The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization

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Cell polarization is required for virtually all functions of T cells, including transendothelial migration in response to chemokines. However, the molecular pathways that establish T cell polarity are poorly understood. We show that the activation of the partitioning defective (Par) polarity complex is a key event during Rap1- and chemokine-induced T cell polarization. Intracellular localization and activation of the Par complex are initiated by Rap1 and require Cdc42 activity. The Rac activator Tiam1 associates with both Rap1 and components of the Par complex, and thereby may function to connect the Par polarity complex to Rap1 and to regulate the Rac-mediated actin remodelling required for T cell polarization. Consistent with these findings, Tiam1-deficient T cells are impaired in Rap1- and chemokine-induced polarization and chemotaxis. Our studies implicate Tiam1 and the Par polarity complex in polarization of T cells, and provide a mechanism by which chemokines and Rap1 regulate T cell polarization and chemotaxis.

Introduction

Cell polarization is required for T cell processes such as transendothelial migration, proliferation, homotypic interactions, activation in response to antigen presentation, and cytotoxity (Sanchez-Madrid and del Pozo, 1999; Dustin and Chan, 2000). Polarized cell migration or chemotaxis in response to chemoattractants stimulates leucocytes to transmigrate through the endothelial barrier to reach secondary lymphoid organs or sites of infection (Sanchez-Madrid and del Pozo, 1999). During the process of T cell polarization, morphological and functional changes result in a bipolar asymmetric shape of T cells, which is mediated by the recruitment of surface receptors, signaling complexes, and cellular organelles to discrete areas of the plasma membrane (Vicente-Manzanares et al., 2002). Polarized T cells are characterized by a leading edge, in which chemokine receptors and activated integrins (such as LFA1) are clustered, and a uropod at the back, in which ICAM-1/3 and CD44 accumulate (del Pozo et al., 1996).

The Ras-like GTPase Rap1 has been implicated in adhesion processes, such as inside-out signaling, integrin-mediated cell–matrix adhesions, and the control of cell polarity (for reviews see Kinashi and Katagiri, 2004; Bos, 2005). Rap1 and its effector protein RAPL are two key proteins that are required for the establishment of T cell polarity. Indeed, inhibition of Rap1 signaling by the overexpression of a GAP for Rap impairs chemokine-induced T cell polarization and transendothelial migration, as well as the adhesion to ICAM-1 and VCAM-1 (Shimonaka et al., 2003). Expression of the truncated mutant RAPL ΔN, which is unable to bind to Rap1, abrogates V12Rap1, as well as chemokine-induced T cell polarization (Katagiri et al., 2003), suggesting that RAPL functions downstream of Rap1. However, little is known about the signaling pathways used by Rap1 and chemokines to induce T cell polarization.

In various cell types and species, three conserved protein complexes, termed the partitioning defective (Par), Scribble, and Crumbs complexes, have been shown to regulate cell polarity (Etienne-Manneville and Hall, 2002; Nelson, 2003). Of these, the Par polarity complex, consisting of a core of Par3, Par6 (for partition-defective), and atypical PKC (aPKCλ/ι and aPKCζ), controls different aspects of cell polarity. These include polarization of astrocytes, asymmetric cell division in yeast, axon specification and synaptogenesis in neuronal cells, and apical–basal polarity in epithelial cells (for reviews see Etienne-Manneville and Hall, 2003; Macara, 2004; Wiggin et al., 2005; Mertens et al., 2006). A recent study has shown that various polarity proteins (e.g., Par3, aPKC, Scribble, Dlg, and Crumbs3) are differentially localized throughout polarized T cells (Ludford-Menting et al., 2005), suggesting that one or more of the polarity complexes may regulate T cell polarization. Whether the Par, Scribble, or Crumbs polarity complexes are

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Abbreviations used in this paper: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; SDF1α, stromal cell–derived factor-1 α; Tiam, T lymphoma invasion and metastasis; WT, wild-type.

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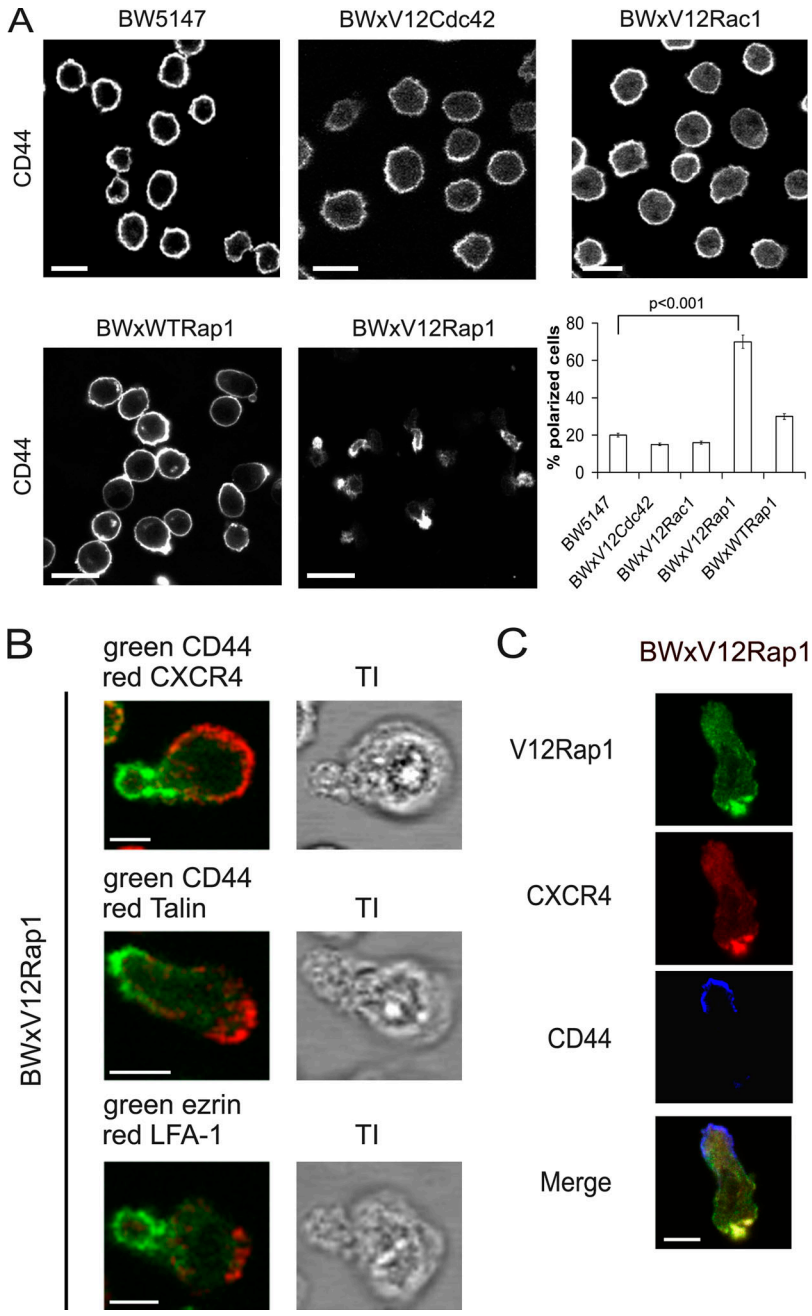

Figure 1. **Constitutively active V12Rap1 induces T cell polarization.** (A) Control, WTRap1-, V12Cdc42-, V12Rac1-, and V12Rap1-expressing BW5147 T lymphoma cells were fixed in suspension and stained for CD44, as described in Materials and methods. The histogram indicates percentage of polarized cells from three independent experiments. Error bars indicate the SD; P indicates the P value. (B) Polarized V12Rap1-expressing BW5147 T lymphoma cells were stained for the leading edge markers CXCR4, talin, and LFA-1, and for the uropod markers CD44 and ezrin. TI, transmission images. (C) V12Rap1 localization. Myc-V12Rap1–expressing BW5147 T lymphoma cells were stained for myc, CXCR4, and CD44. Bars: (A) 10 μm; (B and C) 5 μm.

indeed functionally required for chemokine-induced T cell polarization is unknown.

Rho-like GTPases have been shown to function in the polarization processes of various cells, including T cells (Evers et al., 2000; Etienne-Manneville and Hall, 2002; Raftopoulou and Hall, 2004). In earlier studies, we have identified the T lymphoma invasion and metastasis 1 (Tiam1) gene using retroviral insertional mutagenesis in combination with in vitro selection of invasive T lymphoma variants (Habets et al., 1994). Tiam1 encodes a guanine nucleotide exchange factor (GEF) that specifically activates the Rho-like GTPase Rac (Michiels et al., 1995). However, the physiological function of Tiam1 in lymphoid cells is unknown. We have recently shown, along with other studies, that Tiam1 interacts with Par3 of the Par polarity complex, and thereby is a critical component of Par-mediated regulation of neuronal and epithelial (apical–basal) cell polarity (Chen and Macara, 2005; Mertens et al., 2005; Nishimura et al., 2005; Zhang and Macara, 2006). Moreover, Tiam1 is able to associate with Rap proteins in fibroblasts (Arthur et al., 2004), suggesting that Tiam1 may control Rap1-induced T cell polarization. Therefore, we have investigated the potential function of Tiam1 and the Par polarity complex in T cell polarization. We show here that Tiam1 in conjunction with the Par polarity complex is an important regulator of Rap1- and chemokine-induced polarization and chemotaxis of T cells.

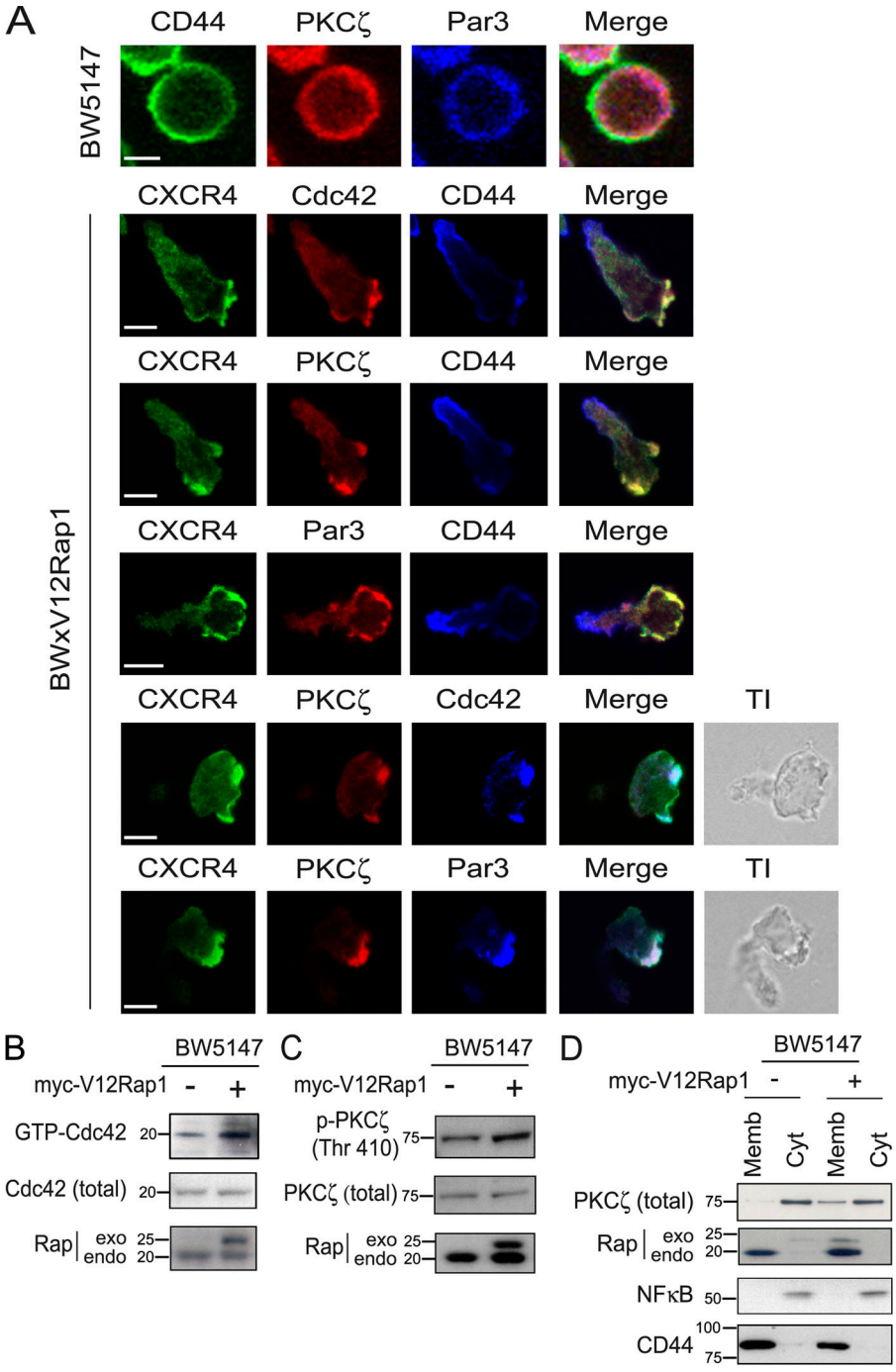

Figure 2. **Intracellular localization and activation of Par polarity proteins in polarized and nonpolarized BW5147 T lymphoma cells.** (A) Intracellular localization of PKCζ, Par3, and Cdc42. Control and polarized V12Rap1-expressing BW5147 T lymphoma cells were stained for Cdc42, PKCζ, Par3, CXCR4, and CD44. Bars, 5 μm. TI, transmission images. (B) V12Rap1 activates Cdc42. The activation of Cdc42 in control and V12Rap1-expressing BW5147 T lymphoma cells was determined by pulldown assays, as described in Materials and methods. (top) Activated GTP-bound Cdc42 and total Cdc42. (bottom) Immunoblot of V12Rap1 expression (exo, exogenous; endo, endogenous). (C) V12Rap1 induces PKCζ phosphorylation. Lysates of starved control and V12Rap1-expressing BW5147 T lymphoma cells were separated by SDS-PAGE and analyzed for the amount of total PKCζ and PKCζ phosphorylation on Thr410 residue. (top) Immunoblots of phosphorylated PKCζ and total PKCζ. (bottom) Immunoblot of Rap1 expression. (D) Lysates of starved control and V12Rap1-expressing BW5147 T lymphoma cells were fractionated in membranous (memb) and cytosolic (cyt) fractions and analyzed for the intracellular localization of PKCζ and Rap1 (top). (bottom) Fractionation control immunoblots Nf-κB (cytosolic fraction) and CD44 (membrane fraction). Sizes are indicated in kilodaltons.

Results

Activated Rap1 induces T cell polarity

GTPases of the Rho and Rap family have been implicated in T cell polarization (del Pozo et al., 1999; Shimonaka et al., 2003). To identify one of the first requirements for T cell polarization, constitutively active mutants of these GTPases (i.e., V12Cdc42, V12Rac1, and V12Rap1) were expressed in BW5147 T lymphoma. None of the constitutively active Rho GTPases tested were able to induce polarization (Fig. 1 A). This indicates that, although necessary (Ratner et al., 2003; Li et al., 2005), activated Rac1 and Cdc42 are not sufficient to induce T cell polarity. In contrast, expression of constitutively active Rap1A (V12Rap1) induced a polarized phenotype in ~75% of the BW5147 T lymphoma cells, as determined by morphological changes and the localization of CD44 in the uropod (Fig. 1 A). A wild-type (WT) form of Rap1A (WTRap1) was unable to induce T cell polarization in BW5147 cells (Fig. 1 A), indicating that the activity of Rap1 was necessary to induce the polarization process. To confirm that V12Rap1 expression induces a fully polarized phenotype, we investigated the localization of additional proteins reported to be restricted either to the leading edge or to the uropod during chemokine-induced T cell polarization. Talin, CXCR4, and LFA-1, were present at the leading edge of

V12Rap1-expressing T cells, and were excluded from the uropod, where CD44 and ezrin specifically accumulated (Fig. 1 B). These results confirm that V12Rap1 expression is sufficient to induce T cell polarization. Interestingly, V12Rap1 was not uniformly distributed at the plasma membrane, but was strongly enriched at the leading edge, where it colocalized with CXCR4 (Fig. 1 C), suggesting that V12Rap1 locally initiates downstream signaling pathways required for T cell polarization. Because Rap1 is activated by chemokines within seconds (Shimonaka et al., 2003), our results suggest that local Rap1 activation is one of the key events initiating T cell polarization. We therefore used V12Rap1-expressing T lymphoma cells as a model to study the biochemical events leading to T cell polarization.

Intracellular localization of Par polarity proteins in polarized T cells

Par3 of the Par polarity complex is asymmetrically localized in the uropod-containing murine T-cell line MD45 (Ludford-Menting et al., 2005). Therefore, we analyzed if proteins of the Par polarity complex and Cdc42, which is a major activator of the Par polarity complex, also differentially localize in polarized V12Rap1-expressing BW5147 T lymphoma cells. Confocal analysis showed that Par3, PKCζ, and Cdc42 were uniformly distributed in nonpolarized control cells (Fig. 2 A and not depicted). In polarized V12Rap1-expressing cells, Cdc42, Par3, and PKCζ were devoid from the uropod where CD44 accumulates, and colocalized at the leading edge where CXCR4 is present (Fig. 2 A). RhoA was not differentially localized in polarized cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200608161/DC1). Moreover, V12Rap1 colocalized with the Par polarity proteins at the leading edge (Fig. S2). These data suggest that Par polarity proteins may function in V12Rap1-induced T cell polarization.

Rap1 activates Cdc42 and the Par polarity complex

To test whether Rap1 can activate Cdc42 and the Par polarity complex in T cells, we investigated if exogenous expression of V12Rap1 in BW5147 T lymphoma cells modifies the activation status of Cdc42 and the Par polarity complex. Expression of V12Rap1 in BW5147 cells enhanced Cdc42 activation compared with untransfected control cells (Fig. 2 B), indicating that Rap1

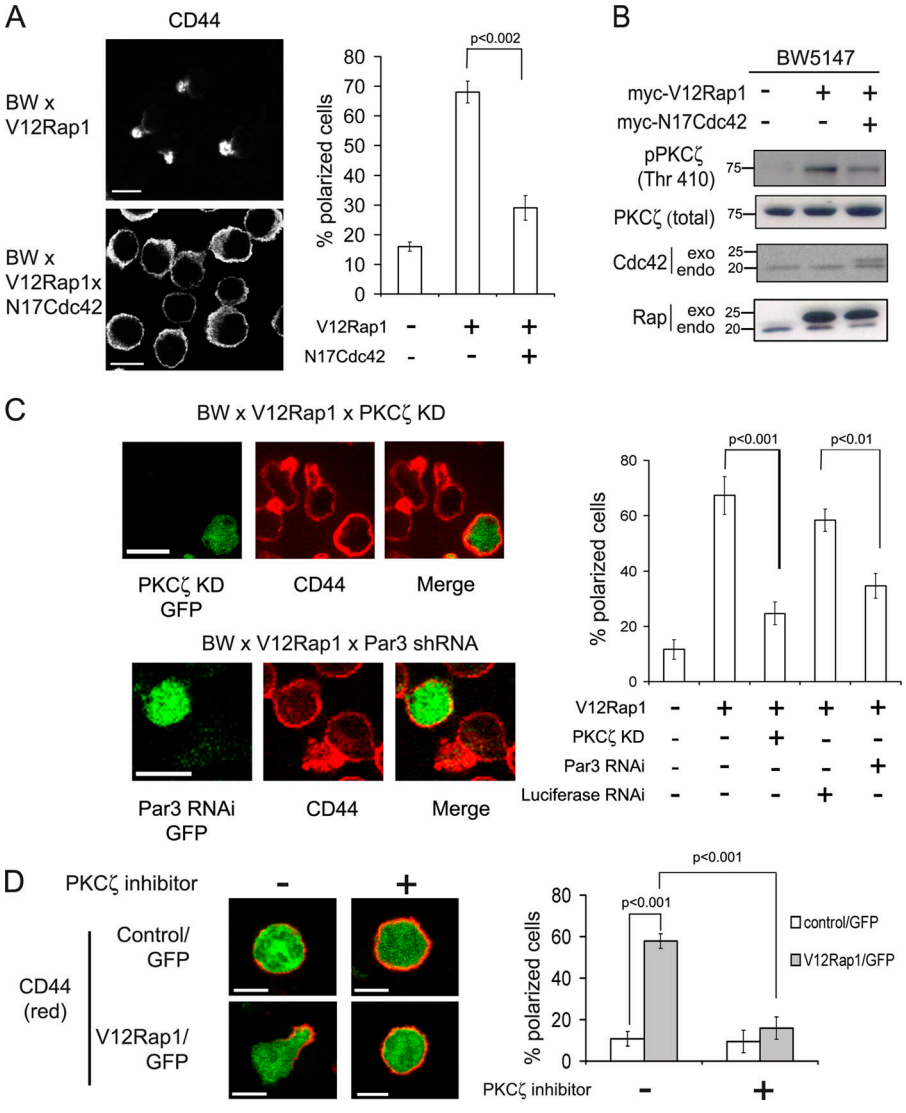

Figure 3. **Activation of Cdc42 and PKCζ is necessary for V12Rap1-induced T cell polarity.** (A) V12Rap1-expressing BW5147 T lymphoma cells were retrovirally transduced with dominant-negative N17Cdc42. Control and transduced cells were fixed in suspension and stained for CD44. Histogram indicates the percentage of polarized cells of three independent experiments. (B) Lysates of the cells described in A were separated by SDS-PAGE and analyzed for total PKCζ and PKCζ phosphorylation on Thr410 residue. (top) Phosphorylated PKCζ and total PKCζ. (bottom) Immunoblots of expressed N17Cdc42 and V12Rap1 (exo, exogenous; endo, endogenous). Sizes are indicated in kilodaltons. (C) Expression kinase-dead PKCζ and knockdown of Par3 inhibit V12Rap1-induced T cell polarization. V12Rap1-expressing BW5147 T lymphoma cells were retrovirally transduced with a kinase-dead mutant of PKCζ (PKCζKD-IRES-GFP), luciferase shRNA, or Par3shRNA (Par3sh-IRES-GFP). 72 h after infection, cells were fixed in suspension and stained for CD44. Polarization was determined in control and GFP-expressing cells. Histogram indicates the percentage of polarized cells from three independent experiments. (D) T lymphocytes derived from FVB mice were retrovirally transduced with a control vector encoding GFP alone or with V12Rap1-IRES-GFP. 48 h after infection, cells were treated with 2 μM PKCζ pseudosubstrate for 1 h and stained for CD44. Histogram represents the quantification of polarized cells from three independent experiments. Error bars indicate the SD; P indicates the P value. Bars: (A and C) 10 μm; (D) 5 μm.

is able to activate Cdc42. Atypical PKCζ is a key protein in the Par polarity complex, and its activation by phosphorylation controls Par-mediated cellular polarization (Suzuki et al., 2002). To determine the activation state of the Par polarity complex, we analyzed the phosphorylation status of PKCζ on Thr410 in polarized and nonpolarized BW5147 cells. As shown in Fig. 2 C, PKCζ phosphorylation was increased in polarized V12Rap1-expressing BW5147 cells when compared with nonpolarized cells, indicating that V12Rap1 activates the Par polarity complex, and thereby PKCζ, in polarized T cells. Because activated PKCζ is localized at the plasma membrane in contrast to non-activated PKCζ (Chou et al., 1998), we also determined the intracellular localization of PKCζ by a biochemical fractionation method. Consistent with the increased phosphorylation observed upon polarization, we found that PKCζ was enriched in the membrane fraction of polarized V12Rap1-expressing BW5147 T lymphoma cells when compared with nonpolarized cells (Fig. 2 D). Together, these data indicate that constitutively active V12Rap1 leads to the activation of Cdc42 and the Par polarity complex, as determined by the activation and membrane translocation of PKCζ.

Cdc42 and the Par polarity complex are required for Rap1-induced T cell polarity

To investigate whether the activation of Cdc42 has a functional effect in V12Rap1-induced T cell polarity, we inhibited Cdc42 activity in BW5147 cells. As shown in Fig. 3 A, expression of dominant-negative N17Cdc42 reduced the number of polarized V12Rap1-expressing cells from 70 to ∼30%. This indicates that Cdc42 activity is required for V12Rap1-induced T cell polarization. To investigate the hierarchical activation of Cdc42 and the Par polarity complex in V12Rap1-expressing cells, we also analyzed the phosphorylation status of PKCζ in the presence of N17Cdc42. We found decreased PKCζ phosphorylation in the nonpolarized cells coexpressing V12Rap1 and N17Cdc42 compared with polarized V12Rap1–expressing cells (Fig. 3 B). This suggests that Cdc42 activates the Par polarity complex, leading to T cell polarization. Indeed, expression of Par3 shRNA, which reduced the Par3 protein levels to ∼50% (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200608161/DC1), impaired V12Rap1-induced T cell polarization when compared with cells expressing control shRNA (Fig. 3 C). In addition, inhibition of PKCζ downstream signaling by the expression of a kinase-dead mutant of PKCζ (PKCζKD) in BW5147 cells (Fig. 3 C), or by a myristoylated PKCζ pseudosubstrate (PKCζ inhibitor; Standaert et al., 1997) in primary T lymphocytes (Fig. 3 D), abrogated V12Rap1-induced polarity in both BW5147 cells and primary T cells. From these data, we conclude that V12Rap1 activates Cdc42, leading to the activation of the Par polarity complex that is required for the establishment of T cell polarity.

Rac activity is required downstream of the Par polarity complex during T cell polarization

The Par complex is known to control changes in the actin and microtubule cytoskeleton that are required for cell polarization. The Rho GTPase Rac controls actin cytoskeleton remodeling during various processes, including cell migration and cell polarization (Ridley et al., 2003; Etienne-Manneville, 2004). Because T cell polarization is dependent on actin remodeling (Serrador et al., 1999), it is likely that Rac has a function in V12Rap1-induced T cell polarization. Indeed, expression of V12Rap1 or PKCζ enhanced Rac activity in BW5147 T lymphoma cells (Fig. 4 A). Most importantly, V12Rap1-mediated Rac activation could be inhibited by the expression of dominant-negative Cdc42 (Fig. 4 B) or PKCζKD (Fig. 4 C). These data indicate that Rap1 regulates Rac activity through the Cdc42–Par–PKCζ pathway, and that Rac acts downstream of PKCζ to mediate T cell polarization. Indeed, N17Rac1 expression inhibited T cell polarization in BW5147 cells induced by

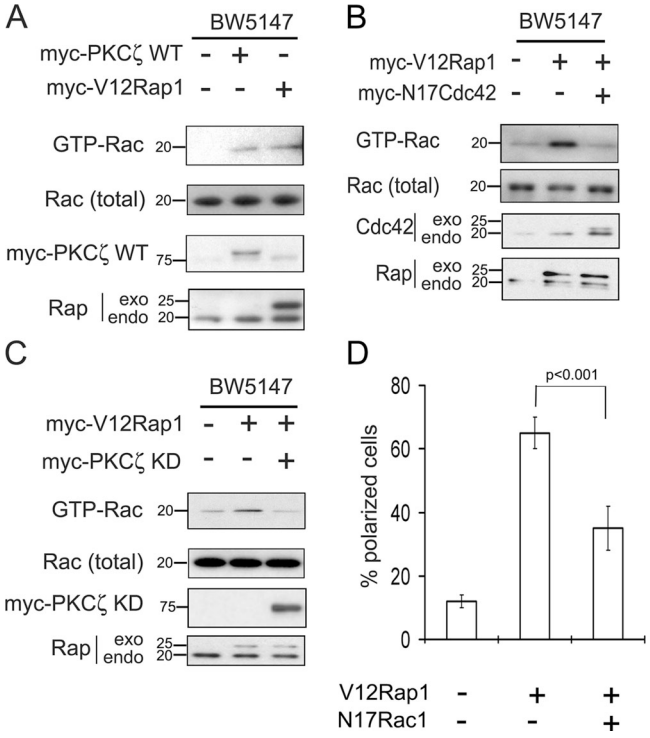

Figure 4. **Rac activity is necessary for V12Rap1-induced polarity.** (A) V12Rap1 and WT-PKCζ activate Rac. Pulldown Rac activity assays were performed with starved control, V12Rap1-, and WT-PKCζ-expressing BW5147 T lymphoma cells. (top) Activated GTP-bound Rac and total Rac. (bottom) Immunoblots of expressed WT-PKCζ and V12Rap1 (exo, exogenous; endo, endogenous). (B) Dominant-negative Cdc42 inhibits V12Rap1-induced Rac activation. V12Rap1-expressing BW5147 T lymphoma cells were retrovirally transduced with dominant-negative Cdc42 (N17Cdc42). 4 d after infection, cells were starved for 16 h and Rac activity was determined. (top) Activated GTP-bound Rac and total Rac. (bottom) Immunoblots of the Cdc42 and V12Rap1 proteins. (C) Kinase-dead PKCζ inhibits V12Rap1-induced Rac activation. V12Rap1-expressing BW5147 T lymphoma cells were retrovirally transduced with a kinase-dead PKCζ (PKCζ KD). 4 d after infection, cells were starved for 16 h and Rac activity was determined. (top) Activated GTP-bound Rac and total Rac. (bottom) Immunoblots of the expressed kinase-dead PKCζ and V12Rap1 proteins. Sizes are indicated in kilodaltons. (D) Dominant-negative N17Rac1 inhibits V12Rap1-induced T cell polarization. V12Rap1-expressing BW5147 T lymphoma cells were retrovirally transduced with N17Rac1 or an empty vector as a control, selected for 3 d, and subsequently analyzed by immunohistochemistry using CD44-specific antibody. Histogram represents the percentage of polarized cells from three independent experiments. Error bars indicate the SD; P indicates the P value.

V12Rap1 (Fig. 4 D), demonstrating that Rac activity is required for V12Rap1-induced polarity in T lymphoma cells. Therefore, we conclude that Rac is activated downstream of the Par polarity complex and PKCζ to mediate the actin remodeling required for the polarization of T cells induced by V12Rap1.

Tiam1 interacts with both Rap1 and the Par polarity complex

Tiam1 is a GEF that specifically activates Rac (Michiels et al., 1995). Tiam1 has recently been shown to act in conjunction with the Par polarity complex in the establishment of neuronal and epithelial cell polarity (Chen and Macara, 2005; Mertens et al., 2005; Nishimura et al., 2005; Mertens et al., 2006). Because Tiam1 is also able to interact with Rap1 in fibroblasts (Arthur et al., 2004), we hypothesized that Tiam1 could control V12Rap1-induced Rac activation in T cells. Interestingly, GST pulldown experiments show that endogenous Tiam1 interacts with activated V12Rap1, but not with WTRap1 (Fig. 5 A), suggesting that the Tiam1–Rap1 interaction is dependent on the activation state of Rap1. Similarly, coimmunoprecipitation experiments show that endogenous Tiam1 interacts with V12Rap1 in polarized T lymphoma cells (Fig. 5 B). Immunoprecipitation of

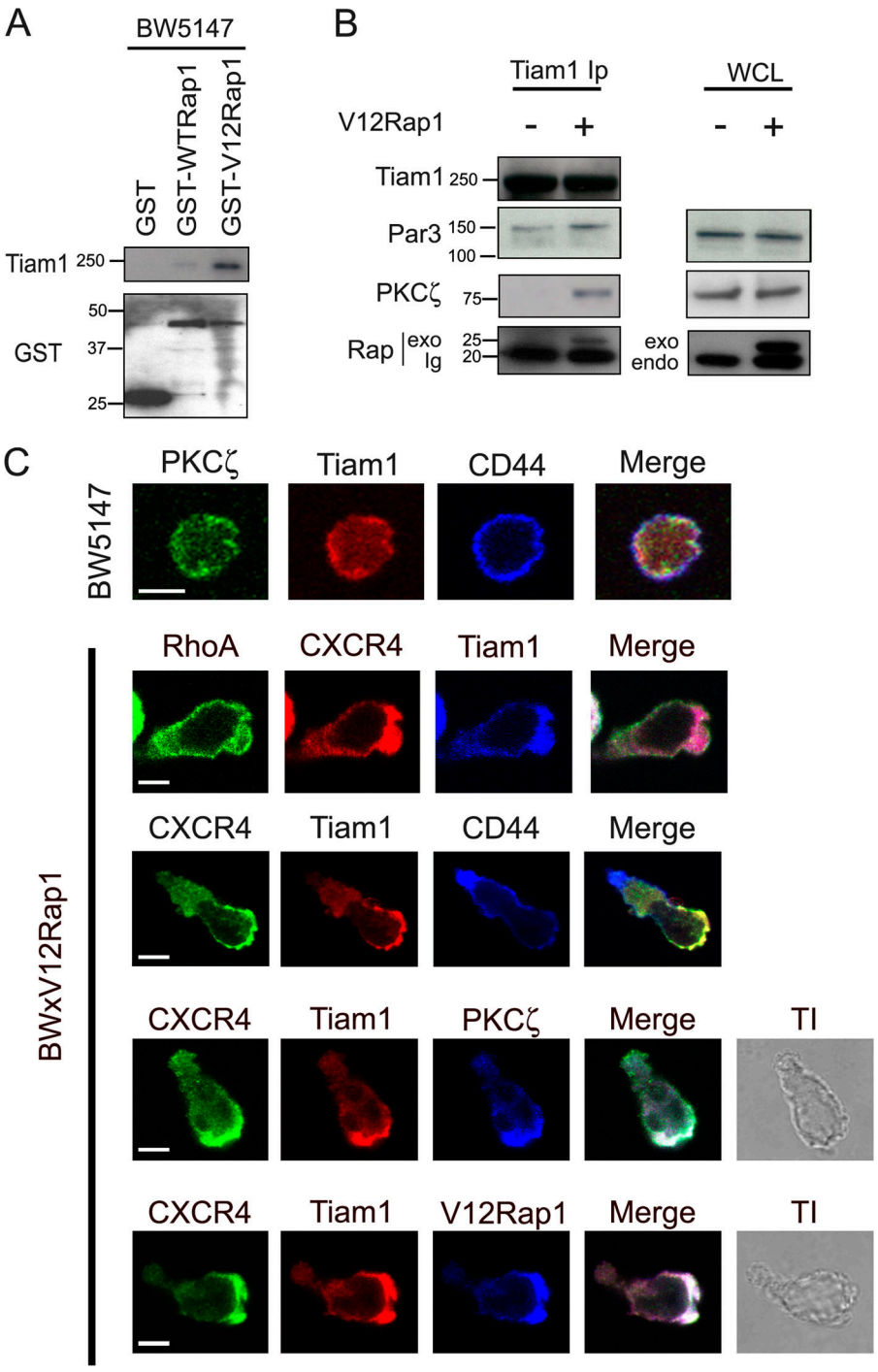

Figure 5. **V12Rap1 interacts with Tiam1 and mediates the association of Tiam1 with the Par polarity complex.** (A) V12Rap1, but not WTRap1, associates with endogenous Tiam1. GST pulldown in lysates of BW5147 T lymphoma cells with purified GST (control), GST-WTRap1, or GST-V12Rap1. Pulldowns were separated by SDS-PAGE, and proteins were identified by Western blotting using Tiam1- and GST-specific antibodies. Sizes are indicated in kilodaltons. (B) Tiam1 associates with Par3, activated PKCζ, and Rap1. Tiam1 was immunoprecipitated from starved control and V12Rap1-expressing BW5147 T lymphoma cells. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting for Tiam1 and coimmunoprecipitated proteins (Par3, PKCζ, and Rap1). (right) Total lysates (exo, exogenous; endo, endogenous). Sizes are indicated in kilodaltons. (C) Intracellular localization of PKCζ and Tiam1. Control and polarized V12Rap1-expressing BW5147 T lymphoma cells were stained for CXCR4, V12Rap1, CD44, RhoA, PKCζ, and Tiam1. TI, transmission images. Bar, 5 μm.

endogenous Tiam1 revealed that Par3 associates with Tiam1 irrespective of the presence of V12Rap1 (Fig. 5 B). Interestingly, Tiam1 interacts with PKCζ only in polarized V12Rap1-expressing T cells, suggesting that Tiam1 is associated with the activated Par complex during T cell polarization. Indeed, Tiam1 colocalizes with PKCζ and V12Rap1 in the front of polarized V12Rap1-expressing cells, whereas it is homogenously distributed in nonpolarized T cells (Fig. 5 C). From these data, we conclude that Tiam1 interacts with V12Rap1 and components of the Par polarity complex, and may thereby have a function in connecting the Par complex and Rac activity at sites where Rap1 is activated and cell polarization is initiated.

Tiam1-mediated Rac activation is required for Rap1-induced polarization of T cells

To investigate whether Tiam1-mediated Rac activation is essential for V12Rap1-induced T cell polarization, we used a dominant-negative mutant of Tiam1, corresponding to the PHnCCex domain of Tiam1 (Stam et al., 1997). This mutant lacks the catalytic DH domain, still contains the Par3 interaction domain, and inhibits Tiam1-mediated Rac activation (Stam et al., 1997). Expression of dominant-negative Tiam1 in BW5147 T lymphoma inhibits both V12Rap1- induced Rac activation (Fig. 6 A) and cell polarization (Fig. 6 B). These results demonstrate that Tiam1 not only associates with Rap1 and the Par polarity complex but is also essential for the Rac activation required for Rap1-induced T cell polarization.

To further substantiate the function of Tiam1 in V12Rap1-induced T cell polarization, we used primary lymphocytes isolated from WT (Tiam1+/+) and Tiam1 knockout (Tiam1−/−) mice (Malliri et al., 2002). V12Rap1-IRES-GFP was transduced into lymphocytes of both genotypes, and the degree of cell polarization was analyzed in the GFP-expressing cells. V12Rap1 induced cell polarization in ∼80% of the GFP-positive Tiam1+/+ T lymphocytes, whereas Tiam1-deficient (Tiam1−/−) cells showed only background polarization (∼20%) as found in nontransduced cells (Fig. 6 C). These data demonstrate that Tiam1 is also required for V12Rap1-induced cell polarization in normal T lymphocytes.

Chemokine-induced activation of the Par complex is dependent on Rap1

The polarized characteristics induced by chemokines and V12Rap1 expression are indistinguishable in terms of morphology and cell surface receptor expression (Shimonaka et al., 2003). Therefore, we investigated whether Rap1 and the Par polarity complex also function in chemokine-induced T cell polarization. First, we analyzed the activation kinetics of Rap1, Cdc42, the Par complex, and Rac1 in primary T lymphocytes upon chemokine stimulation. As shown in Fig. 7 A, Rap1, Cdc42, PKCζ, and Rac1 are rapidly and transiently activated after stromal cell–derived factor-1 α (SDF1α) stimulation. Similar results were found in chemokine-treated Jurkat cells (unpublished data). Upon SDF1α stimulation of Jurkat cells, WTRap1A is recruited to the leading edge (Fig. 7 B), suggesting that upon activation it induces polarity by activation of the Par polarity complex at specific sites in T cells. These data are consistent with the intracellular localization of V12Rap1 in polarized BW5147 T lymphoma cells (Fig. S2). To further substantiate that Rap1 activates the Par polarity complex during chemokine stimulation, Rap1 downstream signaling was inhibited by the expression of Rap1-GAP. As shown in Fig. 7 C,

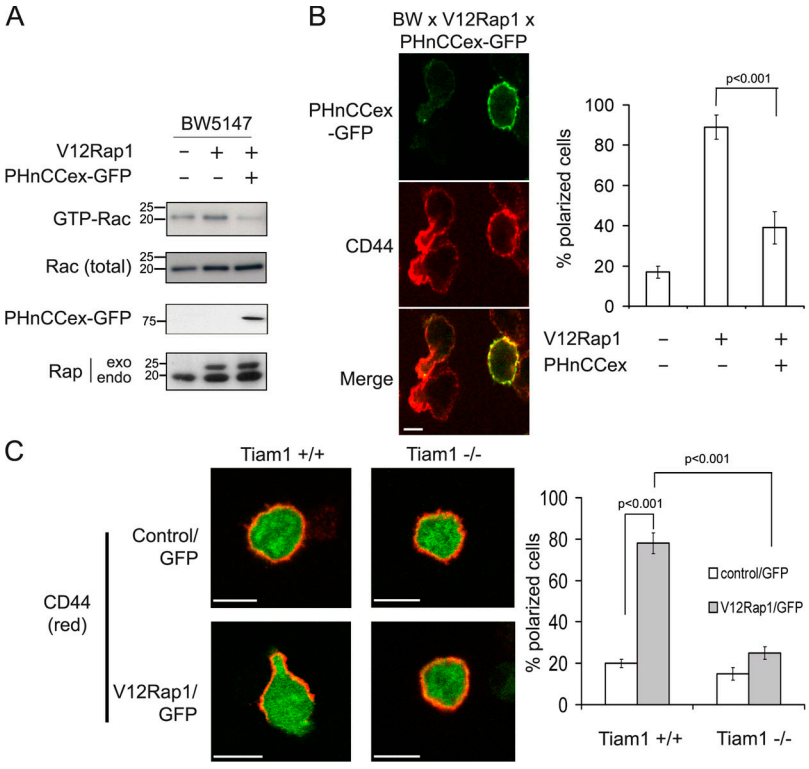

Figure 6. **Tiam1-induced Rac activity is required for induction of T cell polarity.** Dominant-negative Tiam1 (PHnCCex) inhibits V12Rap1-induced Rac activation (A) and cell polarization (B). (A) V12Rap1-expressing BW5147 T lymphoma cells were retrovirally transduced with PHnCCex fused to GFP. 4 d after infection, cells were starved for 16 h and Rac activity was determined. (top) Activated GTP-bound Rac and total Rac. (bottom) Immunoblots of expressed proteins (PhnCCex and Rap1; exo, exogenous; endo, endogenous). Sizes are indicated in kilodaltons. (B) Cells, as described in A, were fixed in suspension for 72 h after infection and stained for CD44. Histogram represents the percentage of polarized cells of three independent experiments. (C) Tiam1−/− T lymphocytes are impaired in V12Rap1-induced cell polarization. T lymphocytes derived from Tiam1+/+ and Tiam1−/− mice were retrovirally transduced with a control vector encoding GFP alone or with V12Rap1-IRES-GFP. 48 h after infection, cells were fixed in suspension and stained for CD44. Histogram represents the quantification of polarized cells of three independent experiments. Error bars indicate the SD; P represents the P value. Bars, 5 μm.

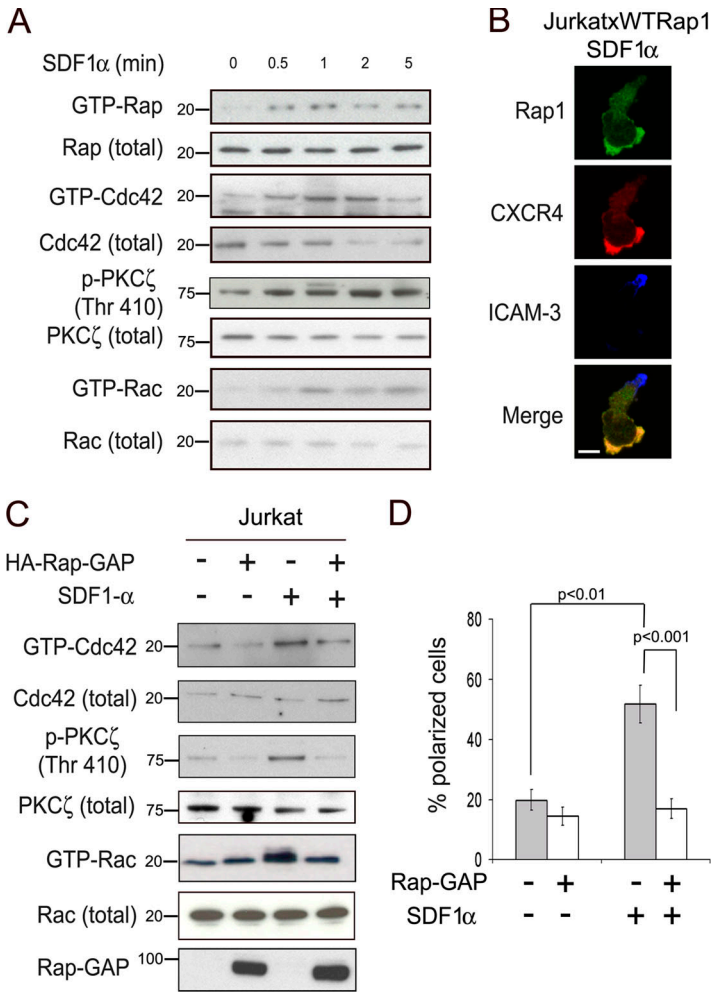

Figure 7. **Rap1 is a major activator of the Par complex upon chemokine stimulation.** (A) Rap1 and the Par complex are activated by SDF1α. Lymphocytes from FVB mice were either nonstimulated or stimulated with 500 ng/ml SDF1α for the indicated time. Subsequently, Rap activity, Cdc42 activity, Rac activity, and PKCζ phosphorylation status were determined. (top) GTP-bound Rap and total Rap. (middle) GTP-bound Cdc42, total Cdc42, phosphorylated PKCζ, and total PKCζ. (bottom) GTP-bound Rac and total Rac. Sizes are indicated in kilodaltons. (B) Rap1 is localized at the leading edge upon chemokine-induced polarity. Myc-WTRap1A–expressing Jurkat cells were stimulated with 200 ng/ml SDF1α for 20 min, and then stained for CXCR4, ICAM-3, and myc. Bar, 5 μm. (C) Rap activity is necessary for chemokine-induced activation of Cdc42, PKCζ, and Rac. Myc-WTRap1A–expressing Jurkat cells were stimulated with 500 ng/ml SDF1α for 2 min. Subsequently, Cdc42 activity, Rac activity, and PKCζ phosphorylation status were determined. (top) GTP-bound Cdc42 and total Cdc42; (middle) phosphorylated PKCζ and total PKCζ; (bottom) GTP-bound Rac, total Rac, and Rap1-GAP. Sizes are indicated in kilodaltons. (D) Rap1-GAP–expressing Jurkat cells or control cells were stimulated for 20 min with SDF1α. Subsequently, the percentage of polarized cells was determined by ICAM3 staining and changes in morphology. Histogram represents the quantification of polarized cells from three independent experiments. Error bars indicate the SD; P represents the P value.

Rap1-GAP inhibited chemokine-induced activation of Cdc42, PKCζ, and Rac in Jurkat T cells. Consistent with these findings, chemokine-induced polarity was strongly impaired in cells expressing Rap1-GAP (Fig. 7 D). From these results, we conclude that Rap1 activity is required for both chemokine-induced T cell polarization and activation of the Par polarity complex.

Tiam1 in conjunction with the Par polarity complex regulates chemokine-induced T cell polarization and chemotaxis

To confirm that Rap-induced activation of the Par polarity complex is necessary for chemokine-induced T cell polarity, we investigated the function of the Par polarity complex and its downstream effector Tiam1 in T cell polarization. First, we analyzed the function of Tiam1 in Rac activation and T cell polarity induced by the chemokine SDF1α. Interestingly, stimulation with SDF1α induced an increase in Rac activation in Tiam1+/+, but not in Tiam1−/−, lymphocytes (Fig. 8 A), indicating that Tiam1 is necessary for SDF1α-induced Rac activation. These data are consistent with the requirement of Tiam1 for Rac activation induced by V12Rap1 (Fig. 6 A). We also determined the SDF1α-induced degree of T cell polarization in Tiam1+/+ and Tiam1–/– T lymphocytes. As shown in Fig. 8 B, SDF1α-induced polarity was ∼50% reduced in Tiam1–/– T cells compared with Tiam1+/+ T cells. Comparable results were found using the secondary lymphoid tissue chemokine (unpublished data). From these data, we conclude that Tiam1-mediated Rac activation controls, to a large extent, the chemokine-induced polarization of T cells. To functionally test the effect of Tiam1 in chemokine-induced T cell polarity, we analyzed the chemotactic migration capacity of T cells of both genotypes in response to SDF1α using a Boyden chamber assay. Tiam1–/– T cells showed ∼50% reduction in their chemotactic response to different concentrations of SDF1α compared with Tiam1+/+ T cells (Fig. 8 C).

Based on our findings in V12Rap1-induced polarity in T lymphoma cells, we also analyzed the association of Tiam1 and the Par polarity complex in primary T lymphocytes upon chemokine stimulation. Similarly, as found for V12Rap1-induced polarization of T lymphoma cells (Fig. 5 B), SDF1α stimulation of primary T cells promoted Tiam1–PKCζ association, whereas interaction between Tiam1 and Par3 was independent of SDF1α (Fig. 9 A). In addition, Tiam1 and PKCζ of the Par complex colocalized at the leading edge of SDF1α-stimulated primary T cells (Fig. 9 B). Interestingly, Tiam1-deficiency did not inhibit the activation of PKCζ induced by SDF1α (Fig. 9 C), whereas inhibition of PKCζ signaling in Tiam1+/+ T lymphocytes inhibited SDF1α-induced Rac activation (Fig. 9 D).

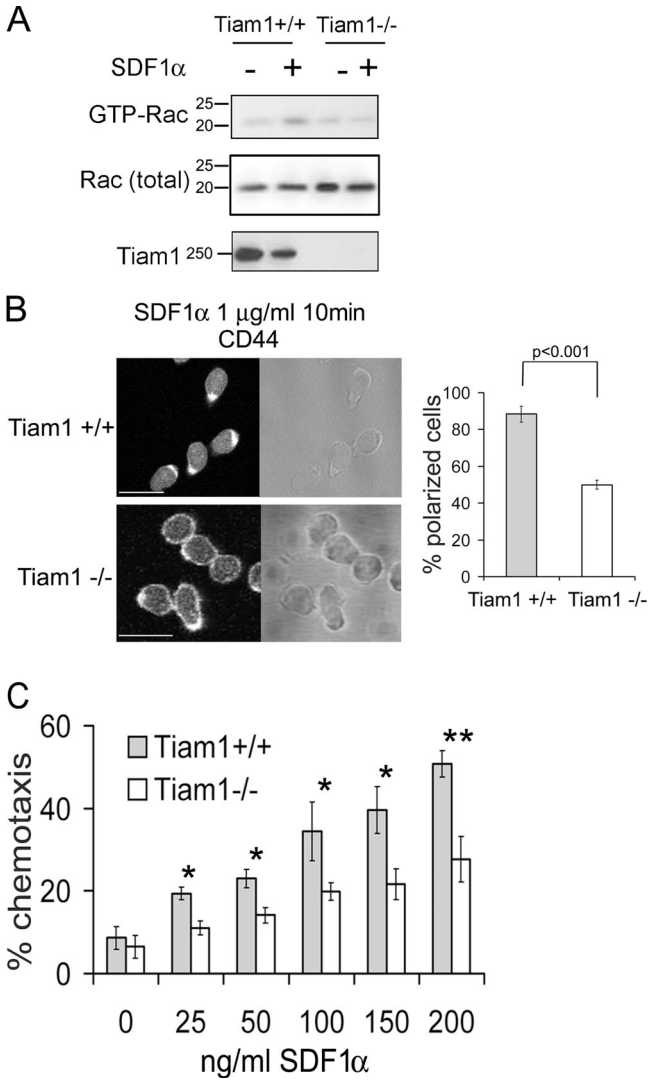

Figure 8. **Tiam1 is necessary for chemokine-induced T cell polarization and chemotaxis.** (A) Rac activation is impaired in Tiam1−/− T lymphocytes upon stimulation with SDF1α. Lymphocytes from Tiam1+/+ and Tiam1−/− mice were either nonstimulated or stimulated with 500 ng/ml SDF1α for 1 min. Subsequently, Rac activity status was determined. (top) GTP-bound Rac and total Rac. (bottom) Tiam1. Sizes are indicated in kilodaltons. (B) Chemokine-induced polarization is strongly reduced in Tiam1−/− lymphocytes. T lymphocytes from Tiam1+/+ and Tiam1−/− mice were stimulated with 1 μg/ml SDF1α for 10 min. Cells were fixed in suspension and stained for CD44. Histogram represents the quantification of polarized cells of four independent experiments. P represents the P value. (C) Chemotaxis is reduced in Tiam1−/− lymphocytes. T lymphocytes from Tiam1+/+ and Tiam1−/− mice were used in Transwell chemotaxis assays. Chemotaxis was measured after 1 h using different concentrations of SDF1α. Results are presented as the percentage of the input cells and are derived from three independent experiments. Error bars indicate the SD. P values are the comparison of the chemotactic capacity of Tiam1+/+ and knockout cells toward the same concentration of SDF1α (*, $P < 0.05$; **, $P < 0.01$).

These findings confirm our earlier conclusion that Tiam1 activates Rac downstream of PKCζ and the Par polarity complex.

Treatment of Tiam1+/+ T cells with PKCζ pseudosubstrate also inhibited SDF1α-induced T cell polarization (Fig. 9 E) and chemotaxis (Fig. 9 F). Intriguingly, the inhibition of PKCζ signaling did not alter the residual 50% polarization and chemotactic migration capacity of Tiam1−/− T lymphocytes (Fig. 9, E and F), indicating that Tiam1 and the Par polarity complex function in the same signaling pathway during chemokine-induced T cell polarization and chemotaxis. Apparently, residual chemokine-induced polarization and chemotaxis, as found in the primary Tiam1−/− T cells, are not dependent on the Par polarity complex.

Discussion

Rap1 and its effector protein RAPL function in chemokine-induced integrin activation (Carman and Springer, 2003), migration, and polarization of T cells (Katagiri et al., 2003; Shimonaka et al., 2003), but how these processes are regulated is unknown. In this study, we show that the Par polarity complex, in conjunction with Tiam1 and Rac, regulates both Rap1-induced T cell polarization and chemokine-induced polarization and migration of primary T cells.

We found that Rap1 triggers polarity by activating Cdc42, which in turn activates the Par polarity complex. Activation of PKCζ through the Par polarity complex leads to the activation of Rac via Tiam1 (Fig. 10). Because V12Rap1-induced Rac activation is inhibited by dominant-negative N17Cdc42 and kinase-dead PKCζ, it is unlikely that V12Rap1 is able to activate Rac directly in T cells, as has been reported in fibroblasts (Arthur et al., 2004). In lymphocytes, Tiam1 mediates V12Rap1-induced Rac activation as a result of the activation of Cdc42 and the Par polarity complex.

Although we show that Cdc42 and Rac are required for T cell polarization, constitutively active V12Cdc42 or V12Rac1 are not sufficient to induce polarization, in contrast to constitutively active V12Rap1. Apparently, specific properties of Rap1 are required to initiate T cell polarity. Similar to Rap1, other proteins that are capable of inducing T cell polarity (i.e., RAPL and Mst1) have been implicated in inside-out signaling of integrins, and are localized in small vesicles in nonstimulated cells (Katagiri et al., 2003, 2006). Upon stimulation, these proteins translocate to a specific site at the plasma membrane, specifically at the future leading edge (Katagiri et al., 2006). Indeed, we found that WTRap1 is present in the leading edge of chemokine-induced polarized T cells upon SDF1α stimulation, where it colocalizes with the proteins of the Par polarity complex (Fig. 7 B).

During axon specification in neuronal cells, Rap1B acts as the primary cue upstream of the Par polarity complex and defines which of the growing neurites becomes the future axon (Schwamborn and Puschel, 2004). The striking similarity by which Rap1 in conjunction with the Par polarity complex determines polarization of T cells (as shown in this study) and axon specification in neuronal cells (Schwamborn and Puschel, 2004; Nishimura et al., 2005) suggests that Rap proteins are able to recruit the Par polarity complex in various cellular systems, and thereby controls the initiation of cell polarity. We found that Rap1, through activation of Cdc42, not only localizes but also activates the Par polarity complex and Rac through Tiam1. Because Tiam1 associates with both activated Rap1 and Par3 (Fig. 10), Tiam1 may also function as a scaffold protein that couples activated Rap1 to the Par polarity complex. In fact, in fibroblasts it has been shown that Rap1 promotes cell spreading

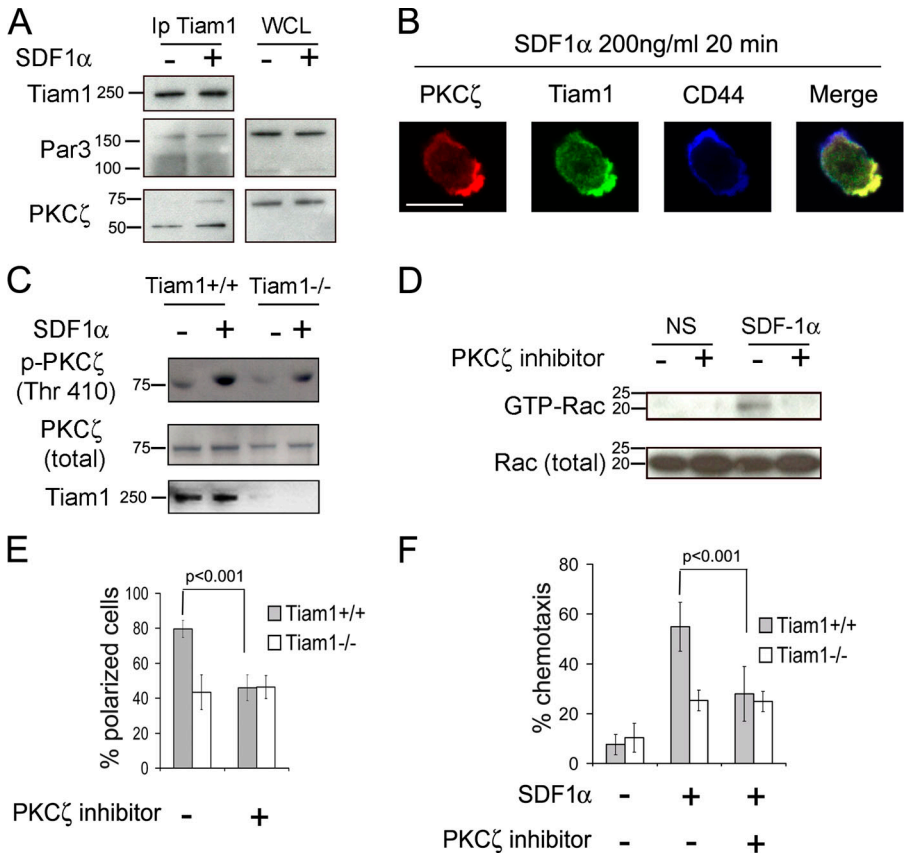

Figure 9. **Tiam1 cooperates with the Par complex in chemokine-induced T cell polarization and chemotaxis.** (A) Tiam1 and the Par complex coimmunoprecipitate after SDF1α stimulation. T lymphocytes from FVB mice were either not stimulated or treated with SDF1α for 3 min. Tiam1 was immunoprecipitated from the cell lysates. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting for Tiam1 and coimmunoprecipitated proteins (Par3 and PKCζ). (right) Total lysates. (B) Colocalization of Tiam1 and PKCζ in chemokine-stimulated T lymphocytes. Lymphocytes from Tiam1+/+ mice were stimulated for 20 min with 200 ng/ml SDF1α and stained for CD44, PKCζ, and Tiam1. Bar, 5 μm. (C) PKCζ activation is not impaired in Tiam1−/− T lymphocytes upon stimulation with SDF1α. Lymphocytes from Tiam1+/+ and Tiam1−/− mice were either nonstimulated or stimulated with 500 ng/ml SDF1α for 3 min. Subsequently, PKCζ phosphorylation status was determined. (top) Phosphorylated PKCζ and total PKCζ. (bottom) Tiam1. (D) PKCζ signaling is necessary for chemokine-induced Rac activation. Lymphocytes from FVB mice were either not treated or treated with 2 μM PKCζ pseudosubstrate for 1 h and stimulated with 500 ng/ml SDF1α for 3 min. Subsequently, Rac activity status was determined. (top) GTP-bound Rac. (bottom) Total Rac. PKCζ pseudosubstrate alters the polarization (E) and chemotactic capacity (F) of Tiam1+/+, but not Tiam1−/−, lymphocytes. (E) Lymphocytes of both genotypes were either nontreated or treated with 2 μM PKCζ pseudosubstrate for 1 h and stimulated with 1 μg/ml SDF1α for 10 min. Cells were fixed and stained for CD44. Histogram represents the quantification of polarized cells of four independent experiments. (F) Chemotaxis of Tiam1+/+ and Tiam1−/− lymphocytes either treated or untreated with 2 μM PKCζ pseudosubstrate was measured in response to 200 ng/ml SDF1α after 1 h. The results are presented as the percentage of the input cells and are based on four independent experiments. Sizes are indicated in kilodaltons. Error bars indicate the SD; P represents the P value.

by binding Tiam1, thereby localizing Rac activity at specific sites in cells (Arthur et al., 2004). Moreover, we found that Tiam1 associates with active, but not inactive, Rap1. Based on these data, it is tempting to speculate that upon a polarization signal in lymphoid cells, Rap1 is activated at a specific site of the plasma membrane and recruits Tiam1 and the Par polarity complex to initiate polarity. Rap1 activates Cdc42 through an associated unknown GEF. Activated Cdc42 binds to Par6 (Joberty et al., 2000; Lin et al., 2000), which leads to activation of the Par polarity complex, including PKCζ, and, subsequently, to activation of Rac through Tiam1. Within this scenario, Tiam1 may have two functions; i.e., to connect the Par polarity to Rap1 at the site where T cell polarity is initiated and to activate Rac downstream of the Par polarity complex to achieve the actin remodeling required for T cell polarization. Consistent with this, it has been shown that PKCζ is able to phosphorylate Tiam1 (Fleming et al., 1997), which could regulate Tiam1 activity. The fact that we identified Tiam1 as a gene that promotes infiltration of T lymphoma cells into fibroblast monolayers (Habets et al., 1994) might be explained by its function in polarity signaling in lymphoid cells. In fact, we have shown that increased Tiam1/Rac signaling promotes the infiltration of T lymphoma cells into fibroblast monolayers in a polarized fashion (Stam et al., 1998).

Our data provide new insights into the mechanisms by which chemokine-mediated polarity is established. However, it is likely that additional signaling pathways play a role. In fact, the incomplete inhibition of SDF1α-induced polarization in T cells lacking Tiam1 or PKCζ activity suggests that other polarity complexes contribute to chemokine-induced T cell polarization. Tiam1–/– mice develop, grow, and reproduce normally (Malliri et al., 2002; unpublished data). In addition, no obvious defects have been found in mice deficient for the Par polarity protein PKCζ, except for a small delay in the formation of secondary lymphoid organs (Leitges et al., 2001; Martin et al., 2002). Apparently, both in vitro and in vivo, other polarization pathways contribute to the polarization process of T cells and/or can overcome the deficiency of the Par3–PKCζ–Tiam1 pathway. Indeed, proteins of the Scribble and Crumbs polarity complexes have been found asymmetrically distributed in polarized T cells (Ludford-Menting et al., 2005), suggesting that they also contribute to T cell polarization. Dlg and Scribble control uropod formation in the uropod-containing T-cell line MD45, and regulate asymmetric distribution of proteins in T cells (Ludford-Menting et al., 2005). In addition, other Rac-specific GEFs have been shown to function in T cell polarization. Deregulation of Vav1-signaling by the expression of a dominant-active or -negative mutant of Vav1 reduces chemokine-induced T cell

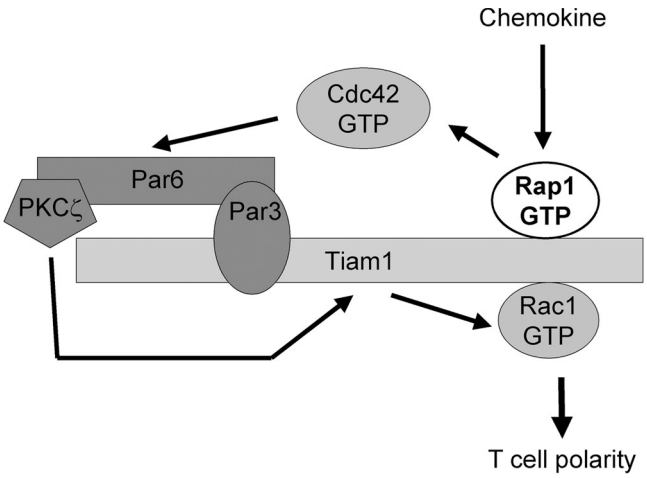

Figure 10. **The Par polarity complex functions in Rap1- and chemokine-induced T cell polarization.** Chemokines are able to activate Rap1 at a specific site on the cell, which is considered as the initiating event for the establishment of T cell polarity. Activated Rap1 recruits the Par polarity complex, which consists of Par3, Par6, and PKCζ. Rap1 activates Cdc42, which is able to activate the Par polarity complex, including PKCζ. The polarity complex subsequently activates Tiam1, and thereby Rac1. Tiam1 associates with active, but not inactive, Rap1 and with components of the Par complex, and may thereby function to connect the Par polarity to Rap1 at the site where T cell polarity is initiated. Furthermore, Tiam1 is required to activate Rac downstream of the Par complex, presumably to regulate actin remodeling required for T cell polarization. Arrows indicate activation steps.

polarization (Vicente-Manzanares et al., 2005). However, T cell polarization is normal in Vav1-deficient T cells compared with Tiam1+/+ cells (Vicente-Manzanares et al., 2005), suggesting that other Vav proteins (e.g., Vav 2 or 3) or Tiam1 compensate for the loss of Vav1. As Vav2 has also been shown to associate with Rap1 in fibroblasts (Arthur et al., 2004), Vav proteins might also have a potential role in Rap1-induced polarity signaling. DOCK2 is an unconventional GEF for Rac that belongs to the CDM family (CED-5 in *Caenorhabditis elegans*, DOCK180 in man, and Myoblast in *Drosophila melanogaster*; Meller et al., 2005). In DOCK2-deficient mice, various defects in T lymphocytes have been described, including migration defects in response to chemokines, lymphocytopenia, and atrophy of lymphoid follicles (Fukui et al., 2001). Deletion of DOCK2 inhibits chemokine-induced formation of a bipolar shape (Nombela-Arrieta et al., 2004; Shulman et al., 2006), but the mechanism by which DOCK2 influences T cell polarization is unknown. Chemokine-induced Rap1 and PKCζ activation is not dependent on DOCK2 (Nombela-Arrieta et al., 2004). Moreover, V12Rap1 induces polarization in BW5147 T lymphoma cells that do not express DOCK2 (Fukui et al., 2001). These data exclude a function of DOCK2 in Par complex–mediated polarization of T cells. It is conceivable that at least two distinct pathways are required for chemokine-induced T cell polarization. One of these pathways involves DOCK2 through an unknown mechanism and the other pathway involves Rap1 and the Par polarity complex in conjunction with Rac activators such as Tiam1 or possibly Vav1–3. Other polarity complexes may also contribute to Rap1- or chemokine-induced polarization of T cells, as they have also a function in other Par complex-mediated polarization processes such as the establishment of epithelial apical–basal cell polarity (Margolis and Borg, 2005).

Collectively, our data implicate Tiam1 in conjunction with the Par polarity complex in Rap1- and chemokine-induced T cell polarization. To achieve T cell polarization, Rap1 is activated by chemokine stimulation leading to activation of Cdc42, and thereby of the Par polarity complex. Tiam1 associates with Rap1 and components of the Par complex and may function to connect the Par polarity to Rap1 at the site where T cell polarity is initiated. Furthermore, Tiam1 is required to activate Rac downstream of the Par complex, presumably to regulate actin remodeling required for T cell polarization.

Materials and methods

Antibodies and reagents

The following antibodies were used in immunoprecipitations, immunoblottings, and immunofluorescent stainings. Antibodies against PKCζ, Tiam1 (C16), Cdc42 (P1), GST, RhoA, and Rap1 were purchased from Santa Cruz Biotechnology, Inc. Antibodies against c-myc (9E10) and Rac1 were purchased from Millipore. Antibodies against Par3 were purchased from Zymed Laboratories. Antibodies against Nf-κB and phospho-PKCζ/λ (Thr 410/403) were purchased from Cell Signaling Technology. Antibodies against talin were purchased from Sigma-Aldrich. Antibodies against ezrin and CD44 were purchased from BD Biosciences. Antibody against GFP was purchased from Roche. Antibody against ICAM3 was purchased from Abcam PVC. Antibody against LFA-1 (M17-4) was provided by E. Roos (The Netherlands Cancer Institute, Amsterdam, Netherlands). All the conjugated secondary antibodies for immunofluorescent staining were purchased from Invitrogen. Poly-L-lysine was purchased from Sigma- Aldrich. Recombinant SDF1α was purchased from Peprotech. The PKCζ pseudosubstrate inhibitor was purchased from Calbiochem.

Expression vectors

Myc-tagged V12Rac1, N17Rac1, V12Cdc42, or N17Cdc42 sequences were cloned into the retroviral vector LZRS-IRES-Neo as previously described (Michiels et al., 2000). Myc-PKCζ-WT and myc-PKCζ-K281W were subcloned into the Swa1 and Not1 sites of the LZRS-IRES-GFP vector (Mertens et al., 2005). Myc-tagged V12Rap1 was subcloned into the XhoI and NotI restriction sites of the retroviral vectors pMX-eGFP and LZRS-IRES-Bsd. Myc-tagged WTRap1 (derived from the University of Missouri-Rolla cDNA Resource Center) was subcloned into the XhoI and NotI restriction sites of the retroviral vector LZRS-IRES-Bsd. GST-WTRap1 and GST-V12Rap1 (derived from UMR cDNA Resource Center) were generated by subcloning WTRap1 and V12Rap1 into the SmaI restriction site of pGEX 6P2. Dominant-negative Tiam1 (PHnCCEx) has been previously described (Stam et al., 1997). pMT2-HA-Rap1-GAP (Rap1-GAP) was provided by J.L. Bos (University Medical Center, Utrecht, Netherlands). The shRNA oligonucleotides targeting Par3 RNA (Par3 shRNA) and luciferase RNA have been designed as previously described (Malliri et al., 2004; Nishimura et al., 2005). Sequences of the primers are as follow: Par3 shRNA sense primer, 5′-GATCCCCGGCATGGAGACCTTGGAAGTTCAAGAGACTTCCAAGGTCTCCATGCCTTTTTGGAAA-3′; Par3 shRNA antisense primer, 5′-AGCTTTTCCAAAAAGGCATGGAGACCTTGGAAGTCTCTTGAACTTCCAAGGTCTCCATGCCGGG-3′; luciferase shRNA sense primer, 5′-GATCCCCCGTACGCGGAATACTTCGATTCAAGAGATCGAAGTATTCCGCGTACGTTTTTGGAAA-3′; and luciferase shRNA antisense primer, 5′-AGCTTTTCCAAAAACGTACGCGGAATACTTCGATCTCTTGAATCGAAGTATTCCGCGTACGGGG-3′.

Oligonucleotides were annealed and cloned into the EcoRI–XhoI restriction sites of the retroviral vector pRetroSuper-GFP.

Cells and retroviral transduction

BW5147 T lymphomas and Jurkat JA16 T cell subclone (provided by J.A. Nunes, Institut National de la Santé et de la Recherche Médicale, Marseille, France; Gerard et al., 2004) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Rac-11P cells were cultured in DME supplemented with 10% fetal calf serum. A laminin-5 matrix was obtained by culturing Rac-11P cells to confluency, after which cells were detached with 10 mM EDTA in PBS containing a mix of protease inhibitors (Sigma-Aldrich) at 4°C. Phoenix retrovirus packaging cells (Michiels et al., 2000)

were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Single T-cell suspensions were isolated from lymph nodes and spleen of 4–8-wk-old Tiam1+/+ and Tiam1−/− mice (Malliri et al., 2002). Negative selection was performed by using a pan T-cell isolation kit (MACS; Miltenyi Biotec), according to the manufacturer's instructions. T cell purity was >95% as determined by flow cytometry.

Jurkat cells (10×10^6) were electroporated at 960 μF, 250 V, for 25 ms with 20 μg of plasmid using a gene Pulser Xcell (Bio-Rad Laboratories). Gene expression was assessed after 24 h.

BW5147 T lymphoma cells were infected with retrovirus containing supernatants, as previously described (Stam et al., 1998). Cells were selected for 2 wk, unless otherwise specified. For retroviral transduction of primary T lymphocytes, single T-cell suspensions were stimulated with 3 μg/ml CD3ε antibody (145-2C11; R&D Systems) and 25 U/ml IL-2 (Peprotech) for 18 h at 37°C. Subsequently, 3×10^6 T lymphocytes were incubated with 1 ml of virus containing supernatant in the presence of 8 μg/ml polybrene (Sigma-Aldrich) and spin-infected for 2 h at 2,000 rpm. After a 5-h incubation, cells were washed and allowed to grow for 48 h. Infection efficiency was between 10 and 30%.

Cell lysates and fractionation

Lysates were prepared in standard NP-40 lysis buffer (10% glycerol, 50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 20 mM NaF, 2 mM $MgCl_2$, 1 mM Na_3VO_4, and 1 mg/ml protease inhibitors cocktail [Sigma-Aldrich]) for 10 min at 4°C and centrifuged at 13,000 rpm for 10 min at 4°C. For fractionation experiments, pellets of BW5147 cells were lysed using the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem) according to the manufacturer's instructions. The efficiency of subcellular fractionation was determined by SDS-PAGE and immunoblotting with selected marker proteins.

Immunoprecipitation, GST pulldown, and immunoblotting

For immunoprecipitation, extracts were clarified by centrifugation and precleared with γ-binding protein G–Sepharose beads (GE Healthcare) for 1 h at 4°C. Precleared lysates were incubated with 1 μg/ml Tiam1-antibody that was preabsorbed on protein G–Sepharose beads for 2 h at 4°C. Immunocomplexes were washed three times, denatured with SDS, and separated by SDS-PAGE.

For GST-pull down experiments, BW5147 lysates were incubated with 2 μg of GST fusion proteins coupled to gluthatione–Sepharose beads for 2 h at 4°C. Pull downs were washed three times, denatured with SDS, and separated by SDS-PAGE.

For immunoblotting, membranes were blocked and probed with specific antibodies, and then incubated with the appropriate secondary antibodies (anti–rabbit IgG or anti–mouse IgG; GE Healthcare), which were horseradish peroxidase conjugated. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce Chemical Co.).

Rac and Cdc42 activity assay

Rac and Cdc42 activity was determined as described previously (Mertens et al., 2005), using a biotinylated Rac1–Cdc42 interactive binding motif peptide of PAK1. For this, BW5147 cells were starved for 18 h in IMDM medium with 0.5% BSA and lysed in standard NP-40 buffer. Purified T cells or Jurkat cells (10×10^6 cells) were stimulated as indicated with 500 ng/ml SDF1α, and lysed in standard NP-40 buffer.

Rap activity assay

Rap activity was determined as previously described (Franke et al., 1997) using a GST-RalGDS-RBD fusion protein. For this, purified T cells (10×10^6 cells) were stimulated as indicated with 500 ng/ml SDF1α, and lysed in standard NP-40 buffer.

Immunofluorescent staining and polarization assay

For intracellular staining, Jurkat cells or primary T cells were stimulated with 200 ng/ml SDF1α and plated on a fibronectin- or collagen I–coated coverslip, respectively, for 20 min at 37°C. BW5147 cells were plated on a laminin-5–coated coverslip for 20 min at 37°C. After plating, cells were fixed with 4% PFA for 15 min at RT, permeabilized in PBS 0.1% Triton X-100 for 10 min, and saturated in PBS 5% BSA for 20 min. Immunostaining was performed with the appropriate primary antibodies and secondary labeled-antibody, as indicated. Polarization was determined after CD44 (or ICAM-3 for Jurkat cells) staining, followed by staining with FITC-, Alexa Fluor 568–, or Cy5-labeled anti–rat (or anti–mouse) antibody and/or CXCR4 staining.

For quantification of polarization, Jurkat cells and purified T lymphocytes were treated with 2 μM PKCζ pseudosubstrate inhibitor for 1 h, when indicated, and then stimulated in suspension with 200 ng/ml SDF1α or secondary lymphoid tissue chemokine for 20 min at 37°C and immediately fixed in 4% PFA for 15 min at RT. BW5147 cells were also fixed in suspension in 4% PFA for 15 min at RT. Cells were stained with CD44 antibody, followed by staining with a secondary FITC-labeled anti–rat antibody for primary T cells and BW5147 cells, or with ICAM-3 antibody followed by FITC-labeled anti–mouse antibody for Jurkat cells. Coverslips were finally mounted on slides using Mowiol. Each experiment was repeated at least three times.

Fluorescence and transmission images (single z slice) were taken at RT using a confocal microscope (TCS SP2 [Leica], HCX PL APO 63×/1.32 NA oil objective [Leica]) and processed using Photoshop CS2 (Adobe).

Chemotaxis assay

The inner and outer face of Transwells (Costar; 5-μm pore size) were coated with 0.5% Ovalbumin (Ova) for 2 h at RT. Purified T cells (10^5 in 150 μl RPMI and 0.1% Ova) were treated with 2 μM PKCζ inhibitor for 1 h, where indicated, and loaded in an Ova-coated transwell, which was placed into a 24-well plate containing 250 μl RPMI supplemented with 0.1% Ova and various concentrations of SDF1α. After 1 h at 37°C, the cells that migrated into the lower chamber were collected and counted.

Statistical analysis

Data were expressed as the mean ± the SD. Comparisons between groups were analyzed with *t* tests. Data were considered as statistically significant when $P \leq 0.05$.

Online supplemental material

Fig. S1 shows the intracellular localization of PKCζ, Par3, and Cdc42 at the leading edge of V12Rap1-expressing BW5147 cells, in comparison with RhoA localization. Fig. S2 shows the colocalization of PKCζ, Par3, and Cdc42 with V12Rap1 at the leading edge of V12Rap1-expressing BW5147 cells. Fig. S3 shows the down-regulation of Par3 expression by shRNA in BW5147 cells. The online version of this article is available at http://www.jcb.org/cgi/content/full/jcb.200608161/DC1.

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