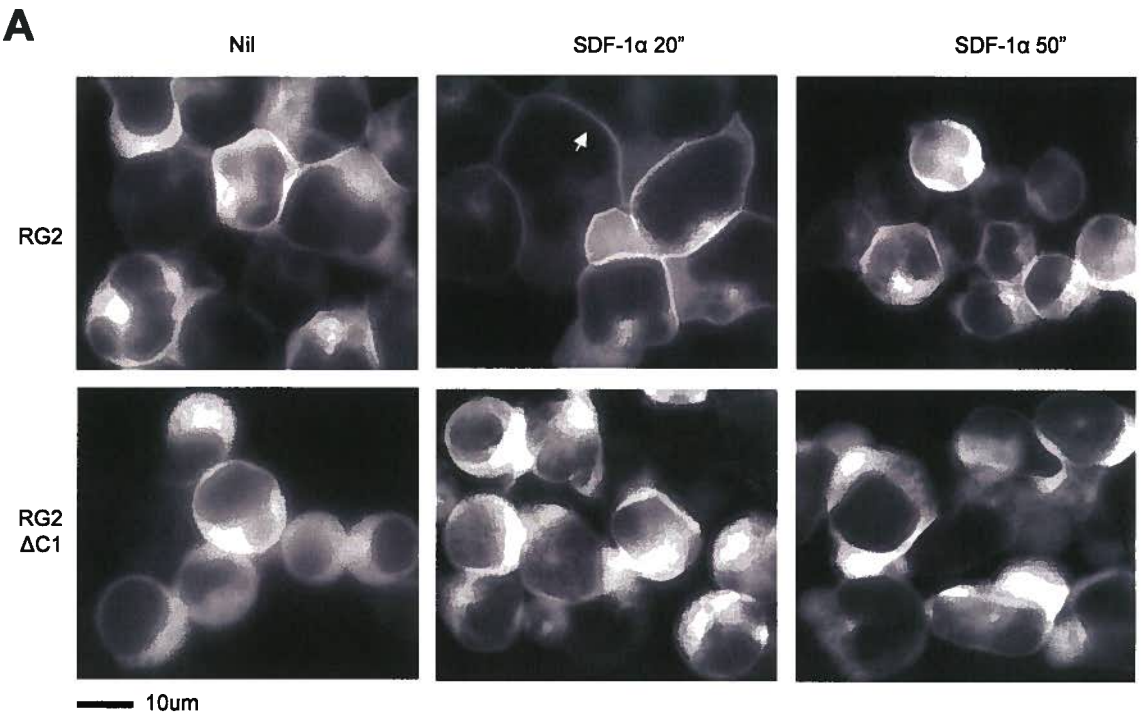

Figure 3.10 RasGRP2 accumulates at the plasma membrane in response to SDF-1α treatment of Jurkat T-cells

A. Jurkat T-cells expressing GFP-tagged RG2 or RG2ΔC1 constructs were starved for 8 hr in serum free media, stimulated with 10 ng/ml SDF-1α for 20 or 50 seconds, fixed photographed by fluorescence microscopy as described in Materials and Methods. Representative fields of cells are shown.

B. Jurkat T-cells expressing GFP alone or GFP-tagged RG2 were starved for 4 hr in serum free media, stimulated with 10 ng/ml SDF-1 α for the 10, 20 or 50 seconds, and then were lysed and assayed for levels of GTP-bound Rap1 by Raf-RBD chromatography and detected by Western blot with anti-Rap1. The expression levels of transduced RasGRP2 protein in each sample, determined by Western blot with an anti-GFP antibody, are shown in the upper blot. Results are representative of two experiments.

3.4 DISCUSSION

Our experiments show that the C1 domain of RasGRP2 is required for full efficiency of Rap1 activation in NIH 3T3 cells, and is also essential for localization of RasGRP2 at the plasma membrane in both NIH 3T3 fibroblasts and Jurkat T-cells. Previous studies have demonstrated that the RasGRP2 C1 domain does not have measurable DAG binding affinity compared to the RasGRP1, 3 and 4α C1 domains (Irie et al., 2004; Johnson et al., 2007). Furthermore, in NIH 3T3 cells the localization of the RasGRP2 C1 domain is distinct from the localization of DAG-binding C1 domains (Johnson et al., 2007). Therefore, DAG production at the plasma membrane cannot be what drives C1-dependent localization of RasGRP2 to the plasma membrane. Independent of DAG binding, all C1 domains in the RasGRP family have substantial affinity for vesicles enriched in anionic phospholipids (Johnson et al., 2007). Therefore it is possible that the C1 domain of RasGRP2 binds to membranes by this mechanism, with its selectivity for the plasma membrane being due to the high concentration of anionic phosphatidylserine at that membrane (Yeung et al., 2008). In contrast, the localization of DAG-binding C1 domains such as those of RasGRP1, 3 and 4α C1 will result in their selective targeting to Golgi membranes which are enriched in DAG (Johnson et al., 2007).

The experiment with isolated monomeric versus tandem C1 domains indicated that the C1 domain of RasGRP2 can directly bind the plasma membrane in NIH 3T3 cells, but does so with an affinity that is too low to support stable localization of RasGRP2 at the plasma membrane. Other parts of RasGRP2 must cooperate with the C1 domain to enable effective plasma membrane binding, either by modifying the affinity of the C1 domain or by providing additional weak interaction sites. The latter is analogous to the situation with RasGRP1, where stable plasma membrane binding requires cooperativity between the PT domain, which specifies plasma membrane targeting, and the C1 domain which provides an additional point of contact with the membrane (Beaulieu et al., 2007). We showed that the C1+C-terminus form of RasGRP2 is sufficient for localization at both the plasma membrane and Golgi, and that the C-terminus is sufficient for Golgi localization. Since the C1 domain is incapable of localizing at the plasma membrane, this indicates that a region in the C-terminus may stabilize C1 domain-mediated RasGRP2 localization.

While the C1 domain specifies plasma membrane localization of RasGRP2, a putative PDZ protein binding site at the C-terminus is required for efficient localization at the Golgi in NIH 3T3 fibroblasts and Jurkat T-cells. The C-terminus could act as a binding site for a Golgi-localized PDZ protein. However, the class II PDZ-binding motif has low complexity, consisting only of two hydrophobic residues, one at position 0 and the other at position -2. Therefore, its presence in a protein cannot be taken as strong evidence that it acts as a PDZ-binding site. It is possible that the C-terminus of RasGRP2 confers Golgi localization by a different mechanism, which is disrupted by the RG2-PBμ mutation.

We showed that RasGRP2 accumulates at the plasma membrane as a rapid response to the chemokine SDF-1α in Jurkat cells, and this is completely dependent on the C1 domain. While this relocalization of RasGRP2 could reflect transient increases in anionic lipids at the plasma membrane, it might also involve the induction of an as yet unidentified ligand for the C1 domain. If so, this ligand is not induced by TCR or BCR ligation, as we found that stimulation of Jurkat T-cells with anti-CD3 plus anti-CD28, or stimulation of DT40 B-cells with anti-IgM, has no effect on RasGRP2 localization.

RasGRP2 expression in NIH 3T3 fibroblasts leads to changes in cell shape and colony structure typified by the acquisition of a tessellated monolayer morphology and tight colony edges. In cells expressing RG2ΔC1, Rap1 activation is reduced compared to wild type RasGRP2, plasma membrane localization is abolished, and morphological changes are not induced. While the RG2-PBμ mutant has loss of Golgi localization and reduced Rap1 activation, this mutant still induces the same morphological changes as wild type RasGRP2. Artificial targeting of RasGRP2 to the plasma membrane via the K-Ras membrane anchor induced Rap1 activation and morphological changes, while artificial targeting of RasGRP2 to the Golgi via the C1 domain of RasGRP1 failed to induce morphological changes despite inducing activation of Rap1. Therefore, RasGRP2 can activate Rap1 when it is localized either to the plasma membrane or Golgi, but only plasma membrane-localized RasGRP2 is capable of inducing morphological changes in NIH 3T3 cells. Rap1 GTPase activation at different membranes has the potential to give rise to different signalling outputs, and thus

different cellular responses (Mor and Philips, 2006). This is known to be the case for Rap1 activation via the GEF Epac1 in PC12 neuronal cells. Epac1 normally localizes and activates Rap1 in the perinuclear region in these cells, but activation of Rap1 at this location does not activate ERK (Wang et al., 2006). When targeted to the plasma membrane via attachment of the K-Ras polybasic and prenylation signal sequence, Epac1 activation of Rap1 leads to ERK activation (Wang et al., 2006). By selective utilization of its C1 domain versus its C-terminus containing the PDZ-binding motif, RasGRP2 also has the potential to modulate where Rap1 is activated in cells, and thus how cells respond to Rap1 activation.

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CHAPTER 4: DISCUSSION

In this chapter, I outline the contributions of this thesis and present questions for future consideration. My aim in this discussion is to expand on previous discussion topics and examine broader concepts not already covered in the discussions of Chapter 2 and Chapter 3.

4.1 C1 DOMAIN MEDIATED LOCALIZATION OF RASGRPS

4.1.1 Not all C1 domains bind DAG

My thesis research on C1 domain mediated localization of RasGRPs has given an insight into the diversity of RasGRP C1 domains. All RasGRP C1 domains have the definitive spacing of two histidine and six cysteines to co-ordinate two zinc atoms, which in turn restrict the domain into a compact configuration. Closer analysis of loops A and B, which insert into membranes and form a binding pocket for DAG, reveals that for RasGRP2 and RasGRP4β C1 domains, the binding pocket is unlikely to be able to tightly bind DAG, if at all. In RasGRP2, crucial residues in loop A and B are divergent, and in RasGRP4β there is a 5 amino acid sequence inserted into the base of the binding pocket just before loop B. My experimental findings in Chapter 2 establish that neither RasGRP2 nor RasGRP4β C1 domains bind DAG in membranes, a result which emphasises that not all RasGRP C1 domains are functionally equivalent. This raises questions concerning the current view of RasGRP2 regulation, and also points to a different mode of regulation for RasGRP4β compared to RasGRP4α. While some researchers have noted that RasGRP2 C1 domain is divergent (Irie et al., 2004; Stone, 2006), for the most part RasGRP2 is often cited as a protein regulated by C1:DAG interactions (Crittenden et al., 2004; Ghandour et al., 2007; Katagiri et al., 2004; Kawasaki et al., 1998b). In the literature, RasGRP2 is frequently referred to as CalDAG-GEFI since it has a pair of EF hands that are thought to be regulated by calcium, and a C1 domain presumed to be regulated by DAG. Moreover, it appears to be a general assumption that DAG, which is produced by receptor mediated PLC activation, acts as a ligand for RasGRP2 (Crittenden et al., 2004; Ghandour et al., 2007; Katagiri et al., 2004; Pasvolsky et al., 2007). No other evidence has come to light, before our research on RasGRP2 and SDF-1α stimulation of T-cells, which demonstrates that RasGRP2 localizes to

membranes in response to receptor stimulation that generates DAG, an event which is now well established for RasGRP1 (Beaulieu et al., 2007; Bivona et al., 2003; Caloca et al., 2003b; Perez de Castro et al., 2004). So although some groups have shown that RasGRP2 is regulated by phorbol ester (PE) in a number of different contexts (Clyde-Smith et al., 2000; Crittenden et al., 2004; Dupuy et al., 2001; Kawasaki et al., 1998a) we propose that this is not likely to occur via PE:C1 mediated translocation of RasGRP2 to membranes. Instead these results may be explained by DAG activation of PKC enzymes, which when active may phosphorylate and activate RasGRP2 (Stone, 2006), as is the case for RasGRP1 and RasGRP3 (Aiba et al., 2004; Brodie et al., 2004; Roose et al., 2005; Teixeira et al., 2003; Zheng et al., 2005) which are phosphorylated at homologous positions, threonine 184 and threonine 133 respectively. RasGRP2 has a threonine at the homologous position 134 (Zheng et al, 2005), and therefore could be similarly regulated. Reduced electrophoretic mobility would indicate whether RasGRP2 was phosphorylated, although we did not observe this in response to TCR, BCR or SDF-1α stimulation (Chapter 3; Figures 3.9 and 3.10), nor has it been reported by others. However, when looking at transduced GFP-tagged RasGRP2 in NIH 3T3s we did observe two bands that ran very closely together in NIH 3T3 cells, which could represent phosphorylated (upper band) and unphosphorylated (lower band) GFP-tagged RasGRP2 (Figure 3.4A; transduced RG2). Treatment of lysates with phosphatases could determine whether the upper band is phosphorylated GFP-tagged RasGRP2, and treatment of cells with PKC inhibitors would determine whether phosphorylation occured via PKC enzymes.

4.1.2 Not all RasGRP C1 domains are targeted to the same membranes

Results from my thesis illustrate that RasGRP C1 domains not only have different affinities for DAG, but they also target different membranes. In fluorescent microscopy experiments, the isolated C1 domains of RasGRP1 and 3 were shown to target the Golgi, while the RasGRP4α C1 domain is less effectively localized to the Golgi and can also be seen localizing in the nucleus, which may reflect reduced affinity of this C1 domain for DAG and/or membranes. In contrast, the isolated RasGRP4β and RasGRP2 C1 domains do not have any detectable localization at membranes. In Chapter 3 the RasGRP2 C1 domain was

however, shown to have affinity for the plasma membrane when tandemized, and RasGRP2 plasma membrane localization was abolished in the absence of the C1 domain.

Results in Chapter 2 showed that all RasGRP C1 domains have affinity for anionic phospholipids including phosphatidylserine (PS). PS is typically at higher concentrations in the cytoplasmic leaflet of the plasma membrane (10-20%) than at other organelles in the cell, and is one of the most abundant anionic phospholipids at this membrane location (Williamson and Schlegel, 1994; Zwaal and Schroit, 1997). PS asymmetry at the plasma membrane is upheld by an ATP-dependent aminophospholipid translocase that catalyzes the transport of selected aminophospholipids from the external leaflet to the internal leaflet of the plasma membrane (Seigneuret and Devaux, 1984). Given that the RasGRP2 C1 domain binds to vesicles containing anionic phospholipids such as PS but does not have DAG affinity (Johnson et al., 2007), this explains why the tandem form of the RasGRP2 C1 domain preferentially localizes at the plasma membrane where PS is in high concentration as opposed to the Golgi, where DAG is found in abundance. It is likely that in the case of the C1 domains of RasGRP1, 3 and 4α, DAG binding at the Golgi sequesters them away from the plasma membrane, however electrostatic interactions of cationic residues within these C1 domains with anionic phospholipids present at the Golgi could provide additional stability for DAG binding. An interesting case though is the C1 domain of RasGRP4β which has reduced affinity for anionic phospholipids compared to that of RasGRP4α and other RasGRP C1 domains. The only difference between RasGRP4α and β is a five amino acid insertion in the DAG binding pocket of the RasGRP4β C1 domain, and it is possible that this insertion not only disrupts the DAG binding pocket, but also decouples the collective interactions of basic residues that are dispersed along the length of the C1 domain with anionic phospholipids. A continuous stretch of basic residues with consistent spacing may be required to facilitate strong anionic phospholipid mediated binding to membranes.

4.2 RASGRP2 LOCALIZATION VIA THE PDZ-BINDING MOTIF

During the course of my thesis research, my intention was to understand how RasGRP2 localized to membranes, and whether such localization was required for its

activity. One major finding was that a putative binding site for PDZ proteins was required for maximal RasGRP2 localization at the Golgi, and also for full activation of Rap1 by RasGRP2. When this PDZ-binding motif is mutated, RasGRP2 has reduced Golgi localization, but retains plasma membrane localization. In my experiments GFP also localized to a minor degree to the Golgi (Chapter 3; Figure 3.1, 3.3 and 3.8), leading to some overlap of signal with the Golgi GM130 marker. Therefore mutation of the PDZ-binding motif may in fact remove all RasGRP2 localization at the Golgi, and what signal remains may simply be GFP signal. These results raise the hypothesis that tethering of RasGRP2 to the Golgi is mediated by a Golgi-localized PDZ protein. This could be tested by using co-immunoprecipitation, at either a candidate or proteome-wide level, to identify PDZ proteins that bind to RG2 and are localized to the Golgi. Identification of Golgi localized partner PDZ protein for RasGRP2 would help to understand how RasGRP2 localization and activation at the Golgi is regulated by functional modulation or differential expression of PDZ proteins. Clustering of RasGRP2 at PDZ protein scaffold structures at the Golgi may cause oligomerization, thus potentially enhancing its binding to membranes via other domains.

4.3 RECEPTOR MEDIATED RASGRP2 ACTIVATION

4.3.1 Constitutive membrane localization of RasGRP2

My results show that RasGRP2 is constitutively localized at the Golgi and the plasma membrane in both NIH 3T3 and Jurkat cells, and that this requires the C1 domain. Perez de Castro et al, demonstrated that while RasGRP1 and 3 translocated to the Golgi in response to T-cell receptor (TCR) stimulation of Jurkat T-cells with antibodies for CD3 and CD28, RasGRP2 and 4α did not (Perez de Castro et al., 2004). We also found that RasGRP2 did not translocate to membranes and did not mediate Rap1 activation in response to TCR stimulation in the same cells. This is intriguing because in Jurkat cells, RasGRP2 has been shown to activate and co-immunoprecipitate with Rap1 and mediate adhesion to ICAM-1 in response to TCR stimulation (Katagiri et al., 2004), although this group did not examine RasGRP2 translocation to membranes. It is quite possible that RasGRP2 is not regulated by translocation to membranes in T-cells, but instead the proportion of RasGRP2 which is

constitutively at membranes is activated in response to TCR stimulation. My results suggest that constitutive localization at the plasma membrane of Jurkat T-cells could be determined by C1:anionic phospholipid interactions, while the Golgi localization via the PDZ-binding motif may involve a PDZ domain-containing protein partner. Although we have not established that TCR signals induce RasGRP2 activation, this may occur at either the plasma membrane or the Golgi. One possibility is that TCR stimulation induces PKC activation at one or both of these membrane sites, which in turn activates RasGRP2 by phosphorylation. Although PKCs have been shown to activate RasGRP1 and 3, they have not been shown to be required for RasGRP1 localization (Aiba et al., 2004) and therefore PKCs may activate constitutively localized RasGRP2. The plasma membrane has been determined to be the predominant site of TCR-stimulated Rap1 activation and no evidence exists for activation of Rap1 at the Golgi of these cells (Bivona et al., 2004). Taking this finding into account, it is likely that if TCR signaling leads to RasGRP2 activation, this would most likely occur at the plasma membrane.

4.3.2 SDF-1α induced plasma membrane localization of RasGRP2

RasGRP2 is required for the chemokine SDF-1α, acting via the G-protein coupled receptor CXCR4, to mediate LFA-1 adhesion to ICAM-1 in primary human T-cells (Ghandour et al., 2007). These studies were interpreted by assuming that RasGRP2 is activated in the context of SDF-1α stimulation via its C1 domain binding to DAG generated by CXCR4 coupled to PLCγ. During my thesis research I determined that SDF-1α treatment of Jurkat T-cells led to rapid concentration of RasGRP2 at the plasma membrane. That this requires the C1 domain is interesting, since the only determinant of C1-mediated localization of RasGRP2 implied by my thesis research thus far is via affinity for the anionic phospholipids of the inner leaflet of the plasma membrane. It is quite possible that SDF-1α stimulation of CXCR4 induces a rapid increase in the content of anionic phospholipids. Phosphatidylserine is already at high concentrations (10-20%) in the inner leaflet of the plasma membrane (van Meer et al., 2008) and its concentration would not be predicted to change as a result of TCR ligation. However, production of other anionic phospholipids at the cytoplasmic leaflet is induced in response to TCR stimulation, for example phosphatidic

acid (PA) and phosphatidylinositol phosphates (PIPs). PA is a relatively minor species with a net negative charge of -1.5. RasGRP C1 domains only bind to a high concentration of PA (30%) in vesicles (Johnson et al., 2007), and PA is not known to be induced to this concentration. PIPs have a higher net charge (-3 to-4 for PI(3,4) P_2 or PI(4,5) P_2 and -4 to -5 for PI(3,4,5)P_3) and therefore may be more likely to interact with the basic residues within the C1 domain of RasGRP2, even if present in the plasma membrane at a low concentration. RasGRP2 translocation in response to SDF-1α stimulation is very rapid, initiated at about 20 seconds and reversed by 50 seconds. Therefore the anionic phospholipids species that the RasGRP2 C1 domain interacts with at the plasma membrane would have to be similarly rapidly induced. By treating cells with SDF-1α, concurrent with the PI3K inhibitors LY294002 and Wortmanin, which reduce production of PIP3, it may be possible to determine whether PIP3 is required for RasGRP2 plasma membrane localization in response to SDF-1α treatment.

While induced anionic phospholipids provide a plausible mechanism for C1-mediated translocation of RasGRP2 to the plasma membrane in SDF-1α stimulated T-cells, it is also possible that RasGRP2 translocation occurs by another, unidentified mechanism.

4.4 HETEROGENEITY OF MECHANISMS FOR MEMBRANE LOCALIZATION OF RASGRPS

My thesis research has shown that the C1 domain is a critical determinant of membrane localization in all forms of RasGRPs, with the sole exception of RasGRP4β which may be a form of RasGRP4 that does not bind membranes. However, my studies of RasGRP2 show that the mechanisms by which RasGRP2 binds to membranes are quite distinct from those of other RasGRPs. RasGRP2 requires its C1 domain for plasma membrane localization but this is not via DAG. Instead, it probably occurs constitutively via affinity of the C1 domain for the anionic phospholipid phosphatidylserine (PS) and perhaps can be induced by generation of other anionic phospholipids such as phosphatidic acid (PA) or phosphatidylinositol phosphates (PIPs). The C1 domain of RasGRP2 is not required for Golgi localization, which is instead mediated via a PDZ-binding motif. This is in striking

contrast to RasGRP1 and RasGRP3, where the C1 domain is sufficient for Golgi localization, via C1: DAG interactions. Like RasGRP2, RasGRP1 is additionally targeted to the plasma membrane, but this occurs via the PT domain (PT). Although the C1 domain cooperates with the PT, specification of plasma membrane targeting is provided by the PT not the C1 domain. RasGRP3 localization has not been as well studied as RasGRP1, but RasGRP3 has homology to the PT and has a DAG-binding C1 domain, so it may share the same modes of regulation as RasGRP1. The RasGRP4α C1 domain demonstrates a weaker affinity for DAG, and a proportionate reduction in Golgi localization via DAG affinity for the C1 domain. RasGRP4α also lacks the PT domain, and therefore it is likely that it cannot localize to the plasma membrane, unless this membrane location is the site of high DAG concentration. The RasGRP4β C1 domain has no DAG-binding affinity, and consequently demonstrates no localization at Golgi or any other membrane. The possibility remains that the RasGRP4β may be capable of plasma membrane localization via C1 binding to PS or other anionic phospholipids, although our in vitro data suggests that its localization by this mechanism will be less efficient in comparison to RasGRP2.

These functional differences in the localization mechanisms of RasGRPs are presumably the outcome of the evolutionary divergence of RasGRPs, which may have proceeded as follows: Starting with a single primordial RasGRP which probably had a C1 domain that bound DAG and anionic phospholipids, a duplication event produced two RasGRPs; RasGRP1/3 and RasGRP2/4. RasGRP1/3 then picked up a PT domain to additionally enable plasma membrane targeting in response to the PT ligand. Secondary duplication events gave rise to RasGRP1 and 3, and RasGRP2 and 4. RasGRP2 was then selected for loss of DAG recognition by its C1 domain with retention of anionic phospholipid recognition. Before or after the loss of DAG binding, RasGRP2 acquired a PDZ-binding motif to enable DAG-independent, but PDZ-binding motif-dependent Golgi localization. Meanwhile, the C1 domain of RasGRP4 diversified by being selected for reduced DAG affinity and additionally acquiring an alternative splice variant that eliminated DAG binding while retaining anionic phospholipid binding. It is possible that RasGRP4β evolved as a negative regulator of RasGRP4α signal transduction. Since RasGRP4α and RasGRPβ are

identical except for a 5 amino acid insertion in the C1 domain, RasGRP4β may be able to bind and sequester the same proteins that RasGRP4α interacts with, leading to attenuation of signal transduction from RasGRPα. However, this is quite speculative given that the only activating interaction currently known for RasGRP4α is the C1:DAG interaction, which does not occur with RasGRP4β.

While this picture of RasGRP evolution is speculative, it is evident from my thesis research that functional divergence of RasGRPs has occurred, via both functional divergence of C1 domains and the acquisition (and perhaps loss) of other membrane-localizing domains. As genes duplicate and diversify, domains may retain functions as seen for RasGRP1 and 3 C1 domains, but they may also diversify their functions as seen for the C1 domains of RasGRP4 and RasGRP2. Diversification of C1 domains may occur as a result of the acquisition of new domains, which in turn may eliminate the requirement to retain the original functionality of the C1 domain. For example if RasGRP2 had to be functional at the Golgi it had to retain DAG binding capability of its C1 domain to achieve this. But once it acquired an alternative mechanism of Golgi localization, by acquisition of the PDZ-binding motif, it was possible for the C1 domain to lose the DAG binding ability and gain the ability to selectively target the plasma membrane via anionic phospholipid binding.

4.5 MEMBRANE SPECIFIC ACTIVATION OF RAP1 BY RASGRP2

Ras and Rap GTPases are relatively simple molecular switches that elicit a plethora of cellular responses. Compartmentalization of Ras and Rap activation at different membranes can serve to increase the complexity of signal transduction by segregating the spatial activation of divergent pathways and providing a distinct output from the same pathway (Mor and Philips, 2006). Most of the evidence that supports this model of compartmentalized signaling comes from experiments using artificial targeting strategies that localize Ras to specific membranes (Chiu et al., 2002). Work in yeast has shown that restricting Ras1 to the ER, as opposed to the plasma membrane abolishes mating, whereas restricting Ras1 to the plasma membrane abolishes the ability to elongate (Papadaki et al., 2002). Others have shown that restricting GEFs to different membranes has effects on

signaling output. For example, artificially targeting the Rap1 GEF Epac1 to the plasma membrane via attachment of the K-Ras4B polybasic and prenylation signal sequence, versus its normal residence at the ER, leads to ERK activation that would otherwise not occur (Bergmeier et al., 2007).

In my thesis research, RasGRP2 was artificially targeted at the Golgi versus the plasma membrane, the two membrane compartments that RasGRP2 normally localizes at. We also studied the signaling output of two mutants of RasGRP2 that in effect similarly restricted RasGRP2 localization at the Golgi and plasma membrane. When RasGRP2 localization was restricted to the plasma membrane it induced morphological changes in NIH 3T3 cells equivalently to wild type RasGRP2. When RasGRP2 was targeted away from the plasma membrane via attachment of the Golgi targeting RasGRP1 C1 domain or by removing the C1 domain, Rap1 activation occurred but did not result in cell morphology changes that were induced by wild type RasGRP2. It should be noted that RasGRP2-mediated Rap1 activation at the Golgi of NIH 3T3 cells is not necessarily without function, but instead could lead to distinct signaling outcomes that have other effects on cells. Co-expression of a fluorescently-tagged RalGDS Rap binding domain (which only binds GTP-loaded Rap) with GFP-tagged RasGRP2, and mutants RG2ΔC1 or RG2-PBμ, could potentially determine whether Rap1 activation via RasGRP2 actually occurs at both the plasma membrane and Golgi in NIH 3T3 cells.

One obvious question that arises from these results is how does plasma membrane-localized RasGRP2 mediate morphological changes in NIH 3T3 cells? RasGRP2-expressing cells have more distinct cell-cell boundaries, and do not migrate independently away from the colony, suggesting that these cells have increased cell-cell adhesion. Rap1 has been implicated as having a role in cell-cell junction formation via the regulation of the actin cytoskeleton and the recruitment of E-cadherin (Kooistra et al., 2007). Expression of Rap1 and the Rap1 GEF Epac1, increases cAMP induced cell-cell junction formation of MDCK cells (Price et al., 2004) and expression of Epac1 also promotes VE-cadherin cell junction formation and actin remodelling in HUVEC (human umbilical vein endothelial cells) (Kooistra et al., 2005). One possibility is that RasGRP2-mediated Rap1 activation at the

plasma membrane of fibroblasts leads to changes in cell-cell adhesion via promotion of actin polymerization. In support of this, RasGRP2 has been shown to interact with F-actin via its N-terminus in COS cells (Caloca et al., 2004). The morpholocial cellular response to Rap1 activation observed in RasGRP2 expressing cells may be compartmentalized at the plasma membrane owing to the concentration of specific Rap1 effectors at this location, e.g. Vav, Vav2 (Caloca et al., 2004) or Tiam1 (Baumeister et al., 2003; Buchsbaum et al., 2003), which could then promote actin polymerization via Rac GTPases. Mediators of actin polymerization e.g. Rac GTPases or their GEFs, are possibly not located or functional in this respect at the Golgi and therefore are not activated by Golgi-localized RasGRP2.

Thus, we have learned that RasGRP2 is capable of activating Rap1 at two different membrane locations. The plasma membrane has been shown before to be a major site of Rap1 localization and activation in different cells (Bivona et al., 2004; Medeiros et al., 2005) while the Golgi has so far only been shown to be a site of localization of Rap1 (Beranger et al., 1991; Nomura et al., 2004). In addition, these experiments further demonstrate that compartmentalization of Rap1 activation at different membranes leads to distinct signaling outcomes, possibly reflecting differential availability of Rap1 effectors. Taken together these results add weight to the hypothesis that compartmentalization of Ras and Rap GTPase signaling is determined by three major factors: a) differential localization of Ras and Rap GTPases via their distinct membrane targeting mechanisms, b) compartmentalization of GEF membrane localization and perhaps also compartmentalization of GAPs, which are negative regulators of Ras and Rap GTPases, and c) compartmentalization of Ras and Rap effectors.

4.6 GENERAL CONCLUSIONS AND IMPLICATIONS FOR RASGRP2 REGULATION

Before I started my thesis research, nothing was known about how RasGRP2 targeted membranes in order to access its substrate Rap1. It was assumed that RasGRP2 behaved the same way as RasGRP1, and therefore research on RasGRP2 has focussed on a model in which the C1 domain binds to DAG that is generated in membranes by receptor stimulation. DAG can be generated in reponse to a number of receptor-mediated signal transduction pathways, for example TCR stimulation, which activates PLC-γ1 that in turn cleaves the plasma membrane phospholipid PIP2 into DAG and IP3. Through the course of my research, I found that the isolated C1 domain of RasGRP2 does not bind to DAG, a finding which supported similar results from another group (Irie et al., 2004). Subsequently, I found that the RasGRP2 C1 domain is however required for plasma membrane localization, which implies that other membrane localization roles for the C1 domain have evolved, other than the conventional DAG affinity function. Another important finding was that RasGRP2 localization at the Golgi is specified by a previously unrecognized PDZ-binding motif. Both the C1 domain and the PDZ-binding motif are required for maximal RasGRP2 activation of Rap1 in NIH 3T3 cells, therefore research which helps to decipher in more detail how the C1 domain and the PDZ-binding motif promote RasGRP2 membrane localization and Rap1 activation, is likely to be important in advancing our understanding of how RasGRP2 is regulated. Findings from my thesis research are a step in this direction.

Changes in RasGRP2 expression are associated with disease phenotypes. Deregulated-increase in expression of RasGRP2 leads to lymphoma (Mikkers et al., 2002; Suzuki et al., 2002) and myeloid leukemia (Dupuy et al., 2001) in mice, while knockout mice have severe platelet (Bergmeier et al., 2007; Crittenden et al., 2004) and leukocyte adhesion defects (Bergmeier et al., 2007). In the case of cancer, RasGRP2 may activate the ERK1/2 pathway through constituitive Rap1 activation of B-Raf, whereas in RasGRP2 deficient leukocytes and platelets, lack of RasGRP2-mediated Rap1 activation in response to receptor stimulation (e.g. the SDF-1α chemokine receptor CXCR4), is thought to lead to a defect in inside-out signaling, which in turn leads to deficient integrin-mediated adhesion. New

studies in leukocyte adhesion deficiency (LAD) III patients that have severely reduced RasGRP2 expression represent the first discovery of a role for RasGRP2 in human disease (Pasvolsky et al., 2007). Thus, advances in the understanding of how RasGRP2 is normally regulated by membrane localization and activation, will provide key knowledge about how this GEF is positioned to promote cancer. Moreover, by learning how membrane localization regulates RasGRP2 to promote integrin-mediated adhesion via Rap1 activation, for example in response to chemokine stimulation of lymphocytes, will better our understanding of this fundamental cell response, and may give new insights into pathologies such as LAD III.

4.6 BIBLIOGRAPHY

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