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Goi2 and ZAP-70 Mediate RasGRP1 Membrane Localization and Activation of SDF-1–Induced T Cell Functions

Kimberly N. Kremer, Ashok Kumar,1 and Karen E. Hedin

RasGRP1, a Ras guanine-nucleotide exchange factor, critically mediates T cell development and function and controls immunodeficiency and autoimmunity. In this study, we describe a unique mechanism of mobilization and activation of RasGRP1 in response to SDF-1, a chemokine that signals via the G protein-coupled receptor CXCR4. Depletion of RasGRP1 impaired SDF-1–stimulated human T cell migration, expression of the activation marker CD69, and activation of the ERK MAPK pathway, indicating that RasGRP1 mediates SDF-1 functions. SDF-1 treatment caused RasGRP1 to localize to the plasma membrane to activate K-Ras and to the Golgi to activate N-Ras. These events were required for cellular migration and for ERK activation that mediates downstream transcriptional events in response to SDF-1. SDF-1–dependent localization of RasGRP1 did not require its diacylglycerol-binding domain, even though diacylglycerol was previously shown to mediate localization of RasGRP1 in response to Ag stimulation. This domain was, however, required for activity of RasGRP1 after its localization. Intriguingly, SDF-1 treatment of T cells induced the formation of a novel molecular signaling complex containing RasGRP1, Goi2, and ZAP-70. Moreover, SDF-1–mediated signaling by both Gi proteins and ZAP-70 was required for RasGRP1 mobilization. In addition, RasGRP1 mobilization and activation in response to SDF-1 was dependent on TCR expression, suggesting that CXCR4 heterodimerizes with the TCR to couple to ZAP-70 and mobilize RasGRP1. These results increase understanding of the molecular mechanisms that mediate SDF-1 effects on T cells and reveal a novel mechanism of RasGRP1 regulation. Other G protein-coupled receptors may similarly contribute to regulation of RasGRP1. The Journal of Immunology, 2011, 187: 3177–3185.
with the TCR, CXCR4 requires at least one TCR ITAM domain to prolong ERK activation, upregulate the AP-1 transcription factor, and mediate gene transcription (4, 5). These SDF-1–mediated signaling pathways also require several conventional TCR pathway signaling proteins, including ZAP-70 and SLP-76 (4, 18), p52Shc, and activity of PI3K (19, 20). Yet other TCR signaling pathway mediators, such as linker for activation of T cells and the proline-rich domain of SLP-76, are not required for SDF-1 to stimulate these same events (4, 18). In addition, SDF-1 binding to CXCR4 of T cells also promotes GTP binding by the α subunits of several heterotrimeric G proteins (21). These Gα subsequently signal by dissociating from the receptor and interacting with downstream effector proteins. Most of the effects of CXCR4 on T cells, including ERK activation and migration, are highly sensitive to PTX, which specifically inactivates the Gi-type G proteins (4, 22).

It has been unclear how CXCR4 integrates signals derived from its interaction with the TCR to mediate downstream physiological outcomes. In this study, we describe an essential role for RasGRP1 in mediating SDF-1–induced T cell responses. In addition, we identify a novel mechanism for mobilizing and activating RasGRP1 that integrates signals from a Gαi protein and a TCR-dependent tyrosine kinase.

Materials and Methods

Cells
Normal human T cells (PBMC T cells) were isolated with 99% purity from peripheral blood. The CD3+ T cells were purified with a CD3+ magnetic bead separation column (Miltenyi Biotec, Auburn, CA). Blood was obtained and used with informed consent and approval by the Mayo Institutional Review Board. Jurkat and PBMC T cells were maintained as described (4, 23). A Jurkat cell line deficient in the proline-rich domain of SLP-76, are not required for SDF-1 to stimulate these same events (4, 18). In addition, SDF-1 binding to CXCR4 of T cells also promotes GTP binding by the α subunits of several heterotrimeric G proteins (21). These Gα subsequently signal by dissociating from the receptor and interacting with downstream effector proteins. Most of the effects of CXCR4 on T cells, including ERK activation and migration, are highly sensitive to PTX, which specifically inactivates the Gi-type G proteins (4, 22).

Assays of active, phosphorylated ERK1 and ERK2 and of short hairpin RNA-mediated protein depletion

for analysis of active ERK, cells were stimulated and assayed via flow cytometry as described (4). Where indicated, 1 h prior to assay the cells were transiently transfected as described (4) with either control plasmid or a plasmid encoding shRNA specific for RasGRP1, N-Ras, or a nontargeting shRNA. Seventy-two hours later, these assays were performed as described (28).

Assay of the subcellular localizations of RasGRP1 and active Ras

Fluorescent expression plasmids were transiently transfected into Jurkat or PBMC T cells at efficiencies of 60–70% or 25–50%, respectively, as described (4), and each experiment was assayed on multiple days. The subcellular locations of fluorescent fusion proteins in live cells was visualized via confocal microscopy with or without SDF-1 as described (27). The RBD–YFP–expressing plasmid was cotransfected with SDF-1 (R&D Systems, Minneapolis, MN). The RBD–YFP–expressing plasmid was cotransfected with SDF-1 (R&D Systems, Minneapolis, MN). The RBD–YFP–expressing plasmid was cotransfected with SDF-1 (R&D Systems, Minneapolis, MN). The RBD–YFP–expressing plasmid was cotransfected with SDF-1 (R&D Systems, Minneapolis, MN).

Results

RasGRP1 is required for SDF-1/CXCL12 signaling to stimulate the ERK MAPK pathway, CD69 expression, and migration of T cells

The molecular mechanisms responsible for CXCR4 regulation of thymocyte development (1), chemotaxis (29), and costimulation of T cell cytokine secretion (4–6) are incompletely characterized. In this study, we address the role of a RasGEF, RasGRP1, in CXCR4 signaling. First, we depleted RasGRP1 protein and investigated the downstream physiological effects in response to SDF-1. Jurkat T cells were transiently transfected with either a control plasmid or a plasmid encoding an shRNA directed against RasGRP1. Both plasmids also express GFP, allowing detection and assay of shRNA-containing cells. Compared with cells transfected with the control plasmid, cells transfected with the plasmid encoding RasGRP1 shRNA displayed significantly lower SDF-1–dependent ERK activation (p < 0.05; Fig. 1A). In multiple experiments,
RasGRP1 protein levels in shRNA-transfected cells were decreased by 80–90% compared with those of control plasmid-transfected cells (Fig. 1A, inset). Similarly, we addressed the role of RasGRP1 in mediating a downstream transcriptional event induced by SDF-1 that requires activation of the Ras–ERK pathway: cell surface expression of the CD69 activation marker (4, 30). Normal, primary, human T cells (T cells derived from PBMCs, or PBMC T cells) were transiently transfected with either the control plasmid or the RasGRP1 shRNA plasmid, leading to substantial RasGRP1 depletion (Fig. 1 inset). Similarly, we addressed the role of RasGRP1 in mediating a downstream transcriptional event induced by SDF-1 that requires activation of the Ras–ERK pathway: cell surface expression of the CD69 activation marker (4, 30). Normal, primary, human T cells (T cells derived from PBMCs, or PBMC T cells) were transiently transfected with either the control plasmid or the RasGRP1 shRNA plasmid, leading to substantial RasGRP1 depletion (Fig. 1 inset). The percentage of cells expressing CD69 in response to SDF-1 increased among transfected control T cells (Fig. 1B). In contrast, PBMC T cells deficient in RasGRP1 were impaired in their ability to upregulate CD69 in response to SDF-1 (Fig. 1B, inset). Thus, RasGRP1 is required for ERK activation as well as for downstream ERK-dependent gene expression. Rho activation after 8 min of SDF-1 treatment was similarly defective upon depletion of RasGRP1 in Jurkat cells, in contrast to Rho activation after 2 min of SDF-1 stimulation (Fig. 1D). Thus, as previously noted for other cell types (31, 32), Ras activation may cross-regulate Rho activation in T cells. Densitometric quantitation of multiple experiments confirmed that RasGRP1 depletion significantly affected SDF-1–dependent Rho activation at 8 min but not 2 min (Supplemental Fig. 1). Consistent with its effects on Ras and Rho activation, RasGRP1 depletion via shRNA also decreased cell migration in response to SDF-1 treatment (Fig. 1E). Thus, RasGRP1 is required for SDF-1 to induce downstream signaling events including ERK activation, gene expression, Rho activation, and T cell migration.

**FIGURE 1.** RasGRP1 is required for SDF-1/CXCL12 signaling to stimulate the ERK MAPK pathway, CD69 expression, and migration of T cells. A, Jurkat T cells transfected with either a RasGRP1 shRNA or vector alone were stimulated with SDF-1 and assayed for ERK activation. Bars denote the fold-increase in ERK activation of SDF-1–stimulated compared with unstimulated cells ± SEM, n = 6. *p < 0.05 (significantly different from control). Inset, Immunoblot of cell lysates showing decreased RasGRP1 compared with a control protein (Vav-1). B, Normal, primary human T cells (PBMC T cells) transfected with RasGRP1 shRNA or vector alone were stimulated for 24 h with SDF-1 and assayed for CD69 expression by flow cytometry. C, Summary of multiple experiments performed as in B using T cells from four donors. Inset, Immunoblot of cell lysates. D and E, Jurkat cells were transfected with RasGRP1 shRNA as in A and assayed for active GTP-bound Rho or total Rho (D) or migration (E) in response to SDF-1. The relative percentage of Rho activation (D) was determined by normalizing to total Rho. Each point in E denotes the mean percentage of cells migrated ± SD, n = 3. Results shown are representative of three independent experiments.

The mobilization and activation of RasGRP1 in response to a chemokine has not been previously described. We used a RasGRP1–YFP fluorescent fusion protein to determine the subcellular location of RasGRP1 before and after SDF-1 treatment of live T cells. RasGRP1–YFP was primarily detected in the cytosol of both PBMC T cells and Jurkat cells prior to SDF-1 treatment; however, RasGRP1–YFP localized to both an intracellular compartment and the plasma membrane after SDF-1 treatment (Fig. 2A, 2D). The plasma membrane-localized RasGRP1 was typically seen only on some portions of the plasma membrane, suggesting that RasGRP1 localizes to particular plasma membrane subdomain(s) (Fig. 2A, 2B). Coexpression of a fluorescent fusion protein of the Golgi marker, GaIT–CFP, revealed that the intracellularly localized RasGRP1 was located at the Golgi (Fig. 2C). Analysis of multiple cells as in Fig. 2A revealed that the percentage of PBMC T cells that localized RasGRP1 to the plasma membrane increased from 7% of untreated cells to 50% of SDF1–treated cells and that RasGRP1 localization to the Golgi increased from 21% of untreated cells to 67% of SDF1–treated cells. Jurkat cells showed similar results, and the percentage of cells showing an SDF1–mediated increase, in the same cell, in RasGRP1 localization to the plasma membrane or Golgi is shown in Fig. 2G. Thus, SDF1 treatment of T cells mobilizes RasGRP1 to both the plasma membrane and the Golgi.

CXCR4 complexes with the TCR upon SDF1 stimulation to prolong ERK activation, an event required for SDF1 treatment to increase gene transcription and cytokine production in activated T lymphocytes (4, 5). We therefore addressed the role of the TCR in the mechanism by which SDF1 regulates RasGRP1. For this purpose, we used a somatic mutant of the Jurkat T cell line
is deficient in TCR-β and is consequently deficient in cell surface TCR molecules (Fig. 2E) (24) and does not form CXCR4–TCR heterodimers in response to SDF-1 (4). Compared with normal Jurkat (Fig. 2D), TCR-β-deficient cells failed to localize RasGRP1 to either the plasma membrane or Golgi in response to SDF-1 (Fig. 2F, 2G).

Because the membrane localization of RasGRP1 regulates its ability to activate Ras isoforms in those locations, we also determined the subcellular location and TCR dependence of Ras isoforms activated in response to SDF-1. First, we showed that SDF-1 stimulation of Jurkat cells increased the GTP-bound (active) levels of all three Ras isoforms expressed in T cells, N-Ras, K-Ras, and H-Ras. For controls, cell lysates were immunoblotted for total N-Ras or actin. The percentage of the indicated active Ras isoform was determined (here and below) by normalizing to the indicated loading control.

FIGURE 2. SDF-1/CXCR4 signaling uses the TCR to mobilize RasGRP1 to enhance activation of N-Ras and K- Ras. A–D, Live, individual PBMC T cells or Jurkat cells expressing a fluorescent fusion protein of RasGRP1 (RasGRP1–YFP) were analyzed by confocal microscopy with or without SDF-1. Arrows indicate RasGRP1–YFP localized to the Golgi and plasma membranes in different z-slices. A differential interference contrast image is included in B. Where indicated, cells also express the Golgi marker, GaIT–CFP (blue). A, n = 29 PBMC T cells. B, n = 7 PBMC T cells. C, n = 4 Jurkat cells. D, n = 49 Jurkat cells.

E, Cell surface expression of the TCR on TCR-β-deficient and TCR-β-reconstituted Jurkat cells. F, TCR-β-deficient cells assayed for RasGRP1 localization as in D. G, Summary of multiple cells assayed as in D and E, n = 24–49 for each bar. H and I, Jurkat, TCR-β-deficient, and TCR-β-reconstituted cells were assayed for active, GTP-bound Ras isoforms in response to SDF-1. For controls, cell lysates were immunoblotted for total N-Ras or actin. The percentage of the indicated active Ras isoform was determined (here and below) by normalizing to the indicated loading control. J, Jurkat or TCR-β-deficient cells expressing a GTP Ras-binding domain fused to YFP (RBD–YFP) and either N-Ras, K-RasB, or H-Ras were imaged as live cells by confocal microscopy with or without SDF-1. K, Results of analyzing multiple cells as in F: n = 15–20 per bar. All scale bars, 2 μm.
and H-Ras (Fig. 2H). In multiple experiments, TCR-β-deficient Jurkat cells responded to SDF-1 by activating less N-Ras and K-Ras, and this defect was corrected by reconstitution of TCR-β (Fig. 2I). In contrast to N-Ras and K-Ras activation, H-Ras activation in response to SDF-1 was not defective in TCR-β-deficient cells compared with that in Jurkat cells (Fig. 2I). These results indicate that SDF-1 signaling via the CXCR4–TCR heterodimer enhances the activation of N-Ras and K-Ras but not H-Ras. Second, we used a fluorescent fusion protein of a GTP Ras-binding domain (RBDF–YFP) to determine the subcellular locations of active, GTP-bound N-, K-, and H-Ras isoforms in SDF-1–treated cells. RBDF–YFP has been shown to localize to the site(s) of active Ras in living cells, in a manner that is detectable by fluorescence microscopy only when the relevant wild-type Ras isoform is overexpressed (33). SDF-1 treatment increased the localization of RBDF–YFP to an intracellular structure in Jurkat cells, but only when the cells were also overexpressing N-Ras (Fig. 2J, 2K, and data not shown). This intracellular structure was determined to be the Golgi apparatus by its colocalization with GaIT–CFP (data not shown). In contrast to cells overexpressing N-Ras, cells overexpressing K-RasB, the K-Ras splice variant in T cells, localized RBDF–YFP to the plasma membrane but not the Golgi in response to SDF-1 (Fig. 2J, 2K). RBDF–YFP localization revealed that H-Ras is activated at both the plasma membrane and Golgi by SDF-1 (Fig. 2J, 2K). Results similar to those in Fig. 2J and 2K were also found using PBMC T cells (data not shown). Using the TCR-β-deficient Jurkat cell line, we additionally found that SDF-1 signaling showed no detectable RBDF–YFP localization, indicative of neither N-Ras nor K-Ras activation at either the Golgi or plasma membranes. Thus, the activation of N-Ras and K-Ras at these locations in response to SDF-1 requires the CXCR4–TCR heterodimer (Fig. 2J, 2K). Consistent with H-Ras activation not requiring the TCR (Fig. 2I), RBDF–YFP localization in response to SDF-1 showed that H-Ras activation at both the Golgi and plasma membranes occurred normally in TCR-β-deficient cells (Fig. 2J, 2K). Together, the results in Fig. 2 indicate that SDF-1 mobilizes RasGRP1 to the plasma membrane and Golgi of T cells via a mechanism requiring the CXCR4–TCR heterodimer, and this enhances the activation of a distinct subset of Ras isoforms: N-Ras at the Golgi and K-Ras at the plasma membrane.

N-Ras and K-Ras mediate ERK activation and migration of T cells via a mechanism requiring RasGRP1 but not SOS-1

We next addressed the role of N-Ras and K-Ras in RasGRP1–mediated ERK activation and migration. First, we confirmed that both N-Ras and K-Ras are downstream targets of RasGRP1. Cells expressing the RasGRP1 shRNA responded to SDF-1 treatment by activating fewer N-Ras and K-Ras molecules (Fig. 3A), indicating that RasGRP1 is required to activate both N-Ras and K-Ras in response to SDF-1. Synergistic signaling by RasGRP1 and SOS has been described in response to direct stimulation of the TCR (17). In response to SDF-1, we found no defect in N-Ras or K-Ras activation despite 80–90% depletion of SOS-1 protein due to transfection with SOS-1 shRNA (Fig. 3B). Thus, SOS-1 is not required for SDF-1–mediated N-Ras and K-Ras activation. Second, we determined the roles of N-Ras and K-Ras in the previously described SDF-1–dependent signal transduction via the CXCR4–TCR heterodimer that is responsible for the prolonged ERK activation after 8–12 min of SDF-1 treatment (4). Importantly, this type of prolonged ERK activation is necessary for downstream transcriptional events in response to SDF-1 (4). Compared with cells transfected with the control plasmid vector alone, Jurkat cells transfected with vectors encoding either N-Ras or K-Ras shRNA reduced protein levels of these Ras isoforms by 65–85% (Fig. 3C, inset). Depletion of either N-Ras or K-Ras significantly impaired SDF-1–dependent ERK activation at 8 min (p < 0.05; Fig. 3C). SDF-1–dependent ERK activation at 12 min was also significantly lower in N-Ras– or K-Ras–deficient cells (data not shown; n = 4; p < 0.05). Finally, because RasGRP1 is also required for migration in response to SDF-1 (Fig. 1E), we additionally showed that Jurkat cells deficient in either N-Ras or K-Ras displayed impaired migration in response to SDF-1 compared with that of cells transfected with plasmid vector alone (Fig. 3D). This result is supported by experiments performed using an additional N-Ras shRNA sequence and a nontargeting shRNA control (Supplemental Fig. 2). Together, the results in Fig. 3 demonstrate that RasGRP1, independently of SOS-1, mediates the activation of both N-Ras and K-Ras in response to SDF-1 treatment of T cells. Furthermore, these results indicate that the downstream effects of SDF-1 on T cells, including ERK activation and migration, require the RasGRP1–mediated activation of N-Ras and K-Ras isoforms.

The DAG-binding domain of RasGRP1 is not required for its membrane localization in response to SDF-1 but is required for its activity

RasGRP1 localization and activation in response to signaling by Ag receptors depends on diacylglycerol binding to the DAG-bind-

FIGURE 3. N-Ras and K-Ras mediate ERK activation and migration of T cells via a mechanism requiring RasGRP1 but not SOS-1. A. RasGRP1 was depleted from Jurkat cells as in Fig. 1A, and N-Ras or K-Ras activation in response to 2 min SDF-1 was assayed as in Fig. 2H. n = 3. B. Middle and bottom gels: SOS-1 was depleted from Jurkat cells via shRNA, and N-Ras and K-Ras activation was assayed as in A. Top gel: Cell lysates immunoblotted for SOS-1. n = 3. C. N-Ras or K-Ras were specifically depleted from Jurkat cells using shRNA, and ERK activation in response to 8 min of SDF-1 was assayed as in Fig. 1A. Bars denote the fold-increase in ERK activation of stimulated compared with unstimulated cells ± SEM, n = 5. *p < 0.05 (significantly different from controls). Inset, Immunoblot of N-Ras and K-Ras compared with actin (control) in cell lysates. D. N-Ras or K-Ras were depleted from Jurkat cells as in C, and migration was assayed as in Fig. 1E. The experiment shown is representative of three independent experiments.
ing domain of RasGRP1 (16, 34). We asked if SDF-1 uses a similar mechanism to localize and activate RasGRP1. First, we determined if the DAG-binding domain of RasGRP1 was necessary for RasGRP1 localization to the membrane. For this purpose, we transfected PBMC T cells with a plasmid expressing a RasGRP1 fluorescent fusion protein lacking the DAG-binding domain (RasGRP1–ΔDAG). Surprisingly, SDF-1 treatment of live PBMC T cells readily induced the plasma membrane localization of the RasGRP1–ΔDAG fluorescent fusion protein, from 3 to 64% of unstimulated or SDF-1–untreated cells, respectively (Fig. 4A). The Golgi localization of RasGRP1–ΔDAG-YFP was constitutive in 84 and 94% of untreated and SDF-1–treated cells, respectively (Fig. 4A). Thus, DAG binding is not necessary for RasGRP1 to localize to either the plasma membrane or the Golgi where its Ras targets are located. Second, we addressed the role of DAG in the activity of RasGRP1 by reconstituting RasGRP1-deficient cells with RasGRP1–ΔDAG. Cells were transfected with an expression plasmid that encoded both a RasGRP1 shRNA and either shRNA-resistant wild-type RasGRP1 or shRNA-resistant RasGRP1–ΔDAG. Compared with cells expressing only RasGRP1 shRNA, cells also expressing the shRNA-resistant wild-type RasGRP1 significantly increased ERK activity in response to SDF-1 (Fig. 4B, 4C). In contrast, cells expressing RasGRP1 shRNA together with RasGRP1–ΔDAG failed to increase ERK activity in response to SDF-1 (Fig. 4B, 4C). Thus, the DAG-binding domain of RasGRP1 is not required for its membrane localization in response to SDF-1 but is required for its subsequent activation of Ras.

Gai2 and ZAP-70 mediate the localization of RasGRP1 and thus lead to the activation of N-Ras and K-Ras

Because SDF-1 mediates the localization of RasGRP1 via a TCR-dependent mechanism that is independent of DAG, we immunoprecipitated RasGRP1 and assayed for other known signaling molecules in the CXCR4–TCR signaling pathway that may regulate the membrane localization of RasGRP1 in response to SDF-1. Intriguingly, we found that SDF-1 treatment induced the formation of a molecular complex detectable by coimmunoprecipitation of RasGRP1 that also contains the Gi protein α subunit, Gai2, and the tyrosine kinase Zap-70 (Fig. 5A, 5B). Similarly, immunoprecipitation of either Gai2 or Zap-70 from SDF-1–treated cells resulted in the copurification of RasGRP1 (Fig. 5A, 5B).

To determine the role of Gai2 in mediating RasGRP1 mobilization to the membrane in SDF-1–treated cells, we used PTX, which specifically inhibits the Gi proteins of T cells. Notably, PBMC T cells pretreated with PTX failed to localize RasGRP1 to either the plasma membrane or Golgi in response to SDF-1, in contrast to T cells treated with the control toxin, PTX-B (Fig. 5C, 5D). To confirm that the disruption of RasGRP1 localization by the inhibition of Gi protein signaling also inhibited the activation of RasGRP1’s downstream targets, we assayed the levels of active N-Ras and K-Ras in the presence of PTX. Cells pretreated with PTX-B activated both N-Ras and K-Ras in a normal manner upon SDF-1 treatment (Fig. 5E). In contrast, cells pretreated with PTX were defective in both N-Ras and K-Ras activation in response to SDF-1 (Fig. 5E). Thus, Gai2 mediates the localization and activation of RasGRP1 in response to SDF-1.

We next addressed the role of Zap-70, which, in addition to Gai2, copurified with RasGRP1 in response to SDF-1 (Fig. 5A, 5B). An inhibitor of Zap-70, piceatannol, markedly decreased the percentage of PBMC T cells that localized RasGRP1 to the plasma membrane upon SDF-1 treatment (Fig. 5F). The effects of piceatannol on the ability of SDF-1 to induce RasGRP1 Golgi localization in PBMC T cells could not be assessed because the vehicle (DMSO) constitutively localized RasGRP1 to this site (data not shown). We therefore analyzed RasGRP1 localization in Zap-70–deficient Jurkat T cells. In contrast to normal Jurkat (Fig. 2D), SDF-1 treatment failed to induce the localization of RasGRP1 to either the plasma membrane or the Golgi of Zap-70–deficient Jurkat cells (Fig. 5G). SDF-1 increased the localization of RasGRP1 to the plasma membrane and the Golgi in only 8% and 0 of Zap-70–deficient cells, respectively. Thus, Zap-70 mediates the localization of RasGRP1 to the plasma membrane and Golgi. Consistent with these results, the Zap-70–deficient cells displayed impaired N-Ras and K-Ras activation in response to SDF-1 compared with that of either normal Jurkat cells or Zap-70–deficient cells with Zap-70 stably re-expressed (Fig. 5H). Together, these results indicate that SDF-1 treatment of T cells induces the formation of a novel molecular signaling complex containing RasGRP1, Gai2, and Zap-70 and that this event localizes RasGRP1 to the plasma membrane and the Golgi.

SDF-1 uses a novel mechanism to regulate RasGRP1 localization and activation to modulate T cell functions

Based on our results, we propose the model shown in Fig. 6. SDF-1 binding to CXCR4 induces formation of the CXCR4–TCR heterodimeric receptor, which signals to cause the binding of Gai2 and Zap-70 to RasGRP1. This RasGRP1–Gai2–Zap-70 complex allows for mobilization of RasGRP1 to the plasma membrane and the Golgi. After localization of RasGRP1, DAG binds to the DAG-binding domain of RasGRP1 thereby activating RasGRP1, which in turn activates K-Ras at the plasma membrane and N-Ras at the Golgi. Active K-Ras and N-Ras then lead to ERK activation and subsequent gene transcription as well as Rho activation that leads to T cell migration.

Discussion

CXCR4 is no longer known solely for its ability to stimulate chemotaxis and mediate HIV-1 infection. CXCR4 is also critical
for developmental cues and for the survival and enhanced activation of T cells (1, 4, 7). In this study, we describe an essential and novel role for RasGRP1 in mediating the SDF-1–induced T lymphocyte functions of ERK activation, gene transcription, and cellular migration.

RasGRP1 has not previously been shown to mediate signal transduction by CXCR4 or other chemokine receptors. We show in this study that RasGRP1 is required in T cells for SDF-1–mediated ERK activation and expression of the T cell activation marker CD69. Intriguingly, we also show that RasGRP1 is necessary for SDF-1–mediated Rho activation and cellular migration, most likely via a mechanism involving the previously published cross-talk between the Ras- and Rho-regulated signaling pathways (31, 32). We demonstrate that SDF-1 treatment mobilizes RasGRP1 to both the plasma membrane and the Golgi complex. We further show that RasGRP1 specifically activates N-Ras and K-Ras downstream of SDF-1–induced CXCR4–TCR complexes. The TCR-independent H-Ras activation that only activates transient ERK and fails to enhance gene transcription appears to depend on a different mechanism involving SOS (K. Kremer and K. Hedin, unpublished observations). Finally, we describe a novel molecular mechanism by which SDF-1 treatment functionally integrates signals derived from both G protein- and tyrosine kinase-coupled receptors to activate RasGRP1 in T cells. Together, these results significantly advance understanding of the molecular mechanisms by which SDF-1 signals to regulate T cell functions.

Our results indicate that SDF-1 induces the formation of a novel molecular signaling complex between RasGRP1 and its upstream regulators in the SDF-1 signaling pathway: Gβ12 and ZAP-70. Signaling by both Gi proteins and ZAP-70 are required to localize RasGRP1 to the plasma membrane and the Golgi in response to SDF-1. Subsequently, RasGRP1 at these locations mediates activation of N-Ras and K-Ras, which are required for several of the downstream effects of SDF-1 on T cells. As a GPCR, CXCR4 couples to activate Gβ12 directly (29). Additionally, CXCR4 likely heterodimerizes with the TCR to couple to ZAP-70 and thereby allow this unique regulation of RasGRP1, because expression of the TCR, in addition to ZAP-70, is required for SDF-1 to mobilize RasGRP1 to membranes to activate N-Ras and K-Ras.

We previously showed that the CXCR4–TCR heterodimer is required for ERK activation in response to SDF-1 (4, 5). In contrast to previously described mechanisms of RasGRP1 mobilization that depend on DAG (14, 16), we found that the SDF-1–induced mobilization of RasGRP1 did not require the DAG-binding

FIGURE 5. Gβ12 and ZAP-70 mediate the localization of RasGRP1 and thus lead to the activation of N-Ras and K-Ras. A and B, Jurkat cells were stimulated with SDF-1, fixed, and either RasGRP1, Gβ12, or ZAP-70 were immunoprecipitated and immunoblotted to reveal copurifying proteins, n = 3. C, PBMC T cells expressing RasGRP1–WT-YFP were pretreated with either the control PTX-B toxin or PTX and assayed for RasGRP1 localization as in Fig. 2A. D, Summary of multiple cells assayed as in C, n = 33–38 for each bar. E, Jurkat cells were pretreated with either PTX-B or PTX and assayed for N-Ras or K-Ras activation in response to 2-min SDF-1 as in Fig. 2H, n = 3. F, Summary of multiple PBMC T cells expressing RasGRP1–WT-YFP that were pretreated with vehicle (DMSO) or piceatannol and assayed for RasGRP1 localization as in Fig. 2A, n = 25–32 for each bar. G, ZAP-70–deficient Jurkat cells were assayed for RasGRP1 localization as in Fig. 2D, n = 12. H, Normal Jurkat, ZAP-70–deficient, or ZAP-70–reconstituted Jurkat cells were assayed for N-Ras or K-Ras in response to 2-min SDF-1 as in Fig. 2H, n = 3. All scale bars, 2 μm.

FIGURE 6. SDF-1 uses a novel mechanism to regulate RasGRP1 localization and activation to modulate T cell functions. Based on our results, we propose the model shown in the figure. SDF-1 binding to CXCR4 induces formation of the CXCR4–TCR heterodimeric receptor, which signals to cause the binding of Gβ12 and ZAP-70 to RasGRP1. This RasGRP1–Gβ12–ZAP-70 complex allows for mobilization of RasGRP1 to the plasma membrane and the Golgi. After localization of RasGRP1, DAG binds to the DAG-binding domain of RasGRP1 thereby activating RasGRP1, which in turn activates K-Ras at the plasma membrane and N-Ras at the Golgi. Active K-Ras and N-Ras then lead to ERK activation and subsequent gene transcription as well as Rho activation that leads to T cell migration.
of RasGRP1. The DAG-binding domain of RasGRP1 was, nevertheless, required for ERK activation in response to SDF-1. Thus, DAG binding to RasGRP1 may be required to relieve intramolecular inhibitory interactions, as is typical for other Ras superfamily GEFs (35). Notably, other GEFs have been shown to use protein–protein interactions to regulate their membrane localization. For example, Vav-1 is recruited to the TCR via src homology region (SH)2 and SH3 domain interactions, and p115RhoGEF is recruited to the membrane by binding to the G protein a subunit Goi3 (35). Whereas RasGRP1 lacks either an SH2 or SH3 domain, RasGRP1 does possess a proline-rich region (36) that could indirectly mediate the interactions with Goi2 and ZAP-70 that mobilize RasGRP1 in response to SDF-1.

We show in this study that RasGRP1 is essential for several SDF-1–induced T cell functions. The RasGRP1-mediated signaling pathways described in this study may, therefore, explain some of the physiological defects of mice deficient in RasGRP1. RasGRP1 deficiency impairs thymocyte positive selection (13, 37, 38). Be-

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