Rac1 Mediates Collapse of Microvilli on Chemokine-Activated T Lymphocytes

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Rac1 Mediates Collapse of Microvilli on Chemokine-Activated T Lymphocytes

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Lymphocytes circulate in the blood and upon chemokine activation rapidly bind, where needed, to microvasculature to mediate immune surveillance. Resorption of microvilli is an early morphological alteration induced by chemokines that facilitates lymphocyte emigration. However, the antecedent molecular mechanisms remain largely undefined. We demonstrate that Rac1 plays a fundamental role in chemokine-induced microvillar breakdown in human T lymphocytes. The supporting evidence includes: first, chemokine induces Rac1 activation within 5 s via a signaling pathway that involves Gαi. Second, constitutively active Rac1 mediates microvilli disintegration. Third, blocking Rac1 function by cell permeant C-terminal “Trojan” peptides corresponding to Rac1 (but not Rac2, Rho, or Cdc42) blocks microvillar loss induced by the chemokine stromal cell-derived factor 1α (SDF-1α). Furthermore, we demonstrate that the molecular mechanism of Rac1 action involves dephosphorylation-induced inactivation of the ezrin/radixin/moesin (ERM) family of actin regulators; such inactivation is known to detach the membrane from the underlying actin cytoskeleton, thereby facilitating disassembly of actin-based peripheral processes. Specifically, ERM dephosphorylation is induced by constitutively active Rac1 and stromal cell-derived factor 1α-induced ERM dephosphorylation is blocked by either the dominant negative Rac1 construct or by Rac1 C-terminal peptides. Importantly, the basic residues at the C terminus of Rac1 are critical to Rac1’s participation in ERM dephosphorylation and in microvillar retraction. Together, these data elucidate new roles for Rac1 in early signal transduction and cytoskeletal rearrangement of T lymphocytes responding to chemokine. The Journal of Immunology, 2004, 173: 4985–4993.
(cpERMs) contributes to the formation of microvilli as this allows ERM proteins to tether the plasma membrane to the underlying actin network (16–18). RhoA results in microvilli formation/maintenance by inducing cpERM phosphorylation (14, 19); in contrast, nothing is known about the role of small G proteins in disassembly of microvilli.

We have previously shown that chemokine stimulation induces dephosphorylation of ERMs which is causally related to the loss of ERM cross-linking function and breakdown of ERM-stabilized microvilli (10). However, the molecular components that connect chemokine signals to ERM inactivation-induced cytoskeletal changes are undefined. We hypothesized that chemokine-induced retraction of microvilli in lymphocytes would be regulated by the family of small G proteins. We therefore investigated 1) whether Rho-GTPases family of actin regulators are involved in SDF-1α-induced microvillar breakdown and 2) if so, how such GTPase signaling relates to ERM dephosphorylation-induced microvillar breakdown. Herein, we report that SDF-1α activates lymphocyte Rac1 very rapidly and transiently. Activated Rac1 plays a central role in the breakdown of microvilli by inducing ERM dephosphorylation.

Materials and Methods

**Human primary T cells**

Human PBT were isolated by leukapheresis of normal human volunteers by immunomagnetic negative selection as previously described (10). PBT were “nucleoporated” using a human T cell nucleofector kit and nucleoporator from Amaxa Biosystems (Gaithersburg, MD) as previously described (10). Cells were held in complete RPMI 1640 with serum for 20 h at 37°C posttransfection before analysis of morphology or functional responses.

**Reagents**

Constructs encoding GFP-tagged constitutively active mutant RhoA (RhoA.L63), Rac1 (Rac1.L61), Cdc42 (Cdc42.L61), and GFP-tagged dominant negative (DN) mutant Rac1 (Rac1.N17), and Cdc42 (Cdc42.N17) were kindly provided by Dr. L. Salazar-Fontana (20) (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Peptides corresponding to the C-terminal regions (without the CAAX motif) of Rho, Rac1 (and mutants thereof), Cdc42, and Rac2 fused to the Tat domain of HIV (Table I) were synthesized as previously described (21), and additional batches thereof were synthesized by Synpep (Dublin, CA). In all studies of inhibition by peptides, they were used at 1 mg/ml and added 5 min before stimulation with SDF-1α (100 ng/ml) for 5 min. Peptide retention by human lymphoid cells was assessed under conditions similar to those used for functional inhibition (1 mg/ml peptide for 5 min) except that peptide was preincubated with streptavidin-PE (Molecular Probes, Eugene, OR). Cells were then immediately fixed with 1% paraformaldehyde for 15 min and washed, and fluorescence was determined by FACs analysis. Fluorescence distributions were unimodal, indicating relatively homogenous uptake by cells but the amount of peptide uptake/retention varied between peptides as indicated by differences in median fluorescence intensity (MFI); the hierarchy of uptake/retention was similar to that observed in other cell types (21). As one approach to determine whether the peptides retained at lower levels were functional inhibitors, their effect on SDF-1α-induced polarization was determined by single-blind scoring of shape change of F-actin-stained peripheral blood T lymphocytes (PBT) after 5 min of SDF-1α exposure. One hundred cells were scored single-blind as either round or “fully polarized” based on F-actin distribution; polarization inhibition was calculated relative to SDF-1α-treated Tat peptide-exposed cells, which was taken as 0%. Peptide retention as low as 6 MFI was associated with inhibition of lymphocyte polarization (RKR→AAA Rac1 peptide). Similarly, low-level intracellular retention of the Cdc42 peptide was sufficient to block cell polarity in epithelial cells (21).

**Microscopy**

A Zeiss LSM 510 meta (Oberkochen, Germany) equipped with a 100× planapochromat optical lens (aperture 1.4) was used to examine cells. For scanning electron microscopy cells after treatment were fixed, processed, observed, and photographed with the Hitachi S-570 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 10 kV as previously described (10).

**Flow cytometry**

For assessing the phospho-ERM levels in transfected PBT, cells, after stimulation with SDF-1α for 30 s, were fixed and permeabilized as described previously (10) and stained with anti-phospho-ERM Ab that reacts with ezrin pT564, radixin pT567, and moesin pT558 (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h. Cells were then incubated with preadsorbed rabbit conjugated secondary IgG conjugated to biotin (Jackson Immunoresearch Laboratories, West Grove, PA) and subsequently streptavidin-PE (Molecular Probes). When analyzing cell populations transfected with GFP-containing constructs, the analysis was performed by gating on GFP-positive cells.

**Biochemical analysis**

PBT whole cell lysates were prepared and resolved by SDS-PAGE as described previously (10). Detection of cpERM and ERM proteins on the gels was done by immunoblotting using anti-cpERM Ab and anti-moesin Ab (Lab Vision, Fremont, CA). The pull-down assay for Rac1 activation used the biotinylated synthetic peptide from the p21-activated kinase Cdc42/Rac interactive binding domain as previously described (22). Human PBT were starved in serum-free RPMI 1640 for 3 h with or without pertussis toxin (100 ng/ml). Cells were then stimulated with SDF-1α, lysed, pull-down performed, and Rac1 detected using anti-Rac1 Ab (BD Transduction Laboratories, San Diego, CA).

**Statistical analysis**

Comparison between two groups was made using a two-sided Student’s t test. Two-sided p < 0.05 were considered to be significant.

**Results**

**Rac1 mediates the SDF-1α-induced microvillar disassembly**

To explore potential contributions of individual small G proteins in the disassembly of microvilli, human peripheral T lymphocytes (PBT) were transfected with GFP-tagged constitutively active versions (CA) of RhoA, Rac1, and Cdc42. The effect of these

<table>
<thead>
<tr>
<th>Peptide</th>
<th>G Protein Source</th>
<th>Peptide Retention (MFI)</th>
<th>% Inhibition of Polarization with SDF-1</th>
<th>Sequence C-terminal to Tat</th>
<th>Previous Functional Validation (21)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat</td>
<td>None</td>
<td>2</td>
<td>0</td>
<td>None</td>
<td>LKARRGKKSG</td>
<td>Blocks serum-induced contraction in fibroblasts</td>
</tr>
<tr>
<td>RhoA</td>
<td>RhoA</td>
<td>17</td>
<td>73</td>
<td>LQRPKEKSR</td>
<td>RKR→AAA Rac1 peptide</td>
<td>Blocks cell polarization in epithelial cells</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cdc42</td>
<td>2</td>
<td>1</td>
<td>LEPEPKEKKRR</td>
<td>Rac2</td>
<td>Unlike Rac1 peptide, does not block epithelial cell adhesion</td>
</tr>
<tr>
<td>Rac2</td>
<td>Rac2</td>
<td>60</td>
<td>0</td>
<td>CPQPTRQQKRA</td>
<td>RKR→AAA Rac1 (mutated)</td>
<td>Blocks membrane ruffling and polarized cell-cell adhesion in epithelial cells</td>
</tr>
<tr>
<td>Rac1</td>
<td>Rac1</td>
<td>25</td>
<td>91</td>
<td>CPFVFKKRRK</td>
<td>PPP→AAA Rac1 (mutated)</td>
<td>Blocks polarized cell-cell adhesion but not ruffling</td>
</tr>
<tr>
<td>RKR→AAA</td>
<td>Rac1 (mutated)</td>
<td>6</td>
<td>81</td>
<td>CPFVFKKAAA</td>
<td>CAAAVKKRRK</td>
<td>Blocks membrane ruffling and polarized cell-cell adhesion</td>
</tr>
</tbody>
</table>

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GTPases on microvilli was studied by scanning electron microscopy. The surface of GFP-transfected T cells (Fig. 1a) was rich in microvilli similar to those of untransfected cells (10). In contrast, transfection with Rac1-CA (Fig. 1d) and Cdc42-CA (Fig. 1c) induced dramatic loss of normal-length microvilli; the residual “pebbled” surface of some such cells may represent the residua of microvilli. Although Rac1-CA and Cdc42-CA shared the ability to reduce loss of microvilli, their surface phenotype differed in other respects. The Rac1-CA-transfected cells had a variety of peripheral processes that could be broadly described as ruffles. In many Rac1-CA-transfected cells, the peripheral processes were multifocal; in others, there was a single peripheral process indistinguishable from an early lamellipod. It should be noted that there is substantial overlap between the range of phenotypes observed with Rac1-CA (Fig. 1d) and those seen in lymphocytes undergoing SDF-1α-induced retraction of microvilli, one needs to block the function of these GTPases to see whether the SDF-1α-induced response is blocked.

FIGURE 1. Constitutively active Rac1 and Cdc42 can both disassemble T cell microvilli. Human PBT were nucleoporated with GFP alone (a) or GFP-tagged constitutively active forms of the Rho family GTPases RhoA-CA (b), Cdc42-CA (c), or Rac1-CA (d). The samples were prepared for conventional scanning electron microscopy after 20 h of transfection. Arrowheads in d indicate lamellipodia and membrane ruffles. All images were acquired at ×6000 and the relative size was maintained during figure composition. Images are representative of the unique phenotypes observed with each construct in three independent experiments.

In certain contexts, DN constructs are powerful tools for investigating the functional role of individual small G proteins in biological responses. However, conceptual and technical issues also described previously (23) made DN constructs uninformative for our scanning electron microscopy studies of the SDF-1α response: 1) the DN construct themselves induced a phenotype in the absence of SDF-1α treatment and 2) a problem in scoring resulted from partial transfection complicated by overlapping phenotypes (i.e., the predicted phenotype of inhibited cells resembles the phenotype of unresponsive cells always present in culture). Recently, a powerful alternative approach has emerged which uses peptides containing sequences unique to a particular GTPase to inhibit function of that GTPase. Peptides from the C termini or effector loop have been particularly effective and are thought to inhibit in part by blocking translocation and/or interaction with effector/adaptor proteins (21, 24, 25). The peptides penetrate the cell rapidly because they contain a transduction motif that facilitates transfer across the plasma membrane (26); these fusion peptides require only brief exposure and show high efficiency of inhibition, making biochemical and morphological analysis simple. Encompassing the unique sequence from the C-terminal of GTPases, the synthetic peptides work as “decoy” by specifically interfering with localization and/or colocalization of relevant interacting proteins of each of these GTPases as previously established (Ref. 21 and Table I). We therefore investigated the potential involvement of four different small G proteins (Table I) in the SDF-1α-induced loss of microvilli by pre-exposure of PBT to corresponding C-terminal peptides for 5 min. Cells stimulated for 1 min with SDF-1α were ruffled and partially polarized (Fig. 2c) as previously described (10). Such cells were devoid of normal-length microvilli but often

FIGURE 2. Rac1 mediates SDF-1α-induced resorption of microvilli in human PBT. Peptides were synthesized using sequences corresponding to the C-terminal regions (without the CAAX motif) of Rho, Rac1, Cdc42, and Rac2 fused to the Tat domain of HIV (Table I). Human T cells were exposed to these selective Rho GTPase inhibitory peptides 5 min before a 1-min stimulation with SDF-1α. The cells were fixed and processed for scanning electron microscopy analysis. Arrowheads point to examples of membrane ruffles characteristic of SDF-1α response in T cells. Both resorption of microvilli and membrane ruffling induced by SDF-1α was blocked specifically by the Rac1 peptide.
residua of microvilli were apparent in cells whose polarization is in a relatively early stage. The C-terminal peptide from Rac1 completely blocked loss of microvilli; it also completely blocked the emergence of membrane ruffles (Fig. 2e). In contrast, neither the control peptide nor the peptides from RhoA, Cdc42, or Rac2 blocked loss of microvilli or ruffle formation (Fig. 2, f–h). Thus, evidence from overexpressed active G protein and inhibition by Trojan peptide establish Rac1 as a fundamental mediator of SDF-1α-induced loss of microvilli.

**Rac1 mediates SDF-1α-induced cpERM dephosphorylation**

We further investigated the molecular basis of those Rac1-mediated changes. Given the crucial function conducted by dephosphorylation of cpERMs in SDF-1α-induced disassembly of microvilli (10) and the present evidence for Rac1 GTPase regulation of microvilli breakdown, we next explored the relationship between cpERM dephosphorylation and Rac1 signaling. We used a flow cytometric approach for quantitative analysis of cpERM phosphorylation of cells transfected with mutant G protein constructs. The results demonstrate that both Rac1-CA and Cdc42-CA induced dramatic cpERM dephosphorylation (Fig. 3a); in contrast, RhoA-CA induced a marked increase in cpERM (Fig. 3a). It is important to note the concordance between the effects of the G protein constructs on cpERM and their effects on lymphocyte microvilli (Fig. 1), which can be explained by a requirement for cpERM in formation of microvilli.

DN constructs were used to determine whether Rac1 and/or Cdc42 mediate the SDF-1α-induced cpERM dephosphorylation. Inhibition by Rac1-DN but not Cdc42-DN constructs indicates that SDF-1α-induced dephosphorylation is mediated by Rac1 (Fig. 3b). In addition, inhibition studies with C-terminal peptides were performed to further establish the link. Rac1 peptide efficiently blocked chemokine-induced dephosphorylation (Fig. 3c); Cdc42, RhoA, and Rac2 peptides did not inhibit, consistent with their lack of effects on surface morphology (Fig. 2). Note that basal cpERM phosphorylation levels were variably influenced by the Rac1 peptide: moderately decreased in some experiments (e.g., Fig. 3c) and unchanged in other experiments (e.g., see Fig. 7a). This somewhat variable basal behavior contrasted with the virtually complete inhibition of SDF-1α-induced dephosphorylation by Rac1 peptide, which was seen in every experiment. Overall, these results confirmed the critical and singular role of Rac1 in mediating SDF-1α-induced cpERM dephosphorylation.

We have previously demonstrated that SDF-1α-induced cpERM dephosphorylation involves a calyculin A-sensitive phosphatase (10). Therefore, inhibition of G protein-mediated cpERM dephosphorylation by Calyculin A would be expected if those G proteins are involved in SDF-1 signaling upstream of the phosphatase. Consistent with this prediction, calyculin A inhibited cpERM phosphorylation induced by Rac1 and Cdc42, as well as SDF-1α (Fig. 3a). This model predicts that blocking dephosphorylation will overcome the Rac1-induced loss of microvilli. Scanning electron microscopy studies demonstrated that within 5 min of calyculin A treatment there is marked reversal of the Rac1 phenotype of decreased microvilli (Fig. 4 and legend). Thus, Rac1-induced loss of microvilli depends on dephosphorylation.

**SDF-1α induces rapid and transient Rac1 activation**

We measured the activation of Rac1 during SDF-1α stimulation of PBT with a pull-down assay using a biotinylated peptide corresponding to the Cdc42/Rac active interaction binding domain of p21-activated kinase. The results demonstrate that the peak of Rac1 activation by SDF-1α occurred early (15 s) and that active Rac1 levels fell to ~2-fold above baseline by 30 s (Fig. 5a). Previous studies of SDF-1α-stimulated lymphocytes have been inconsistent in detection of Rac1 activation (27, 28); a possible explanation is provided by the present findings that Rac1 activation on SDF-1α stimulation occurs very early and is transient. One notable characteristic of cpERM dephosphorylation is its onset within seconds

![FIGURE 3. SDF-1α-induced cpERM dephosphorylation is regulated by Rac1. a. Effect of Rho GTPases on cpERM phosphorylation: Human PBT were nucleofected with GFP alone or GFP-tagged constitutively active forms of the Rho family GTPases, treated (or untreated) with calyculin A for 5 min, and stained for cpERM. Levels of phosphorylation of ERM proteins in GFP-transfected cells were measured by flow cytometry. Data shown represent the median cpERM levels in each preparation expressed relative to the level present in GFP-transfected control cells. The horizontal dashed line represents the baseline level (100% of control). n = 2, mean ± SD. cpERM in cells transfected with Cdc42-CA and Rac1-CA was lower than in the controls (p < 0.01); cpERM was increased by 5-min exposure to calyculin A in the Cdc42-CA- and Rac1-CA-transfected cells (p < 0.01) as well as in the SDF-1α-treated control cells. b. DN Cdc42-DN and Rac1-DN constructs were transfected into PBT to study their effects on SDF-1α-induced cpERM dephosphorylation. Cells were processed as above after SDF-1α stimulation for 30 s. Data shown represent the median cpERM levels in each SDF-1α-stimulated preparation expressed relative to the level present in the same preparation of cells before stimulation. n = 3, Mean ± SD, *, p < 0.01; **, p < 0.05. c. Influence of shRNA on cpERM phosphorylation. Human PBT were untransfected or exposed to Tat, RhoA, Rac1, Rac2, or Cdc42 peptides (Table I) 5 min before SDF-1α stimulation for 30 s. The cells were lysed and immunoblotted for both cpERM and total moesin. Moesin is abundant in PBT (lower band), ezrin is moderately expressed (upper band) while radixin is absent in lymphocytes (10).
of SDF-1α stimulation (10). To ascertain whether Rac1 activation was sufficiently rapid to mediate cpERM dephosphorylation, we analyzed the earliest time point that is technically feasible, 5 s. The results (Fig. 5, b and c) demonstrated that Rac1 activation was already at its maximum value by 5 s; at that time, marked dephosphorylation of cpERM was also evident. Thus, the rapid kinetics of Rac1 activation was fully consistent with a role in inducing cpERM dephosphorylation.

Inhibition of Rac1 activation by pertussis toxin (Fig. 5d) confirms that Rac1 activation by SDF-1α is via the conventional Grα-dependent pathway as previously demonstrated for SDF-1α-induced cpERM dephosphorylation (10). Collectively, the data show that Rac1 is rapidly activated by SDF-1α in primary T cells and establishes that the SDF-1α-induced collapse of microvilli results from Rac1-mediated signaling.

**Rac1 mediates the effects through basic residues in C terminus**

The Rac1 C-terminal peptide has two distinct sequence elements: a PPP motif that interacts with Src homology 3 (SH3) containing proteins such as Crk and a RKR motif that is involved both in interaction with membrane and with phosphatidylinositol 4,5-phosphate kinase (PPI4P5K) (21). To shed light on potential effectors in SDF-1α-induced Rac1 signaling, we investigated inhibition by C-terminal Rac1 mutant peptides that each contain only one of the two motifs (Table I). Note that the PPP→AAA peptide retains an intact RKR motif and acts as a competitive inhibitor of RKR functions; similarly, RKR→AAA blocks PPP motif-binding effectors. The results demonstrated that the PPP→AAA peptide (intact RKR motif) blocked the SDF-1α-induced dephosphorylation, while the RKR→AAA peptide had no effect (Fig. 6a). The ability of the PPP→AAA peptide to block dephosphorylation indicates that the PPP motif (and therefore interactions with Crk) is not essential and underscores the importance of the RKR motif in mediating cpERM dephosphorylation. Since cpERM dephosphorylation is required for loss of microvilli, the Rac1 RKR element should be required for SDF-1α-induced loss of microvilli. Results of scanning electron microscopy analysis (Fig. 6b) confirmed this prediction; the PPP motif (RKR→AAA peptide) containing peptide was unable to block loss of microvilli but the RKR residues (PPP→AAA peptide) containing peptide competitively blocked all morphological changes. Altogether, basic residues of Rac1 C terminus are critical to SDF-1α-induced dephosphorylation, which in turn is critical to microvillar breakdown.

**Discussion**

Rac1 is a multifunctional switch that influences diverse cellular functions. It is involved in proliferation, differentiation, gene expression as well as formation of membrane ruffles and/or lamellipodia (29, 30). The present study documents a new cytoskeletal function of Rac1 in T cells and sheds light on the molecular basis of this function. We show that Rac1 mediates loss of microvilli upon chemokine stimulation of circulating lymphocytes. Moreover, in a functional sense, ERM proteins are Rac1 effectors that mediate microvillus breakdown.

ERM proteins, specifically phosphorylated ERMs, play a fundamental role in maintenance of microvilli. The structural organization of an ERM protein is well suited to its role in reversibly connecting membrane proteins to microfilaments: an N-terminal FERM domain that binds to the tails of transmembrane proteins; an α helical rod-like region; and a C-terminal region that binds to F-actin (for reviews, see Refs. 16–18). ERM proteins exist in two conformationally regulated forms: open and closed. In the closed (inactive) form, intramolecular association between the N-terminal FERM domain and C-terminal tail masks the membrane binding as well as actin binding sites. ERM proteins are conformationally induced into an open active form by phosphoinositide binding and phosphorylation (31). Phosphorylation of a conserved C-terminal threonine stabilizes ERM proteins in an open conformation and thereby facilitates binding to actin and cytoplasmic tails of membrane proteins (32–34). Studies from several groups have established strong functional connections between ERM phosphorylation and the establishment/maintenance of microvilli in certain cell types (35–37). Conversely, dephosphorylation of ERM proteins is associated with release of ERM proteins from cytoskeleton, thus contributing to disassembly of microvilli (10, 38, 39). Findings from the current morphological and biochemical analyses establish a strong connection between Rac1 and ERM in the SDF-1α response. Specifically, 1) SDF-1α induces Rac1 activation (Fig. 5, a and b); 2) the speed of the Rac1 activation is sufficiently rapid to account for cpERM dephosphorylation (Fig. 5b); 3) overexpression of constitutively active Rac1 induces both cpERM dephosphorylation and loss of microvilli; (Figs. 1 and 3a); 4) DN Rac1 blocks cpERM dephosphorylation (Fig. 3b); 5) disruption of Rac1 function by a Trojan peptide both abolishes cpERM dephosphorylation and loss of microvilli (Figs. 2 and 3c); and 6) blocking...
cpERM dephosphorylation with calyculin A reverses Rac1-induced microvilli retraction (Fig. 4). Thus, the present studies establish that ERM proteins act as control points for Rac1-induced disassembly of microvilli.

The rapidity and duration of Rac1 activation during signal transduction ranges from very rapid to rather prolonged, depending on the cell type and stimulus (40–43). The Rac1 activation seen in our studies is both very rapid and transient, with a peak as early as 5 s; Rac1 activation such as this, which peaks earlier than 15 s, is physiological but rare in the literature (43, 44). Such rapidity of Rac1 activation in response to SDF-1/H9251 is a logical necessity in order for Rac1 to play a role in early events of lymphocyte adhesion to endothelium (4), including initiation of the loss of microvilli and of polarization. Although dephosphorylation of ERM is a critical initiating step in disassembly of microvilli, the physical process of disassembly requires both time and participation of other biochemical processes. Two considerations raise the possibility that disassembly of microvilli in vivo may be much more rapid than is reflected in this in vitro model system (roughly 30 s).

First, our approach is a reductionist approach in which only a single activation signal is used, SDF-1/H9251. In contrast, in vivo binding of lymphocyte to endothelium involves additional key signals such as integrin binding to endothelium and flow-generated shear forces which can dramatically augment signals (45). Second, the...
changes we observe in microvilli are in the absence of applied compressive force. In contrast, under physiological conditions, microvilli present in the contact region between lymphocytes and endothelium are subject to compression since shear-mediated forces push the lymphocyte toward the endothelium.

Activation of small G proteins results in release of GDP and formation of a GTP-bound complex, whose unique conformation mediates downstream signaling. In addition, dynamic regulation of spatial localization is a second key mechanism controlling these small G proteins and their interactions with effector molecules (21, 46–48). The hypervariable C termini of small G proteins are of pivotal importance in their specific function, both by controlling localization within the cell and by facilitating interactions with particular effectors (21, 49, 50). The C terminus of Rac1 contains two different binding motifs: a proline-rich sequence and a basic RKR sequence. Those two motifs contribute to binding to different partners and thereby different functions of Rac1. Proteins with the SH3 domain such as Crk, Nck, and Grb2 have been found to interact with the proline-rich motif of Rac1. The basic residues, in contrast, can bind to PI4P5K and protein kinase C-related kinase 1 (21, 51). SH3-mediated interactions with the Rac1 C terminus may not be critical in the SDF-1α response since the mutant Rac1 peptide retaining the PPP motif cannot inhibit. In contrast, the basic residues in the C terminus must be present in the blocking peptide in order for it to inhibit function (Fig. 6). The requirement for basic residues is likely to reflect 1) their role in Rac1 association with the distinct membrane domain and/or 2) their role in Rac1 association with partners such as PI4P5K and protein kinase C-related kinase 1, both of which can participate in actin reorganization.

Rac2, which is abundantly expressed in hemopoietic cells, is highly homologous to Rac1 but differs markedly in the polybasic region C terminus. We anticipated that Rac2 would not be involved in cpERM dephosphorylation or resorption of microvilli given the importance of the Rac1 C terminus in this function and the difference between Rac1 and Rac2 in their C termini. Results with the Trojan peptide confirm the prediction that Rac2 is not required for these SDF-1α responses. These findings are consistent with the fact that their divergent C termini contribute to 1) preferential Rac2 localization to endomembranes and Rac1 predominant localization to plasma membrane (52) and 2) distinct roles of Rac1 and Rac2 in other cellular responses (46–48).

Our data also indicate that Cdc42 is not a primary mediator of SDF-1α-induced cpERM dephosphorylation and microvillus collapse. Two approaches fail to detect such a role for Cdc42. First, Cdc42 DN does not block SDF-1α-induced cpERM dephosphorylation, which is commonly viewed as sufficient evidence to discount Cdc42 involvement. Second, Cdc42 C-terminal peptide does not block SDF-1α-induced cpERM inactivation or microvillar collapse. These findings do not conflict with data showing that Cdc42 can be forced to induce cpERM dephosphorylation and collapse of microvilli by long-term overexpression of its constitutively active form. Rather, such function of the overexpressed Cdc42 is consistent with shared effectors of Rac1 and Cdc42 (53, 54). Importantly, the failure of Cdc42 blocking agents to inhibit the SDF-1α response indicates that Cdc42 is not the physiological mediator in this response. By the same logic, these findings do not support a role of Cdc42 upstream of Rac1, as has been demonstrated in other responses (11, 55).

Previous studies in diverse cell types including ours in lymphocytes indicate that cpERM dephosphorylation is in dynamic equilibrium with ERM phosphorylation (boxed region in Fig. 7) and that the equilibrium is shifted by external signal. For instance, following SDF-1α stimulation, dephosphorylation dominates, but under other circumstances, such as thrombin activation of platelets, phosphorylation dominates (33). Studies in migrating neutrophils show that the balance of ERM phosphorylation and dephosphorylation is dynamically regulated in space and time (56). Previous studies in some cell types demonstrate that RhoA promotes phosphorylation; however, the corresponding mediator for dephosphorylation has not been identified (14, 19). The present findings and our recent related studies of TCR-induced activation (57) reveal that Rac1 is a major stimulus in lymphocytes for cpERM dephosphorylation. Thus, in a functional sense, Rac1 and Rho play reciprocal roles in regulating cpERM phosphorylation. In other cell types, RhoA induces moesin phosphorylation via one or more moesin kinases (35, 58). For reasons of simplicity, we favor a model in which a counteracting ERM phosphatase is activated by Rac1 (directly or indirectly); more complex modes are also possible given the extensive reciprocal regulation between Rac1 and Rho (59). Inhibition by calyculin A implicates a PP1/PP2A phosphatase in both the SDF-1α-induced and the Rac1-induced dephosphorylation of cpERM; this indicates that the phosphatase acts downstream of Rac1 and is consistent with studies implicating myosin L chain phosphatase as the moesin phosphatase (56, 60).

Rac1-mediated ERM dephosphorylation may be a mechanism of general importance in signaling pathways involving cytoskeletal remodeling. We have recently demonstrated that Rac1 mediates...
Rac1-TRIGGERED MICROVILLAR BREAKDOWN

References


