Regulation of RasGRP1 by B Cell Antigen Receptor Requires Cooperativity between Three Domains Controlling Translocation to the Plasma Membrane

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RasGRP1 is a Ras-activating exchange factor that is positively regulated by translocation to membranes. RasGRP1 contains a diacylglycerol-binding C1 domain, and it has been assumed that this domain is entirely responsible for RasGRP1 translocation. We found that the C1 domain can contribute to plasma membrane-targeted translocation of RasGRP1 induced by ligation of the B cell antigen receptor (BCR). However, this reflects cooperativity of the C1 domain with the previously unrecognized Plasma membrane Targeter (PT) domain, which is sufficient and essential for plasma membrane targeting of RasGRP1. The adjacent suppressor of PT (SuPT) domain attenuates the plasma membrane-targeting activity of the PT domain, thus preventing constitutive plasma membrane localization of RasGRP1. By binding to diacylglycerol generated by BCR-coupled phospholipase C γ 2, the C1 domain counteracts the SuPT domain and enables efficient RasGRP1 translocation to the plasma membrane. In fibroblasts, the PT domain is inactive as a plasma membrane targeter, and the C1 domain specifies constitutive targeting of RasGRP1 to internal membranes where it can be activated and trigger oncogenic transformation. Selective use of the C1, PT, and SuPT domains may contribute to the differential targeting of RasGRP1 to the plasma membrane versus internal membranes, which has been observed in lymphocytes and other cell types.

INTRODUCTION

RasGRP1 is a guanine nucleotide exchange factor that couples antigen receptors to the activation of Ras GTPases (Dower *et al.*, 2000; Ebinu *et al.*, 2000; Priatel *et al.*, 2002; Bivona *et al.*, 2003; Caloca *et al.*, 2003b; Layer *et al.*, 2003; Norment *et al.*, 2003; Oh-hora *et al.*, 2003; Guilbault and Kay, 2004; Perez de Castro *et al.*, 2004; Reynolds *et al.*, 2004; Zugaza *et al.*, 2004; Coughlin *et al.*, 2005; Roose *et al.*, 2005). Deletion of the RasGRP1 gene perturbs the immunological selection and activation of lymphocytes (Dower *et al.*, 2000; Priatel *et al.*, 2002, 2006; Layer *et al.*, 2003) and mast cells (Liu *et al.*, 2007), whereas aberrant expression of RasGRP1 initiates oncogenic transformation of lymphocytes (Li *et al.*, 1999; Mikkers *et al.*, 2002; Suzuki *et al.*, 2002; Kim *et al.*, 2003; Dupuy *et al.*, 2005; Klinger *et al.*, 2005), fibroblasts (Ebinu *et al.*, 1998; Tognon *et al.*, 1998) and keratinocytes (Oki-Idouchi and Lorenzo, 2007).

To be active as an exchange factor, RasGRP1 must be localized to cell membranes where Ras GTPases reside. This requirement provides an opportunity for positive or negative regulation. RasGRP1 contains a C1 domain that binds the lipid second messenger diacylglycerol (DAG), or DAG-

mimetic phorbol esters (Ebinu *et al.*, 1998; Lorenzo *et al.*, 2000; Shao *et al.*, 2001; Rong *et al.*, 2002; Carrasco and Merida, 2004; Madani *et al.*, 2004). Treatment of cells with DAG or phorbol esters results in the translocation of RasGRP1 to membranes (Ebinu *et al.*, 1998; Tognon *et al.*, 1998; Bivona *et al.*, 2003; Rambaratsingh *et al.*, 2003; Caloca *et al.*, 2004; Stone *et al.*, 2004), and it stimulates Ras activation via RasGRP1 (Ebinu *et al.*, 1998; Kawasaki *et al.*, 1998; Tognon *et al.*, 1998; Priatel *et al.*, 2002; Rambaratsingh *et al.*, 2003; Caloca *et al.*, 2004). In serum-stimulated NIH 3T3 fibroblasts (Tognon *et al.*, 1998) and COS cells (Caloca *et al.*, 2003a), RasGRP1 translocation to internal membranes (endoplasmic reticulum [ER] and Golgi) requires its C1 domain, and phorbol ester-induced transformation of fibroblasts by RasGRP1 is entirely dependent on the C1 domain (Tognon *et al.*, 1998). In combination, these observations support a model of RasGRP1 regulation involving C1 domain-mediated recruitment to membranes enriched in DAG and containing Ras GTPase substrates. This mechanism has the potential to positively regulate RasGRP1 in response to any receptor that couples to a DAG-generating phospholipase C (PLC). In addition to the C1 domain, RasGRP1 has a pair of EF-hands that have been implicated as calcium-responsive positive regulators (Ebinu *et al.*, 1998; Kawasaki *et al.*, 1998; Guilbault and Kay, 2004), although no involvement of the EF-hands in regulating RasGRP1 localization has been established.

In the DT40 B cell line, B cell antigen receptor (BCR) ligation induces translocation of RasGRP1 to the plasma membrane (Caloca *et al.*, 2004). Membrane translocation of RasGRP1 is also induced by T cell antigen receptor (TCR) ligation in the Jurkat T cell line, although this is variably reported to be directed to the plasma membrane (Carrasco

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Abbreviations used: BCR, B cell antigen receptor; DAG, diacylglycerol; GFP, green fluorescent protein; PLC, phospholipase C; TCR, T cell antigen receptor.

and Merida, 2004; Zugaza *et al.*, 2004) or to Golgi membranes (Bivona *et al.*, 2003; Perez de Castro *et al.*, 2004). Membrane-selective localization of RasGRP1 may play a critical role in modifying the responses of T cells to TCR ligation. RasGRP1 is localized primarily to Golgi in thymocytes undergoing positive selection, but it is at the plasma membrane in thymocytes undergoing negative selection (Daniels *et al.*, 2006). In mature T cells, RasGRP1 translocates to the plasma membrane after engagement with antigen presenting cells, but it is predominantly at internal sites when the T cells are anergized before antigen presentation (Zha *et al.*, 2006). Differential spatial activation of DAG kinases, which convert DAG to phosphatidic acid, has been suggested as one physiological mechanism by which RasGRP1 could be switched between internal versus plasma membrane localization (Zha *et al.*, 2006). Alternatively, DAG generation at the plasma membrane via integrin-induced activation of phospholipase D and phosphatidic acid phosphatase can relocate RasGRP1 from Golgi to plasma membranes in TCR-stimulated T cells (Mor *et al.*, 2007).

There is extensive evidence that a C1 domain-driven mechanism of translocation is essential for activation of RasGRP1 by antigen receptors. Deletion of the C1 domain eliminates TCR-induced activation of RasGRP1 (Roose *et al.*, 2005). BCR- or TCR-induced RasGRP1-dependent activation of Ras is dependent on PLC γ (Ebinu *et al.*, 2000; Caloca *et al.*, 2003b; Perez de Castro *et al.*, 2004; Reynolds *et al.*, 2004; Zugaza *et al.*, 2004) and in the Jurkat T cell line is suppressed by DAG kinases (Sanjuan *et al.*, 2003). The requirement for PLC and DAG does not necessarily involve the translocation step of activation, because catalytic activation of RasGRP1 is dependent on phosphorylation by protein kinase Cs (PKCs) (Aiba *et al.*, 2004; Roose *et al.*, 2005; Zheng *et al.*, 2005), which are themselves dependent on DAG generated by receptor-coupled PLCs. However, PLC inhibition impairs TCR-induced accumulation of RasGRP1 in insoluble structures, which could represent membrane translocation (Ebinu *et al.*, 2000; Reynolds *et al.*, 2004), and overexpression of DAG kinase α reduces the plasma membrane translocation of RasGRP1, which is induced by TCR ligation in mature T cells (Zha *et al.*, 2006).

All of these experiments indicate that antigen receptor-induced activation of RasGRP1 includes a membrane translocation step that is driven by the C1 domain binding to DAG generated in membranes via receptor-coupled PLCs. However, when expressed in the Jurkat T cell line, the isolated C1 domain of RasGRP1 remained at internal membranes after TCR ligation, whereas the same stimulation induced translocation of either full-length RasGRP1 or a DAG-binding C1 domain from PKC θ to the plasma membrane (Carrasco and Merida, 2004). This unexpected result demonstrated that under some circumstances the C1 domain of RasGRP1 can fail to confer stable localization at the plasma membrane even when antigen receptor ligation results in generation of DAG predominantly at the plasma membrane. As pointed out by Carrasco and Merida (2004), this experiment also suggested that the full-length RasGRP1 protein can receive signals that override internal membrane targeting via the C1 domain. This motivated us to examine in detail the role of the C1 domain in BCR-induced activation of RasGRP1. We demonstrated that the C1 domain is essential for efficient BCR-induced translocation of RasGRP1 to the plasma membrane in DT40 cells. However, the C1 domain by itself is unable to direct RasGRP1 to the plasma membrane. Instead, it is the previously unrecognized Plasma membrane Targeter (PT) domain that specifies the plasma membrane as the site of translocation of RasGRP1.

The C1 domain serves a secondary role as an enhancer of PT domain-dependent translocation of RasGRP1 to the plasma membrane, with cooperativity between these domains being required to confer stringent quantitative and spatial regulation of RasGRP1 by BCR.

MATERIALS AND METHODS

Cells and Reagents

Wild-type and PLC γ 2-deficient DT40 cells were obtained from Mike Gold (University of British Columbia, Vancouver), and they were originally from T. Kurosaki (RIKEN Research Center, Yokohama, Japan). All 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. DT40 cells were cultured in RPMI 1640 medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2% chicken serum (Invitrogen, Carlsbad, CA), and 50 μ M 2-mercaptoethanol. NIH 3T3 cells from American Type Culture Collection (Manassas, VA) were cultured in DMEM (Stem Cell Technologies) containing 10% bovine calf serum (Hyclone). The origin and culture of WEHI-231 cells are as described previously (Guilbault and Kay, 2004). DO11.10 cells (Morgan *et al.*, 1999) were obtained from Barbara Osborne (University of Massachusetts), and they were cultured in DMEM/10% fetal bovine serum. Anti-chicken IgM polyclonal antibody was from Bethyl Laboratories (Montgomery, TX), anti-murine IgM was from Jackson ImmunoResearch Laboratories (West Grove, PA), and anti-CD3 ϵ and anti-CD28 were from eBiosciences (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA) was from Sigma-Aldrich (St. Louis, MO). Anti-extracellular signal-regulated kinase (ERK)1/2 anti-phospho-specific ERK1/2 antibodies were from Cell Signaling Technology (Danvers, MA), anti-green fluorescent protein (GFP) was from Abcam (Cambridge, MA), anti-hemagglutinin (HA) was from Covance (Princeton, NJ), and anti-pan Ras was from Calbiochem (San Diego, CA). Alexa fluor 488-conjugated anti-GFP polyclonal antibody was from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories.

Construction of Modified Forms of RasGRP1

The N-terminally GFP-tagged form of full-length murine RasGRP1 (RG1) was derived from the XFL construct described previously (Tognon *et al.*, 1998), with the GFP coding sequences from pEGFP-C1 (Clontech, Mountain View, CA) fused to amino acid 2 of RasGRP1 (GenBank accession no. NP_035376). The sequence at the fusion site is DELYKSGLRSSAQSEGLTGKAR, with the sequences that do not naturally occur in enhanced (e)GFP or RasGRP1 in italics. The following constructs are modifications of this RG1 construct with the indicated changes (sequences that do not naturally occur in RasGRP1 are italicized; C terminus indicated by star). RG1-GEF μ has a single R271E point mutation that disrupts binding to Ras GTPases (Park *et al.*, 1994; Tognon *et al.*, 1998). RG1 Δ C1 = deletion of amino acids 538–595; sequence around deletion = YSKLGSTKSPAIS. RG1 Δ PT = deletion of amino acids 717 to C terminus; sequence N-terminal to deletion = ASPCSPAST*. RG1 Δ C-term = deletion of amino acids 597 to C terminus; sequence around deletion = FECKKRIKPT*EA*. RG1 Δ C1+SuPT = deletion of amino acids 538–694; sequence around deletion = YSKLGSTPRKSAQ. RG1 Δ SuPT = deletion of amino acids 646–694; sequence around deletion = VDHSEESTPRKSAQ. RG1/Pren = replacement of RasGRP1 amino acids 538 to C terminus with an HA epitope tag plus the prenylation signal of K-Ras; sequence from fusion to C terminus = YSKLGSTEAYPYDYASGRKHKEKMSKDGGKKKKKSKTKCV-IM*. The isolated C1 domain construct consisted of amino acids 540–596 of RasGRP1, fused at the N terminus to GFP (sequence around fusion = DELYKSGLRSLKSTFPNHF) and with the C-terminal sequence FECKKRIKPT*EA*. The sequence around the GFP fusion is DELYKSGLRSLKSTFPNHF for the C1+C-term construct, and DELYKSGLRSLKST for the C-term, C-term Δ 1, and C-term Δ 2 constructs, with the N-terminal boundaries of the RasGRP1 sequences in the latter three constructs indicated in Figure 4C.

Construction of PKC C1 Domains

The C1b domain of murine PKC δ encoding amino acids 229–280 (GenBank 45330876) was attached to an N-terminal GFP tag derived from EGFP-C1. The sequence around the fusion is RSLKSTMPHREKVF and the sequence at the C terminus is KVANLCKKRIKPT*. The tandem C1a+C1b domain segment of murine PKC ϵ encoding amino acids 168–294 (GenBank accession no. 6755084) was attached to an N-terminal GFP tag derived from EGFP-C1. The sequence around the fusion is RSLKSTNGHKFMA and the sequence at the C terminus is VAPNCGVEA*.

Construction of GFP-tagged Ras Binding Domain and C1 Domain of Raf-1 (GFP-RBDL)

cDNA encoding amino acids 51–220 of human Raf-1, as described previously (Bondeva *et al.*, 2002), were amplified by polymerase chain reaction (PCR) and

fused to the C terminus of GFP. The resulting encoded sequence at the N-terminal Raf boundary is STPSKTSNT and the Raf-1 C-terminal sequence is LTMRRMRESA* where italicized letters are not natural Raf-1 sequence.

Retroviral Transduction of Cell Lines

Transfection of BOSC23 ecotropic packaging cells with retroviral vector plasmid DNA was performed as described previously (Pear *et al.*, 1993). Virus-containing medium was supplemented with polybrene to 20 $\mu\text{g}/\text{ml}$ and then added to an equal volume of DT40, WEHI-231 or DO11.10 cells ($2 \times 10^6/\text{ml}$) in appropriate medium. After 5–10 h of culture, 2–3 volumes of medium was added. Transduced cells were selected by addition of puromycin 30–48 h after 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.

Fluorescence Microscopy

Cells expressing different mutants of RasGRP1 were plated on poly-L-lysine-coated glass coverslips (using poly-D-lysine for WEHI-231 cells). Before stimulation, DT40, WEHI-231 and DO11.10 cells were cultured in serum-free medium for 3–4, 6, or 3 h, respectively. Cells were stimulated in Hank's buffer (Stem Cell Technologies) with 5 $\mu\text{g}/\text{ml}$ anti-chicken immunoglobulin (Ig)M or 500 ng/ml PMA (DT40 cells), 5 $\mu\text{g}/\text{ml}$ anti-mouse IgM (WEHI-231 cells), or 10 $\mu\text{g}/\text{ml}$ each of anti-CD3 ϵ + anti-CD28 (DO11.10 cells). Cells were then fixed with 4% formaldehyde in PBS. In Figures 2B and C, 3A and B, 4A and B, 6, and 7B and F, DT40 cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) and stained with Alexa Fluor 488-conjugated anti-GFP antibody to increase sensitivity of detection of GFP. Fluorescence microscopy used a 450- to 485-nm excitation filter and a 500- to 545-emission filter. Images were captured using OpenLab imaging software. Images were used to score at least 100 cells for plasma membrane localization of the GFP fusion proteins. Only cells showing fluorescence well above autofluorescence levels were scored. The individual cells displayed in the figures were chosen to be representative of the majority of the population of cells, unless otherwise noted in the figure legends. Histograms of fluorescence intensities along segments were drawn by the profile function of Scion Image software.

To mark Golgi membranes, fixed cells were stained with mouse anti-GM130 followed by Alexa Fluor 647-conjugated anti-mouse IgG. ER was marked by treating unfixed cells with glibenclamide BODIPY-Texas Red (ER Tracker; Invitrogen), followed by fixation with formaldehyde in PBS as described above.

Stimulation and Lysis of Cells for Detection of P-ERK2 or Ras-GTP

DT40 cells (2.5×10^7) 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. Anti-chicken IgM was then added to a concentration of 5 $\mu\text{g}/\text{ml}$ for the indicated times. Cells were immediately lysed with 2.5 volumes 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, and 1 mM NaMoO_4) containing 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM activated Na_3VO_4 .

Affinity Purification of Activated Ras GTPases by Raf-RBD Chromatography

Lysates prepared as described above were incubated for 30 min with glutathione S-transferase (GST)/Raf-RBD fusion protein, which had been prepared and prebound to glutathione-agarose beads as described previously (Taylor *et al.*, 2001). After washing, samples were eluted in Bio-Rad XT sample buffer (Bio-Rad, Hercules, CA), electrophoresed, and detected by Western blot as described below, using anti-HA or anti-pan Ras antibodies.

Western Blot Analysis

Samples containing equal quantities of total protein (measured by the BCA protein assay; Pierce Chemical, Rockford, IL) were denatured using Bio-Rad XT sample buffer, separated by gel electrophoresis on 12% XT-Criterion acrylamide gels (Bio-Rad), and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) by electroblotting. After blocking the membranes overnight at 4°C in Tris-buffered saline/Tween 20 (TBST) (25 mM Tris-HCl, pH 7.4, 3 mM KCl, 150 mM NaCl, and 0.05% Tween 20) containing 5% BSA, primary antibodies were applied to the membrane for 90 min at room temperature in 2% BSA TBST, and horseradish peroxidase-conjugated secondary antibodies were applied for 45 min at room temperature in TBST containing 1% BSA. Membranes were exposed to substrate/enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA) and chemiluminescence was detected using the VersaDoc 5000 imaging system (Bio-Rad). P-ERK2 was quantified by band volume analysis using Quantity One software (Bio-Rad). In all Western blot figures, each panel is from a single blot,

Figure 1. Domain structures of GFP-tagged RasGRP1 proteins used in this study. GEF, guanine nucleotide exchange domain that catalyzes GTP loading of Ras GTPases. EF, EF-hands. PT and SuPT are the plasma membrane targeting regulatory domains identified in this study. The black circle represents prenylation.

with gaps in the image indicating intervening segments of the blot that are not displayed. For Figure 7D, the activities of RasGRP1 mutants relative to RasGRP1 within the same experiment were calculated as follows, using RG1ΔC-term as an example: (P-ERK2 in cells expressing RG1ΔC-term-P-ERK2 in cells expressing GFP control)/(P-ERK2 in cells expressing RG1 – P-ERK2 in cells expressing GFP control). These relative values from multiple experiments were then displayed as means $\pm$ SD. Two-tailed p values were calculated by one-sample *t* tests.

RESULTS

BCR Ligation Induces Translocation of RasGRP1 to the Plasma Membrane, and RasGRP1 Activation

GFP-tagged forms of RasGRP1 (Figure 1) were expressed in the DT40 B cell line, to assess how RasGRP1 localization and activation are affected by signaling from the BCR. In all unstimulated cells, full-length RasGRP1 was cytoplasmic, and it was predominantly colocalized with endoplasmic reticulum as well as Golgi membranes (Figure 2A). As described previously (Caloca *et al.*, 2003b), ligation of BCR with anti-IgM induced a radical relocalization of RasGRP1 to the plasma membrane in all of the cells (Figure 2B). The plasma membrane also was the predominant site of BCR-induced activation of Ras GTPases in DT40 cells (Figure 2C), as detected by a probe (GFP-RBDL) consisting of GFP fused to the extended Ras-GTP binding domain of Raf-1 (Bondeva *et al.*, 2002).

RasGRP1 activation was detected by comparing GTP loading of Ras GTPases in RasGRP1-transduced versus control DT40 cells. Measured in this way, RasGRP1 was weakly active in unstimulated cells and much more strongly activated after BCR ligation (Figure 2D). Phosphorylation of ERK2 at T193 and Y195, a signal transduction output of RasGRP-catalyzed Ras GTP loading in DT40 cells (Oh-hora *et al.*, 2003), was used as an alternative means for detecting and quantifying activation of RasGRP1. Transduction of DT40 cells with RasGRP1 led to a two- to threefold increase in BCR-induced ERK2 phosphorylation (Figure 2E). A mutant of RasGRP1 that is defective for Ras binding (Tognon *et al.*, 1998) failed to increase BCR-induced ERK2 phosphory-

Figure 2. Plasma membrane translocation and activation of RasGRP1 in response to BCR ligation. (A) Unstimulated DT40 cells expressing GFP-tagged RasGRP1 (RG1) were stained with either ER Tracker to mark ER or anti-GM130 to mark Golgi membranes, as described in *Materials and Methods*. Individual cells showing fluorescence from the GFP-tagged RG1 and either ER Tracker or GM130 staining are shown. (B) DT40 cells transduced with GFP alone or RG1 were untreated (nil) or treated with 5 μ g/ml for 15 min. Cells were then fixed and photographed by fluorescence microscopy. The single cells shown are representative of the typical appearance of the population of cells. Histograms of fluorescence intensities along the indicated segments are shown to the top right of each cell image. The edge of the cell is indicated by the vertical line on the segment. The percentages of cells in each population with detectable GFP at the plasma membrane are listed to the bottom right of each cell image. (C) DT40 cells expressing GFP-RBDL were untreated (nil) or treated with 5 μ g/ml anti-IgM for 15 min. Cells were then fixed and photographed by fluorescence microscopy. The cells are displayed as described for B. (D) DT40 cells transduced with a retroviral vector expressing HA epitope-tagged wild-type N-Ras, and untransduced (nil) or transduced with RG1, were stimulated with anti-IgM for the indicated times. Activated Ras GTPases were purified by Raf-RBD chromatography and detected by Western blot, by using anti-HA to detect the transduced N-Ras and an anti-Ras antibody to detect endogenous K-Ras and H-Ras. (E) DT40 cells expressing either GFP as a control, RG1, or RG1 with a point mutation that prevents Ras binding (RG1-GEF μ) were treated with anti-IgM for the indicated times, and levels of phosphorylated ERK2 were quantified by Western blot. The numbers below the P-ERK blot are relative quantities of each band. The expression levels of transduced RasGRP1 protein in each sample, determined by Western blot with an anti-GFP antibody, are shown in the lower blot.

lation (Figure 2E), confirming that the ERK2 phosphorylation coupled to RasGRP1 transduction was the output of RasGRP1-catalyzed Ras-GTP loading.

The C1 Domain Is a Major but Not Exclusive Contributor to BCR-induced Translocation of RasGRP1 to the Plasma Membrane, and It Is Incapable of Independently Mediating Plasma Membrane Targeting

Either deletion of the C1 domain (Figure 3A) or PLC γ 2 deficiency (Figure 3B) reduced translocation of RasGRP1 to the plasma membrane in response to BCR ligation. This confirmed that the C1 domain is needed for efficient translocation of RasGRP1 to the plasma membrane, via binding to DAG generated by PLC γ 2. However, in a substantial percentage of cells there was still detectable BCR-induced translocation of RasGRP1 to the plasma membrane in the absence of either the C1 domain or PLC γ 2 (examples shown in Figure 3, A and B). This was the first indication that RasGRP1 translocation to the plasma membrane can be achieved, albeit with reduced efficiency, through a mechanism that does not require the C1 domain.

To determine whether the C1-dependent component of RasGRP1 translocation occurred through a mechanism that involved only the C1 domain, we expressed the isolated C1 domain as a GFP fusion. In unstimulated DT40 cells, the RasGRP1 C1 domain was partially localized to internal membranes (Figure 3C). After BCR ligation, there was no detectable translocation of the RasGRP1 C1 domain to the

plasma membrane, whereas there was a minor increase in localization at internal membranes (Figure 3C). The lack of observable plasma membrane localization of the RasGRP1 C1 domain was not due to either nonfunctionality of this C1 domain or to its active exclusion from the plasma membrane, because stimulation of DT40 cells with the C1 domain ligand PMA induced partial translocation of the RasGRP1 C1 domain to the plasma membrane (and to the nuclear envelope) in all cells (Figure 3C).

To understand why the RasGRP1 C1 domain concentrated at internal membranes and failed to translocate to the plasma membrane in response to BCR ligation, we examined the distribution of DAG in DT40 cells by using a fusion of GFP to the tandem C1a + C1b domains of PKC ϵ (Stahelin *et al.*, 2005). The pairing of DAG binding sites provided by the tandem C1 domains considerably increases the avidity of DAG binding (Codazzi *et al.*, 2001; Giorgione *et al.*, 2003). The tandem PKC ϵ C1 domains localized intensely to internal membranes in unstimulated DT40 cells (Figure 3D), indicating that these membranes are enriched for DAG even in the absence of stimulation. BCR ligation induced translocation of the tandem PKC ϵ C1 domains to the plasma membrane and the nuclear envelope, accompanied by partial retention at ER and Golgi (Figure 3D). Thus, the absence of translocation of the RasGRP1 C1 domain is not due to lack of BCR-induced DAG generation at the plasma membrane, but instead it could reflect insufficient avidity of the monomeric RasGRP1 C1 domain for DAG at the plasma membrane. To

Figure 3. The C1 domain promotes but is insufficient for BCR-induced plasma membrane translocation of RasGRP1. DT40 cells expressing the indicated proteins were untreated (nil) or treated with anti-IgM 5 μ g/ml anti-IgM for 15 min. Cells were prepared for fluorescence microscopy, analyzed, and displayed as described for Figure 2B. In this and subsequent figures, the percentages of cells in each population with detectable GFP at the plasma membrane are indicated at the bottom right of each cell image as % pm⁺. (A) DT40 cells expressing RG1 or RG1ΔC1. For the anti-IgM-stimulations, a cell representative of those showing plasma membrane localization of RG1ΔC1 is shown. (B) PLCγ2-deficient DT40 cells expressing RG1 were treated as described for A and prepared for fluorescence microscopy, analyzed, and displayed as described for Figure 2B. For the anti-IgM-stimulation, a cell representative of those showing plasma membrane localization of RG1 is shown. (C) DT40 cells expressing the isolated C1 domain of RasGRP1 or the isolated C1b domain of PKCδ, each with an N-terminal GFP tag. PMA treatment (500 ng/ml) was for 5 min. (D) DT40 cells expressing the tandem C1a + C1b domains of PKCε with an N-terminal GFP tag.

further examine this, we used the C1b domain of PKCδ, which binds DAG with an affinity similar to that of RasGRP1 (Tognon *et al.*, 1998; Giorgione *et al.*, 2006) and which has a moderate preference for localizing to the plasma membrane versus internal membranes (Stahelin *et al.*, 2005). This C1 domain was similar to that of RasGRP1 in being partially localized to internal membranes in unstimulated cells, and increasing its localization to internal membranes in response to BCR ligation, but the C1b domain of PKCδ additionally showed faint plasma membrane localization (Figure 3C). The RasGRP1 C1 domain seems to have two distinct deficiencies that make it incapable of mediating plasma membrane targeting in response to BCR; insufficient avidity for DAG when expressed in isolation (as seen also for the C1b domain of PKCδ), and a preference for binding to internal membranes rather than the plasma membrane (in contrast to the C1b domain of PKCδ). The latter could reflect higher affinity of the RasGRP1 C1 domain for saturated DAGs, which can be more abundant in internal versus plasma membranes (Carrasco and Merida, 2004).

These experiments contradict the expectation that binding of the C1 domain to DAG generated at the plasma membrane by BCR-coupled PLCγ2 should provide an effective and sufficient mechanism for translocating RasGRP1 exclusively to the plasma membrane. Nonetheless, either deletion of the C1 domain or PLCγ2 deficiency partially impedes translocation of RasGRP1 to the plasma membrane. The C1 domain must cooperate with other domains of RasGRP1 to drive BCR-induced RasGRP1 translocation, and one or more of these other domains must be responsible for the preferential targeting of RasGRP1 to the plasma membrane rather than internal membranes.

The PT Domain of RasGRP1 Mediates BCR- and TCR-induced Plasma Membrane Targeting

The EF-hands of RasGRP1 (Figure 1) were the initial lead candidates for providing cooperativity with the C1 domain in maximizing BCR-induced plasma membrane localization, because they were the only other recognized regulatory domains within RasGRP1, and their deletion compromises RasGRP1-mediated apoptosis induction by BCR (Guilbault and Kay, 2004). However, a C-terminally deleted form of RasGRP1 that retains both the C1 domain and the EF-hands (RG1ΔC-term) failed to translocate to the plasma membrane in response to BCR ligation (Figure 4A). In contrast, an N-terminally truncated form of RasGRP1 that lacks the EF-hands and retains only the C1 domain and the C-terminal region efficiently translocated to the plasma membrane in response to BCR ligation (C1+ C-term in Figure 4B). When expressed without the C1 domain, the C-terminal region translocated to the plasma membrane in response to BCR ligation, but with reduced efficiency in terms of the percentage of cells showing translocation and the quantity of plasma membrane translocation in those cells (Figure 4B). Thus, the C-terminal region is what cooperates with the C1 domain to achieve efficient plasma membrane targeting of RasGRP1. Progressive truncation of the C-terminal region localized its plasma membrane targeting activity to the 101 amino acids at the C terminus (C-termΔ2 in Figure 4, B and C). We refer to this as the PT domain. Deletion of the PT domain from RasGRP1 eliminated all detectable plasma membrane translocation in response to BCR ligation (Figure 4D). Thus, the PT domain is the component of RasGRP1 that confers plasma membrane targeting, and it is able to achieve this even in the absence of the C1 domain.

Figure 4. The region C-terminal to the C1 domain contains the PT and SuPT domains that control BCR-induced translocation of RasGRP1 to the plasma membrane. (A and B) DT40 cells expressing the indicated RasGRP1 constructs were untreated (nil) or treated with anti-IgM for 15 min. Cells were prepared for fluorescence microscopy, analyzed, and displayed as described for Figures 2 and 3. (B) Cells representative of those showing plasma membrane localization of the RasGRP1 constructs are shown. (C) Sequence of the C-terminal region of murine RasGRP1, from amino acid 571 to the C terminus (GenBank accession no. NP 035376). Bases conserved between murine and chicken RasGRP1 are in bold. The potential leucine zipper is underlined. The arrows show the N-terminal boundaries of the indicated RasGRP1 constructs, and the regions deleted in RG1ΔSuPT and RG1ΔPT. (D) DT40 cells expressing RG1 or RG1ΔPT were untreated (nil) or treated with anti-IgM for 15 min. Cells were prepared for fluorescence microscopy, analyzed, and displayed as described for Figures 2 and 3. (E) DT40 cells expressing RG1 or RG1ΔSuPT were untreated (nil) or treated with anti-IgM for 15 min. Cells were prepared for fluorescence microscopy, analyzed, and displayed as described for Figures 2 and 3. In the experiment shown in the bottom panels, BCR stimulation was relatively weak, resulting in only a low level of plasma membrane translocation of RG1.

Figure 5. PT domain-mediated plasma membrane localization of RasGRP1 in murine B and T cell lines. (A) WEHI-231 B cells expressing GFP or the indicated RasGRP1 constructs were untreated (nil) or treated with 5 μ g/ml anti-IgM for 15 min. Cells were prepared for fluorescence microscopy, analyzed, and displayed as described for Figures 2 and 3. (B) DO11.10 T cells expressing GFP or the indicated RasGRP1 constructs were untreated (nil) or treated with 10 μ g/ml anti-CD3 ϵ plus 10 μ g/ml anti-CD28 for 15 min. Cells were prepared for fluorescence microscopy, analyzed, and displayed as described for Figures 2 and 3. For the anti-CD3 ϵ + α -CD28 treated cells expressing RG1, the displayed cell is representative of those showing detectable plasma membrane localization.

The role of the PT domain in plasma membrane targeting of RasGRP1 was even more dominant in the WEHI-231 murine B cell line. In these cells, RasGRP1 was predominantly at internal membranes in unstimulated cells, with a low level of plasma membrane localization (Figure 5A). BCR ligation induced strong translocation of RasGRP1 to the plasma membrane. In contrast to DT40 cells, deletion of the C1 did not impede BCR-induced translocation to the plasma membrane, whereas in unstimulated WEHI-231 cells deletion of the C1 domain eliminated internal membrane localization and increased plasma membrane localization (Figure 5A). Deletion of the PT domain eliminated plasma membrane localization without affecting internal membrane localization. The isolated PT domain (C-term Δ 2) was strongly localized to the plasma membrane in both unstimulated and stimulated WEHI-231 cells (Figure 5A). In this B cell line, it seems that the PT domain is fully sufficient for plasma membrane localization, whereas the C1 domain specifies internal membrane localization.

In the murine DO11.10 T cell line, TCR plus CD28 ligation was modestly effective at inducing translocation of RasGRP1, with about one third of the cells showing detectable localization of RasGRP1 at the plasma membrane after stimulation with anti-CD3 ϵ + anti-CD28 (Figure 5B). The percentage of DO11.10 cells showing detectable TCR-induced plasma membrane translocation was greatly reduced by deletion of the PT domain. When expressed in isolation, the PT domain was weakly localized to the plasma membrane in most DO11.10 cells, and TCR/CD28 ligation induced an increase in both the percentage of cells showing plasma membrane localization and the intensity of plasma membrane localization (Figure 5B). Therefore, the PT domain confers plasma membrane targeting of RasGRP1 in this T cell line, although with reduced efficiency in comparison to the DT40 and WEHI 231 B cell lines.

A Suppressor Domain Attenuates Constitutive Plasma Membrane Translocation via the PT Domain

The PT domain differed from full-length RasGRP1 by being partially plasma membrane localized in the absence of antigen receptor ligation (RG1 in Figure 4A vs. C-term Δ 2 in Figure 4B), implying that full-length RasGRP1 contains one or more domains that negatively regulate plasma membrane targeting by the PT domain. This suppressor of PT (SuPT) function was conferred by a 51-amino acid segment lying between the C1 and PT domains (Figure 4C). When this SuPT domain was attached to the PT domain, it reduced both constitutive and BCR-induced plasma membrane localization (comparing C-term Δ 1 to C-term Δ 2 in Figure 4B). Deletion of the SuPT domain from RasGRP1 resulted in enhanced translocation to the plasma membrane in response to BCR ligation, which was particularly evident when BCR ligation induced only partial translocation of wild-type RasGRP1 (Figure 4E, bottom). The SuPT domain is thus effective at quenching the propensity of the adjacent PT domain to drive constitutive localization of RasGRP1 to the plasma membrane, but it does so at the cost of impeding the efficiency of BCR-induced translocation via the PT domain. As a result, the SuPT + PT complex provides only a partial solution to the problem of attaining stringent translocational regulation of RasGRP1 by BCR.

The C1 Domain Counteracts the SuPT Domain and Makes Translocation via the PT Domain Responsive to Regulation by PLC γ 2

Attachment of the C1 domain to the C-terminal region served to override the effect of the SuPT domain, resulting in high efficiency of BCR-induced plasma membrane translocation while still minimizing plasma membrane localization in the absence of BCR ligation (comparing C1+C-term to

Figure 6. Plasma membrane targeting by the PT domain occurs independently of PLC γ 2, whereas the ability of the C1 domain to counteract the SuPT domain requires PLC γ 2. PLC γ 2-deficient DT40 cells expressing the indicated RasGRP1 constructs were untreated (nil) or treated with anti-IgM for 15 min. The cells were then prepared for fluorescence microscopy, analyzed, and displayed as described for Figures 2 and 3. Cells representative of those showing plasma membrane localization of the RasGRP1 constructs are shown.

C-term in Figure 4B). Although BCR-induced plasma membrane translocation of the PT domain with or without the SuPT domain occurred independently of PLC γ 2 (comparing C-term and C-term Δ 2 in Figure 4B vs. Figure 6), the ability of the C1 domain to enhance plasma membrane translocation was dependent on PLC γ 2 (comparing C1+C-term in Figures 4B vs. Figure 6). These results indicate that the C1 domain acts as a DAG-dependent positive mediator of translocation that counterbalances the ability of the SuPT domain to impede plasma membrane targeting by the PT domain. This concept was further tested by examining how the C1 domain and the SuPT domain functionally interact within full-length RasGRP1. As shown in Figure 3A, deletion of the C1 domain greatly reduced BCR-induced plasma membrane translocation, and it also eliminated activation of RasGRP1 in response to BCR ligation (Figure 7A). However, when the SuPT domain is deleted along with the C1 domain, BCR-induced plasma membrane localization and activation are both partially restored (Figure 7, B–D). Therefore, PT domain-mediated RasGRP1 activation at the plasma membrane can be achieved either by positive cooperativity with the C1 domain, or by inactivation of the SuPT domain.

BCR-induced Activation of RasGRP1 Can Occur Away from the Plasma Membrane and Independently of the PT Domain

When the C-terminal region containing the PT and SuPT domains was deleted (RG1 Δ C-term), there was no detectable localization at the plasma membrane after BCR ligation (Figure 4A). Nonetheless, RG1 Δ C-term was activated by BCR ligation (Figure 7, D and E), demonstrating that plasma membrane localization is not required for BCR-induced RasGRP1 activation in DT40 cells. RG1 Δ C-term was quite diffusely distributed in the DT40 cells both before and after BCR ligation (Figure 3A), but it was not as delocalized as GFP alone (Figure 2B) and it was partially in the cytoplasmic regions occupied by ER and Golgi (Figure 2A). This distribution resembles that of the isolated C1 domain of RasGRP1 (Figure 3C). In the absence of the PT domain, the C1 domain apparently provides interactions with internal membranes that are sufficient to enable productive encounters of RasGRP1 with its Ras substrates.

RG1 Δ C-term requires BCR ligation to be activated (Figure 7E), even though its localization does not overtly change with BCR ligation (Figure 4A). When the C1 domain of

RG1 Δ C-term was replaced by a prenylation signal that provides constitutive membrane localization (Figure 7F), BCR ligation was still needed for activation (Figure 7G). This demonstrates that membrane localization, although critical for providing access to membrane-bound Ras GTPases, is insufficient for RasGRP1 activation. This is expected, given that RasGRP1 requires catalytic activation by PKCs, and PKCs are themselves activated by antigen receptor ligation (Roose *et al.*, 2005; Zheng *et al.*, 2005).

In NIH 3T3 Fibroblasts, the PT Domain Does Not Confer Plasma Membrane Localization or Contribute to Oncogenic Transformation by RasGRP1

RasGRP1 was initially identified as an oncogene by its ability to induce transformation of fibroblast cell lines (Ebinu *et al.*, 1998; Tognon *et al.*, 1998). This experimental system was subsequently used to define the roles of DAG and the C1 domain in driving membrane localization of RasGRP1, and in activating RasGRP1 as an oncogene (Tognon *et al.*, 1998). Having identified the PT domain as a critical mediator of RasGRP1 localization and activation in DT40 cells, we wanted to determine whether the PT domain influences RasGRP1 localization in fibroblasts and to examine the potential of this domain to provide a DAG-independent mechanism by which RasGRP1 can trigger oncogenesis.

RasGRP1 localizes to the ER and Golgi in serum-stimulated NIH 3T3 fibroblasts (Figure 8A) and its constitutive activation at these sites results in oncogenic transformation, evident by high cell refractility and loss of contact inhibition (Figure 8B). Deletion of the PT domain had no discernible effect on either the localization of RasGRP1 at internal membranes or its ability to induce transformation (Figure 8B). When expressed in isolation, the PT domain was distributed throughout NIH 3T3 fibroblasts, with no detectable plasma membrane localization (Figure 8C), indicating that lack of plasma membrane targeting is not imposed by the SuPT domain.

In contrast to the PT domain, the C1 domain was both necessary and sufficient for RasGRP1 localization in NIH 3T3 cells. The isolated C1 domain had the same internal membrane distribution as RasGRP1 (Figure 8C), whereas deletion of the C1 domain caused loss of internal membrane localization of RasGRP1 and loss of transforming activity (Figure 8B). Constitutive membrane targeting by prenylation enabled transformation in the absence of the C1 domain (Figure 8B), confirming that the role of the C1 domain in enabling oncogenic transformation by RasGRP1 is to provide membrane localization (Tognon *et al.*, 1998).

These results demonstrate that the PT domain is intrinsically nonfunctional as a plasma membrane targeter in NIH 3T3 fibroblasts, whereas the C1 domain serves to target RasGRP1 to internal membranes. The lack of plasma membrane targeting by the PT domain causes wild-type RasGRP1 in NIH 3T3 fibroblasts to behave equivalently to PT domain-deleted RasGRP1 in B cells.

DISCUSSION

Our experiments demonstrate that the C1 domain plays an essential role in the activation of RasGRP1 by BCR in DT40 cells. In the absence of the C1 domain, BCR-induced translocation of RasGRP1 to the plasma membrane is reduced, and activation is undetectable. This supports the hypothesis that the interaction of the C1 domain with DAG provides a mechanism for translocating RasGRP1 to membranes, where it can find its Ras GTPase substrates. However, the C1 domain is unable on its own to mediate translocation, be-

Figure 7. Activation of RasGRP1 by BCR can occur at the plasma membrane independently of the C1 domain, or at internal membranes independently of the PT domain. (A) The C1 domain is required for RasGRP1 activation by BCR. DT40 cells transduced with GFP as a control or with the indicated RasGRP1 constructs were treated with anti-IgM for the indicated times. Levels of phosphorylated ERK2 were quantified by Western blot, with relative amounts indicated below each sample. Transduced RasGRP1 protein, detected by Western blot with anti-GFP, is shown in the lower blot. (B) RasGRP1 translocates to the plasma membrane in response to BCR ligation when both the C1 and SuPT domains are absent. DT40 cells expressing RasGRP1 with deletion of the C1 and SuPT domains were untreated (nil) or treated with anti-IgM for 15 min. The cells were then prepared for fluorescence microscopy, analyzed, and displayed as described for Figures 2 and 3. (C) RasGRP1 can be activated by BCR when both the C1 and SuPT domains are absent. DT40 cells transduced with GFP as a control or with the indicated RasGRP1 constructs were treated with anti-IgM for the indicated times. Levels of phosphorylated ERK2 were quantified by Western blot, with relative amounts indicated below each sample. Transduced RasGRP1 protein, detected by Western blot with anti-GFP, is shown in the lower blot. (D) ERK2 activation by RG1ΔC-term and RG1ΔC1+SuPT in response to BCR ligation, relative to ERK2 activation by RG1. P-ERK2 levels were measured in DT40 cells expressing RG1, RG1ΔC-term, or RG1ΔC1+SuPT and stimulated with αIgM for the indicated times. The P-ERK2 levels induced by RG1ΔC-term or RG1ΔC1+SuPT are displayed relative to the P-ERK2 levels induced by RG1, calculated as described in *Materials and Methods*. Results are from four experiments with RG1ΔC-term and three experiments with RG1ΔC1+SuPT. Bars show SD. The p values are for comparison to hypothetical mean of 0 (no activation of ERK2 by the RasGRP1 mutants). (E) RasGRP1 can be activated in the absence of the PT domain. DT40 cells transduced with GFP as a control or with the indicated RasGRP1 constructs were treated with anti-IgM for the indicated times. Levels of phosphorylated ERK2 were quantified by Western blot, with relative amounts indicated below each sample. Transduced RasGRP1 protein, detected by Western blot with anti-GFP, is shown in the lower blot. (F) Membrane localization of RasGRP1 by prenylation. DT40 cells expressing prenylated RasGRP1 were untreated (nil) or treated with anti-IgM for 15 min. The cells were then prepared for fluorescence microscopy, analyzed and displayed as described for Figures 2 and 3. (G) RasGRP1 can be activated by BCR when the C1 and PT domains are absent, if membrane localization is provided by prenylation. DT40 cells transduced with GFP as a control or with the indicated RasGRP1 constructs were treated with anti-IgM for the indicated times. Levels of phosphorylated ERK2 were quantified by Western blot, with relative amounts indicated below each sample. Transduced RasGRP1 protein, detected by Western blot with anti-GFP, is shown in the lower blot.

cause the concentration of DAG generated at the plasma membrane by BCR-coupled PLCγ2 is not high enough to

draw the C1 domain away from internal membranes. The plasma membrane targeting function is instead provided by

Figure 8. The PT domain does not contribute to membrane targeting of RasGRP1 in fibroblasts, and it is not required for activation of RasGRP1 as an oncogene. (A) NIH 3T3 cells expressing RG1 were stained with either ER Tracker to mark ER or anti-GM130 to mark Golgi membranes, as described in *Materials and Methods*. Individual cells showing fluorescence from GFP-tagged RG1 and either ER Tracker or GM130 staining are shown. (B) The top pictures show localization of the indicated RasGRP1 constructs in transduced NIH 3T3 cells. Subconfluent cells were fixed and photographed by fluorescence microscopy. Representative cells are shown. The RG1/pren construct serves to highlight the plasma membrane. The bottom pictures are low-magnification views of NIH 3T3 cells cultured for 5 d after confluence. High refractility, elongation, high cell density, and loss of contact inhibition are indicative of oncogenic transformation. (C) Localization of the isolated C1 domain and PT (C-term Δ 2) domain of RasGRP1 in NIH 3T3 cells. Subconfluent cells were fixed and photographed by fluorescence microscopy. Representative cells are shown.

a previously unrecognized part of RasGRP1, the PT domain. A ligand for this domain is apparently localized at or close to the plasma membrane, and it is able to attract the PT domain to this site, weakly in the absence of BCR signaling and strongly once BCR is ligated. The PT domain contains a putative α helical segment capable of forming a leucine zipper (Figure 3C) (Tognon *et al.*, 1998), which could contribute to recognition of its ligand. A third regulatory element, the SuPT domain, serves to partially attenuate plasma membrane targeting by the PT domain, thereby raising the threshold of signaling required for RasGRP1 translocation to the plasma membrane and preventing constitutive plasma membrane localization of RasGRP1.

Although the PT domain is solely responsible for specifying the plasma membrane as the site of translocation of RasGRP1, correct quantitative and spatial regulation of RasGRP1 translocation in DT40 cells is only achieved by the

combined action of the PT, C1 and SuPT domains. We propose the following model for BCR-induced translocation of RasGRP1 (Figure 9). In unstimulated DT40 cells, there is negligible translocation of RasGRP1 to the plasma membrane because DAG is absent, the quantity of functional PT ligand is low, and the SuPT domain attenuates interaction of the PT domain with its ligand. Some RasGRP1 is free in the cytoplasm, whereas the remainder is weakly bound to internal membranes via the C1 domain. This interaction could be mediated by DAG constitutively found in internal membranes (Figure 9), or by interaction of the C1 domain with negatively charged phospholipids (Hurley and Meyer, 2001; Cho and Stahelin, 2005). After BCR ligation, two events occur that promote translocation of RasGRP1. The first is an increase in the quantity of functional PT ligand at the plasma membrane, which enables weak binding by RasGRP1 via the PT domain. The second BCR-induced event is generation of

Figure 9. Proposed mechanism for BCR-induced translocation of RasGRP1 to the plasma membrane. (A) In unstimulated cells, the C1 domain of RasGRP1 is attracted predominantly to internal membranes, via DAG binding. A hypothetical ligand for the PT domain is present at the plasma membrane but at functionally low levels. Due to the suppressive action of the SuPT domain, binding of RasGRP1 to the PT ligand is minimal, relative to its binding to internal membranes via the C1 domain. (B) BCR ligation induces high levels of functional PT ligand. Binding of this ligand to the PT domain, partially counteracted by the suppressive effect of the SuPT domain, provides an interaction site at the plasma membrane. Once attracted to the plasma membrane by this interaction, RasGRP1 can acquire an additional interaction via binding of its C1 domain to the local pool of DAG generated at the plasma membrane by BCR-coupled PLC γ 2. The combination of these two interactions is sufficient to confer stable binding of RasGRP1 at the plasma membrane, despite the persistent capability of the C1 domain to bind to internal membranes.

DAG at the plasma membrane by PLC- γ 2. RasGRP1, which has been transiently recruited to the plasma membrane via the PT domain, can thus acquire an additional interaction via the C1 domain. The combination of the PT-ligand and C1-DAG interactions confers enough stability to maintain RasGRP1 at the plasma membrane, where it can be further activated by protein kinase Cs and access its Ras GTPase substrates.

In this model, each domain has a distinct role: the PT domain specifies the plasma membrane as the site of RasGRP1 translocation, the SuPT domain prevents constitutive localization of RasGRP1 to the plasma membrane via the PT domain, and the C1 provides an essential second interaction site at the plasma membrane and imposes on RasGRP1 regulation by PLC- γ 2. The latter is critical for ensuring that RasGRP1 activation is DAG dependent and thus can be negatively regulated by DAG kinases (Topham and Prescott, 2001; Sanjuan *et al.*, 2003; Regier *et al.*, 2005; Topham, 2006).

The finding that RasGRP1 uses more than its single C1 domain to take the critical translocation step in the activation process fits with the precedents set by other C1 domain-containing proteins (Brose *et al.*, 2004). In conventional PKCs, selective binding to cellular membranes is achieved by cooperativity between a pair of C1 domains interacting with DAG and negatively charged phospholipids, in conjunction with a calcium-dependent phospholipid-binding C2 domain (Cho, 2001). C1 domains also have contributory rather than deterministic roles in membrane localization of β -chimerin and protein kinase D1 (Oancea *et al.*, 2003; Hall *et al.*, 2005). The dependence of C1 domains on cooperation with other regulatory domains may increase the stringency of regulation of these proteins, by ensuring that their quantity and locale of activation is not simply a linear function of the concentration of diacylglycerol in membranes.

The membranes to which RasGRP1 is translocated in response to receptor ligation vary among different lymphoid cell lines or sublines, in some cases being at the plasma membrane (Caloca *et al.*, 2003b; Carrasco and Merida, 2004; Zugaza *et al.*, 2004) and in others at the Golgi and/or ER (Bivona *et al.*, 2003; Perez de Castro *et al.*, 2004). RasGRP1 is localized predominantly to Golgi in thymocytes undergoing TCR-induced positive selection but is at the plasma membrane in thymocytes undergoing negative selection driven by more intense TCR signaling (Daniels *et al.*, 2006). Similarly, RasGRP1 translocates to the plasma membrane after TCR engagement via antigen-presenting cells, but it remains at internal sites when the TCR signaling is modulated by anergization (Zha *et al.*, 2006). Although TCR ligation alone can induce RasGRP1 localization to Golgi, combined ligation of TCR plus the integrin lymphocyte function-associated antigen 1 can induce relocation of RasGRP1 to the plasma membrane (Mor *et al.*, 2007). Membrane-selective localization of RasGRP1 could be determined by differences in the spatial distribution of diacylglycerol generation or degradation, e.g., via variable localization of phospholipase Ds (Mor *et al.*, 2007) or diacylglycerol kinases (Topham, 2006). Alternatively or additionally, selective use of the C1, PT, and SuPT domains could provide a mechanism for varying the site of activation of RasGRP1. Using RasGRP1 mutants, we have demonstrated that this occurs in DT40 cells. Under the same stimulatory conditions, RasGRP1 can be localized to and active at either the plasma membrane or internal membranes, with the site of localization being determined by the availability of DAG for C1 domain-mediated binding to either the plasma membrane or internal membranes, the capability of the PT domain to redirect RasGRP1 specifically to the plasma membrane, and the capability of the SuPT

domain to suppress plasma membrane targeting. In WEHI-231 B cells, the PT domain is exceptionally effective as a plasma membrane targeter, whereas the C1 domain contributes only to internal membrane localization. Plasma membrane targeting by the PT domain is less effective in DO11.10 T cells, resulting in only partial translocation of RasGRP1 to the plasma membrane in response to TCR ligation. In NIH 3T3 fibroblasts, the PT domain is completely ineffective, and localization of RasGRP1 is determined by the C1 domain, which targets it to internal membranes. All nonlymphoid cells examined thus far have internal membrane localization of RasGRP1 (Tognon *et al.*, 1998; Bivona *et al.*, 2003; Caloca *et al.*, 2003a). This could be a result of nonfunctionality of the PT domain itself or lack of expression or availability of the hypothetical ligand for the PT domain.

The combined actions of the C1, PT, and SuPT domains can have a predominant role in controlling access of RasGRP1 to membranes, but additional modes of regulation of RasGRP1 have been demonstrated or can be anticipated. Phosphorylation of RasGRP1 by PKCs seems to be essential for RasGRP1 activation, although this may act strictly at the level of stimulating exchange activity, because mutation of the major PKC phosphorylation site does not affect BCR-induced membrane translocation of RasGRP3 to the plasma membrane in DT40 cells (Aiba *et al.*, 2004). Previous studies have indicated that the EF-hands of RasGRP1 are required for its activation in B cells but not in fibroblasts (Tognon *et al.*, 1998; Guilbault and Kay, 2004). In DT40 cells, efficient plasma membrane translocation of RasGRP1 via the PT domain can occur in the absence of the EF-hands, although this does not rule out the EF-hands acting in a more subtle or separate way as calcium-responsive modulators of RasGRP1 membrane binding or activation. It is notable that the RasGRP1 mutant that lacked the C1 and SuPT domains but retained the PT domain had reduced plasma membrane targeting in comparison with the isolated PT domain. This implies that an additional plasma membrane targeting suppressor may be located in the portion of RasGRP1 N-terminal to the C1 domain.

RasGRP1 is normally under strict transcriptional regulation in lymphocytes (Norment *et al.*, 2003), and additionally it requires antigen receptor ligation for its activation (Dower *et al.*, 2000; Coughlin *et al.*, 2005). Deregulated transcription of wild-type RasGRP1 in immature T cells initiates oncogenic transformation even in the absence of functional antigen receptors (Klinger *et al.*, 2005). B cells malignancies are also induced by deregulated expression of nonmutated RasGRP1 (Mikkers *et al.*, 2002; Suzuki *et al.*, 2002). The stringent antigen receptor-induced regulation of RasGRP1 that is mediated by cooperativity of the C1, PT, and SuPT domains may be lost in these cells. Although NIH 3T3 cells are a dubious model for lymphocyte transformation, they may provide some relevant insight into how biochemical deregulation of RasGRP1 can occur. In NIH 3T3 cells, the C1 domain is sufficient to localize RasGRP1 to internal membranes and to activate it as an oncogene, with no requirement for regulatory input from the PT or SuPT domains. This highlights the potential hazard of ectopic expression of RasGRP1, if it occurs in a cell in which the C1 domain can constitutively target RasGRP1 to membranes populated by Ras GTPases. Alternatively, there is potential for RasGRP1 deregulation to occur in lymphocytes via constitutive presentation of the PT ligand in combination with inactivation of the SuPT domain. This would result in antigen receptor-independent activation of RasGRP1 accompanied by resistance of RasGRP1 to negative regulation by DAG kinases (Topham and Prescott, 2001; Sanjuan *et al.*, 2003; Regier *et al.*,

2005). Identifying the mechanism of activation of RasGRP1 as a physiological oncogene will require analyses of the location and domain dependence of RasGRP1 in normal versus transformed lymphocytes.

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