The C-terminal Domain of Rac1 Contains Two Motifs That Control Targeting and Signaling Specificity*§

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Rho-like GTPases control a wide range of cellular functions such as integrin- and cadherin-mediated adhesion, cell motility, and gene expression. The hypervariable C-terminal domain of these GTPases has been implicated in membrane association and effector binding. We found that cell-permeable peptides, encoding the C termini of Rac1, Rac2, RhoA, and Cdc42, interfere with GTPase signaling in a specific fashion in a variety of cellular models. Pull-down assays showed that the C terminus of Rac1 does not associate to either RhoGD1 or to Pak. In contrast, the C terminus of Rac1 (but not Rac2 or Cdc42) binds to phosphatidylinositol 4,5-phosphate kinase (PIP5K) via amino acids 185–187 (RKK). Moreover, Rac1 associates to the adapter protein Crk via the N-terminal Src homology 3 (SH3) domain of Crk and the proline-rich stretch in the Rac1 C terminus. These differential interactions mediate Rac1 localization, as well as Rac1 signaling, toward membrane ruffling, cell-cell adhesion, and migration. These data show that the C-terminal, hypervariable domain of Rac1 encodes two distinct binding motifs for signaling proteins and regulates intracellular targeting and differential signaling in a unique and non-redundant fashion.

Rho-like GTPases drive temporally and spatially coordinated actin polymerization to control cell motility, cadherin-based cell-cell adhesion, and cytokinesis (1–4). To relay their signals, Rho-like GTPases interact with a wide range of effector proteins. These interactions are generally thought to occur at the plasma membrane to which GTPases are translocated prior to activation by guanine nucleotide exchange factors. This membrane association is dependent on lipid modifications of specific cysteine residues in the extreme C terminus (i.e. prenylation), as well as on the C-terminal polybasic region (5).

Rho-like GTPases share a high level of homology, despite the fact that their effects on cellular morphology or function can be very distinct. This has been attributed to sequence diversity in the effector domain (residues 26–45) and the so-called insert region (amino acids 124–135). However, the most divergent region between otherwise very homologous GTPases (e.g. Rac 1066 CX, and Rac2) is the C-terminal polybasic region. This domain was shown to be required for activation of the neutrophil NADPH oxidase (6) and was claimed for Rac1 to drive activation of PAK1 by some (7) but not by others (6). Most of the studies that have addressed the role of this domain relied on deletion or mutation strategies, testing the role of this region in the context of, for instance, a constitutively active variant of the GTPase.

Tao et al. (5) showed recently that deleting the C-terminal polybasic region affects the efficiency of prenylation of Rac2. Moreover, del Pozo et al. (8) have shown that mutation of the crucial cysteine residue to prevent Rac prenylation blocked activation of PAK. This means that mutating or deleting the C-terminal domain may affect downstream signaling in an indirect manner, complicating the interpretation of this type of analysis.

In an extensive study using GFP-tagged versions of a range of Rho-like GTPases, Michaelson et al. (9) showed the differential intracellular localization of various Rho-like GTPases to the plasma membrane, the Golgi, etc. These authors suggested that the hypervariable domain determines which membrane compartment the GTPases are targeted, although the mechanism of this targeting was not identified.

To study the role of the hypervariable C-terminal domain in the targeting and signaling specificity of Rho-like GTPases, in particular of Rac1, we hypothesized that this region may act as a selective, targeted inhibitor when brought into living cells. To test this hypothesis, we fused the C-terminal domains of Rac1, Rac2, RhoA, and Cdc42, excluding the CAXX region, to the protein transduction domain (PTD) of the human immunodeficiency virus TAT protein (10, 11) and tested modulation of signaling by these GTPases in a variety of assays in adherent and non-adherent cells. The results show that the C-terminal domain of Rho-like GTPases is differentially targeted to intracellular sites and specifically interferes with GTPase function because of differential associations with effector, as well as adapter, proteins.

MATERIALS AND METHODS

Cell Culture—The HL60, KG1a, NIH3T3, MDCKII, and COS7 cell lines were maintained in Issviv’s modified Dulbecco’s medium (Bio-
Whittaker) containing 2 mM l-glutamine, 100 units/ml penicillin, 100 
µg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Invitro-
gen) at 37 °C/5% CO2. Adherent cells were passaged by trypsinization. 

Immortalized human umbilical vein endothelial cells (HUVEC) were 
cultured as described (12).

Peptides, Loading, Quantification, and Localization—For this study,
we made use of synthetic peptides (peptide synthesis was performed on
a Syro II, using Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid phase
chemistry) encoding the C-terminal domain of Rac1, Rac2, RhoA, and 
Cdc42, excluding the CAA
X box, fused to a protein transduction domain
of human immunodeficiency virus, from here on denoted as TAT (see
Fig. 1A). HL60 or trypsinized MDCKII cells in a concentration of 1
× 10^6 cells/ml were incubated with 200–1,000 
µg/ml biotinylated TAT-
Rac1, TAT-Rac2, or TAT-Cdc42 C-terminal peptides for various times.
At the indicated time points, 100 µl of the cell suspension was trans-
ferrred to 100 µl of fixation solution (Intraprep Permeabilization Rea-
gen; Coulter Immunotech). After 15 min at room temperature, the cells
were washed with phosphate-buffered saline/0.5% BSA (Sigma), resus-
spected in 100 µl of permeabilization solution (Intraprep Permeabiliza-
tion Reagent; Coulter Immunotech), and incubated for 10 min at room
temperature. The cells were subsequently exposed to Streptavidin-
fluorescein isothiocyanate (1:100; Immunopure; Pierce) or Streptavidin-
Alexa 488 (1:100; Molecular Probes) on ice and in the dark during 45
min. F-actin in MDCKII cells was made visible by Texas Red Phalloidin (1:500; Molecular Probes). Phase
contrast and fluorescent confocal images and movies were recorded
with a Zeiss LSM 510 confocal laser scanning microscope.

**Determination of the C-terminal Peptide Specificity**—The specificity
of the TAT-RhoA and the TAT-Rac1 C-terminal peptides was deter-
mined in three separate, established models. In the first model, serum-
starved NIH3T3 fibroblasts were exposed to the 400 
µg/ml of the var-
ious TAT C-terminal peptides during 30 min at 37 °C before adding 10%
heat-inactivated fetal calf serum. The control situation, cells respond
to fetal calf serum with RhoA-mediated contraction. In the second
model, GFP-Rac1-expressing HUVEC were exposed to 1 mg/ml of the
C-terminal peptides, and the effect on the spontaneous, Rac1-mediated
membrane ruffling, cell spreading, and contractility was determined.
The third model comprised GFP-Rac1 transduced MDCKII cells exposed to 10 ng/ml HGF during 30 min, which induces Rac1-mediated membrane ruffling. Subsequently, the various C-terminal peptides in a concentration of 1 mg/ml were added to the cells, and the effect on the stimulus-induced ruffling, cell spreading, and contractility was assessed. In all models, the effect of the C-terminal peptides was recorded using time-lapse fluorescence or phase contrast microscopy.

**Cell Migration and Actin Polymerization Assays**—Transmigration assays (13) were performed in 6.5-mm, 5-μm pore Transwell plates, coated overnight with 20 μg/ml fibronectin (Sigma) at 4 °C. The cells used were pre-treated with 200 μg/ml of the indicated TAT C-terminal peptides (TAT sequence YGRKRRQRRR) for 30 min and washed with Iscove’s modified Dulbecco’s medium/0.25% BSA. KG1a cells were exposed to the peptides during the 30-min pre-treatment and during the migration assay. Pretreated, freshly isolated CD34+ cells from cord blood or mobilized peripheral blood (13), B-CLL blasts, HL60, or KG1a cells (100,000 cells/Transwell) were added to the upper compartment and were allowed to migrate toward 100 ng/ml SDF-1 (HL60) or to medium (KG1a), present in the lower compartment, for 4 h. Actin polymerization assays were performed as described previously (14). Briefly, after a 30-min pre-treatment with 200 μg/ml of the C-terminal peptides, HL60 cells were exposed to 100 ng/ml SDF-1. At the indicated time points 100 μl of the cells was transferred to 100 μl of fixation solution (Intraprep Permeabilization Reagent) for at least 15 min. The cells were washed in phosphate-buffered saline/0.5% BSA and resuspended in 100 μl of permeabilization reagent (Intraprep Permeabilization Reagent). After 5 min 1 unit/ml Alexa 488 phallolidin (Molecular Probes) was added to visualize the F-actin. After 20 min the cells were centrifuged and resuspended in phosphate-buffered saline/0.5% BSA.

Mean fluorescence intensity was measured by fluorescence-activated cell scan (BD Biosciences) and used as a measure for the amount of polymerized actin. The fold increase actin polymerization was calculated by dividing the mean fluorescence intensity generated at a particular time point by the mean fluorescence intensity at t = 0 of that particular condition.

**Pull-down Assays**—To assay the activity of Rac1 and RhoA in MDCKII cells (5 × 10⁶ cells) following incubation with the different C-terminal peptides (15 min, 200 μg/ml), pull-down assays were performed with GST-PAK-CRIB and GST-Rhotekin, respectively. Cells were lysed in Lysis Buffer A (50 mM Tris/His, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol, and 1% Nonidet P-40) and incubated with one of the GST fusion proteins and glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 1 h while rotating. After incubation, samples were washed five times in Lysis Buffer A and resuspended in 25 μl of SDS sample buffer. Rac1 or RhoA binding was determined by Western blot analysis as described (15).

To determine the association of signaling proteins with the different C-terminal peptides, 5 × 10⁶ MDCKII or Cos7 cells were seeded 1 day prior to the assay. Cells were lysed in Lysis Buffer B (50 mM Tris/HCl, pH 7.5, 400 mM NaCl, 10 mM MgCl₂, 10% glycerol, 1% Nonidet P-40, and 0.1% SDS), centrifuged for 10 min at 14,000 rpm at 4 °C. The supernatant was then incubated with 10 μg of one of the C-terminal peptides and with Streptavidin-coated beads (Sigma) at 4 °C for 1 h while rotating. In these experiments, equal amounts of the different peptides were added to cellular lysates to exclude that differences in peptide loading of cells would cause variations in the detection of peptide-protein complexes. Samples were then washed in Lysis Buffer A five times and either resuspended in 25 μl of SDS sample buffer or used for the PIP5K activity assay (16). Ori binding to the various peptides was determined by pull-down assays as described above followed by Western blot analysis. For analysis of binding of the C terminus of Rac1 to the different...
v-Crk mutants, the v-Crk, v-Crk 1 SH2, and v-Crk 3 SH3 proteins were expressed in Cos7 cells, prior to the pull-down assays.

**Lipid Raft Visualization**—Lipid rafts in HL60 cells were stained with cholera toxin subunit B (CTX; 10 µg/ml final concentration, 15 min, 37 °C; Molecular Probes) after a 5-min incubation with TAT-Rac1 WT, TAT-Rac1 PPP, and TAT-Rac1 RRK (1 mg/ml) at 37 °C.

**Statistical Analysis**—Significance of differences was determined with a two-sided Student’s t test. Two-sided p values less than 0.05 were considered significant.

**RESULTS**

**Differential Loading of the Cell-permeable C Termini**—To determine the kinetics and levels of cell loading for the various C-terminal peptides used in this study (Fig. 1A), HL60 leukemic (Fig. 1, B and C) and MDCKII epithelial cell lines (not shown) were transduced with biotinylated versions of the peptides. Already within 1 min, 100% of the exposed cells were positive for the transduced peptide (Fig. 1B). Interestingly, although the percentage of positive cells was similar for all peptides used, the relative level of cell loading differed. The Rac1 and Rac2 C termini accumulated to higher amounts, compared with the Cdc42 peptide, even though cells were exposed to equal concentrations of peptide (Fig. 1C). The RhoA C terminus was transduced as efficiently as Rac1 (not shown).

Peptide loading remained detectable for 2–4 h, after which the levels of fluorescence declined, possibly because of proteolytic breakdown. Detection of peptide loading required cell permeabilization, indicating that the peptides were present inside the cell, rather than associated with the cell surface. These data suggest that these C-terminal peptides are transduced rapidly into adherent, as well as non-adherent, cells and are selectively sequestered, indicating that the C-terminal domains of Rho-like GTPases differentially associate to intracellular proteins.

**Rho-GTPase C Termini Mediate Selective Inhibition of Cell Contraction, Membrane Ruffling, and Cell Polarization**—We next tested whether the C-terminal peptides could be used to dissect intracellular GTPase signaling. RhoA-mediated, serum-induced contraction of NIH3T3 fibroblasts was selectively blocked by the TAT-RhoA C-terminal peptide (TAT-RhoA) but not by the PTD or the TAT-Rac1 peptide (Fig. 2A, movie 1, a–c). TAT-Cdc42 also did not inhibit serum-induced contraction (data not shown). Conversely, in GFP-Rac1-expressing HUVEC, the TAT-Rac1 peptide induced a rapid loss of spontaneous membrane ruffling and a concomitant loss of GFP-Rac1 localization, whereas cell spreading and contractility were not impaired (Fig. 2B, movie 2).

To further confirm the specificity of the effects of these C-terminal peptides, GFP-Rac1-expressing MDCKII cells were exposed to the various peptides following treatment with HGF (17), which induced formation of prominent lamellae and peripheral membrane ruffles, brightly positive for GFP-Rac1 (Fig. 2C). The TAT-Rac1 or a TAT-Rac1 17–32 peptide (a Rac-inhibiting peptide (18)) blocked membrane ruffling within 1–3 min and induced a diffuse GFP-Rac1 distribution (Fig. 2C, movie 3). Cell spreading was unaffected, but membrane ruffling and GFP-Rac1 localization to these ruffles was blocked. Apparent, both the effector loop and as the C terminus of Rac1 are required, in a non-redundant fashion, for growth factor-induced membrane ruffling and proper Rac1 localization. The levels of GFP-Rac1 at cell-cell junctions remained unaffected (data not shown). In contrast to the Rac1, the RhoA, Rac2, and Cdc42 C-terminal peptides did not affect either HGF-induced ruffling or GFP-Rac1 localization, further underscoring the specificity of the effects exerted by these C termini.

Rho-like GTPases, in particular Rac1 and Cdc42, control polarization of cadherin-based cell-cell adhesion in epithelial cells (4, 19). In agreement with this notion, transduction of MDCKII cells with both TAT-Rac1 and TAT-Cdc42 peptides reduced cell polarity and induced cell spreading (Fig. 2D). In addition, increased stress fiber formation in the lower region of the TAT-Rac1 transduced cells was observed, in line with the antagonism between Rac1 and RhoA in epithelial cells (15, 20). The TAT-RhoA peptide induced increased cell spreading but had no major effects on cell polarization. The TAT-Rac2 C-terminal peptide did not affect either MDCKII cell-cell adhesion or spreading (data not shown).
Summary of the inhibition of SDF-1-induced migration of various cell types by the indicated peptides as compared to the migration of TAT-treated cells

* Significant \( (p < 0.05) \) inhibition in comparison with TAT-treated cells.

| Cell Type | Rac1 | Rac2 | RhoA | Cdc42 |
|-----------|------|------|------|-------|
| HL60 (n = 3-4) | 98.6 (± 0.5)* | 50 (± 45.1) | 69 (± 12.7)* | 29 (± 2.51)* |
| KG1A (n = 3-4) | 92 (± 2.9)* | 20 (± 28.4) | ND | ND |
| CB CD34+ (n = 1) | 83 | 52 | 60 | ND |
| MPB CD34 (n = 3-4) | 49 (± 13.4)* | 11 (± 10.2) | 21 (± 9.0) | 34 (± 7.1)* |
| B-CLL (n = 3-4) | 39 (± 14.9)* | 0.8 (± 34.6) | 29 (± 6.5)* | ND |

**The Rac1 C-terminal Domain Inhibits Actin Polymerization and Cell Migration**—The above results show that the C-terminal peptides act as selective inhibitors of GTPase signaling. This allowed us to use these peptides to assess the contribution of different Rho-like GTPases to chemokine-induced actin polymerization and directional migration of normal and malignant hematopoietic cells.

SDF-1-induced actin polymerization and cellular motility in HL60 cells (14) was significantly blocked by the Rac1 but not the RhoA, Rac2, Cdc42, or PTD peptides (Fig. 3A, movie 4, a and b). SDF-1-induced migration of HL60 cells and of primary normal and leukemic hematopoietic progenitor cells was blocked by TAT-Rac1 in a dose-dependent fashion (Fig. 3B). These data were obtained using pre-incubation followed by washing of the cells; when peptides were also present during the migration assay, lower concentrations were sufficient for complete inhibition. The inhibition of cell migration was not because of peptide-induced toxicity, as we found no signs of reduced cell growth or viability after prolonged and repetitive transduction with the various peptides (data not shown). Table I depicts an overview of a large series of migration experiments in various cell types, which shows that chemotaxis is significantly and differentially inhibited by the Rac1 and RhoA C-terminal peptides, e.g. for 98 and 69%, respectively, in HL60 cells. Together, these data indicate that in normal and malignant human hematopoietic cells, primarily Rac1 and RhoA, and not Rac2 or Cdc42, mediate chemotaxis.

The Rac1 C-terminal Domain Associates with Effector and Adapter Proteins—In MDCKII, HL60, COS7, or KG1a cells, the Rac1 or RhoA C-terminal peptides did not inhibit GTP loading of either endogenous Rac1 or RhoA. In addition, HGF-induced PAK activation was also not affected by transduction with TAT-Rac1 (data not shown). Clearly, the inhibitory effects presented above are mediated through a different, GTP loading-independent mechanism.

The differential cellular loading of the C termini suggested that these domains mediate specific interactions with cellular proteins, rather than with lipids. In line with this, we were unable to find associations between phosphoinositides and the Rac1 C-terminal peptide. The Tat-Rac1 C terminus (or the RhoA, Rac2, Cdc42, and PTD peptides) did not associate with RhoGDI, which has been suggested to bind to Rac via the C terminus (21). This may be because of the absence of the CAAX region and thus the lack of lipidation of the peptides. Similarly, the Tat-Rac1 peptide did not bind to the Rac effector PAK (not shown). This finding is in line with the above data that the Tat-Rac1 C-terminal peptide does not affect PAK activation and with the notion that the interaction of PAK with Rac is mediated by the effector loop.

Tolias et al. (22) have shown that the lipid kinase PIP5K binds to a GST-Rac 165–192 fusion protein, which comprises the C-terminal domain (22). Using pull-down experiments followed by lipid kinase activity assays (23), we found that indeed the Tat-Rac1 C terminus (amino acids 178–188) bound PIP5K activity when used as a bait to fish in HL60 cell lysates (Fig. 4). The Rac2 and Cdc42 C-terminal domains were less efficient in binding to PIP5K, but the RhoA C-terminal peptide also associated to PIP5K, which confirms earlier data by Ren et al. (24). Tat-Rac1 also bound PIP5K in KG1a, MDCKII, and COS7 cells (data not shown). Apparently, the Rac1 178–188 region, encoded by the peptide used in this study, is already sufficient to mediate association of Rac1 with PIP5K.

We next examined the binding of the Rac1 peptide to other signaling proteins, implicated in cell migration and cell-cell adhesion. We found that the adapter proteins NCK and Crk bound specifically to the Rac1 C-terminal peptide. Although these adapter proteins have been implicated in cell migration (25, 26), a direct association with Rho-like GTPases has not been reported previously. The association of Crk to the C termini of Rac2 or Cdc42 was much less or even absent, compared with the Rac1 peptide (Fig. 5A). To confirm that the association between the Rac1 C-terminal peptide and Crk was also mediated by full-length Rac protein, we used the CRIB domain of PAK to isolate Rac1 from cell lysates. We indeed found Crk to be associated to this complex (Fig. 5B). Moreover, in the presence of the Rac1 C-terminal peptide, Crk binding to Rac was no longer observed, although the Rac-CRIB association remained unaltered. The RhoA C-terminal peptide did not compete for Crk binding to endogenous Rac (Fig. 5B). Subsequently, using transient transfection of v-Crk mutants that were mutated in the SH2 or the N-terminal SH3 domain (27), we could show that the association of Crk with the Rac1 C terminus is mediated by the N-terminal SH3 domain of Crk and is not dependent on an intact SH2 domain (25).
Having established at least two signaling proteins that associate directly with the Rac1 C terminus, we tested whether these interactions would involve identical residues within the Rac1 C-terminal sequence. To this end, two mutants of the TAT-Rac1 C-terminal peptide were synthesized. In the first one, the three prolines (amino acids 179–181) were replaced by alanine residues (Rac1 PPP-AAA). In the second, the RKR sequence (amino acids 185–187) was replaced by alanine residues (Rac1 RKR-AAA) (Fig. 6A). The interaction of the Rac1 C terminus with PIP5K was shown previously (28) to be mediated by basic amino acids, in particular Lys-186. In line with these data, the TAT-Rac1 RKR-AAA peptide no longer associated with PIP5K activity in the pull-down assay (Fig. 6B).

Because the interaction with the Crk adapter protein involved the Crk SH3 domain, we tested whether the three consecutive prolines in the Rac1 C terminus mediated Crk binding. When using the Rac1 PPP-AAA peptide, the binding to Crk was abolished, confirming that these prolines were required for the interaction (Fig. 6C). This peptide was still capable of associating to PIP5K (Fig. 6B). The Rac1 RKR-AAA mutant peptide also no longer associated to Crk, indicating that, in addition to the proline stretch, the adjacent basic amino acids are also important for Crk binding. These data establish the presence of two binding motifs for signaling proteins within the Rac1 C terminus.

Next, the wild type and the two mutant peptides were used to determine the relation between the presence of either binding site and localization and effects of the peptides. Phosphatidylinositol 4,5-biphosphate, a product of PIP5K, is present in lipid rafts from where actin polymerization is mediated (29, 30). In line with this, the wild type Rac1 C terminus co-localized with lipid rafts in HL60 cells (Fig. 7A), suggesting that the inhibitory effect of this peptide on in vitro migration is because of interference with localized PIP5K/phosphatidylinositol 4,5-biphosphate-dependent, Rac-mediated signaling. This idea is further supported by the finding that SDF-1-induced HL60 migration was abolished following disruption of lipid rafts by methyl-β-cyclodextrin (data not shown). The Rac1 PPP-AAA peptide also localized to lipid rafts; the Rac1 RKR-AAA peptide showed less prominent co-localization with lipid rafts, suggesting that the basic motif in the C terminus is involved, perhaps
via PIP5K, in mediating associations with raft-resident proteins.

In MDCKII cells, the Rac1 C terminus showed a clear localization to both cell-cell junctions and peripheral membranes (Fig. 7B). The peptide did not associate to endomembranes or to the nuclear membrane. This localization was mimicked by the Rac1 PPP-AAA peptide, but the Rac1 RKR-AAA peptide showed a marked loss of accumulation at peripheral membranes. This reduction of the staining intensity at the cell periphery was shown to be significant (Fig. 7B). The Rac1 RKR-AAA peptide no longer inhibited HGF-induced membrane ruffling, whereas the Rac1 PPP-AAA peptide did (Fig. 7C). This suggests that the RKR sequence in the Rac1 C terminus controls membrane ruffling and Rac1-localization to these ruffles, possibly through the association with PIP5K. On the other hand, both peptides were able to inhibit the formation of polarized cell-cell adhesion, in line with the localization to cell-cell junctions (Fig. 7D). These data further underscore the relationship between the amino acid sequence of the C-terminal domain of Rho-like GTPases and their intracellular targeting and suggests that binding partners for Rac1 are also differentially distributed over distinct regions of the plasma membrane (i.e. cell-cell junctions versus peripheral plasma membrane).

Finally, the different Rac1 peptides were characterized for their effects on SDF-1-induced actin polymerization and chemotaxis. The Rac1 PPP-AAA peptide efficiently blocked SDF-1-induced actin polymerization, whereas the Rac1 RKR-AAA peptide did not (Fig. 7E). Similarly, the Rac1 RKR-AAA peptide no longer interfered significantly with SDF-1-induced chemotaxis of HL60 cells. In contrast, the PPP-AAA mutant peptide inhibited migration as efficiently as the wild type peptide (Fig. 7F). This indicates that, in addition to the Crk binding proline-rich motif, it is primarily the basic, PIP5K binding motif that contributes to the control of actin polymerization and cell migration by Rac1.

**DISCUSSION**

Despite their high level of sequence identity, the various members of the Rho-like GTPase family display remarkable
specificity in their effects on the actin cytoskeleton and cellular morphology. This signaling specificity has been largely ascribed to differences in the effector loop (amino acids 25–40) and the insert region (amino acids 124–136), domains that are divergent among the different family members. However, the most variable region within the closely related Rho-like GTPases is the C terminus. This hypervariable part of the protein is generally assumed to mediate membrane localization because of the lipid modification on the C-terminal cysteine residue and the presence of a polybasic region. However, it is not clear how a lipid anchor and a series of basic residues could account for the observed signaling specificity and differential cellular localization of the Rho-like GTPases.

The current study was designed to further analyze the role of the hypervariable C-terminal domain of Rho-like GTPases, in particular Rac1, in the control of cell adhesion and migration. This was done with cell-permeable versions of the C termini of Rac1, Rac2, Cdc42, and RhoA. The results obtained with a variety of cellular assays show that these domains behave as selective inhibitors of Rho GTPase signaling, acting rapidly (i.e. within min) in transduced (primary, as well as transformed) cells. Thus, these peptides represent a valuable tool to study GTPase signaling.

In addition to confirming the selective inhibition of contractility or membrane ruffling by RhoA or Rac1 C termini, these peptides were used to show that primarily Rac1 and, to a lesser extent, RhoA were required for efficient SDF-1-induced chemotaxis. The differential effects of the Rac1 and Rac2 peptides, both on actin polymerization and chemotaxis, are remarkable, considering there is more Rac2 than Rac1 in leukocytes (31) and that Rac2 has been implicated in migration of neutrophils, B-cells, and T-cells (32–34). Yet introduction of the C terminus of Rac1 is sufficient to block chemotaxis, further underscoring the role of the C terminus in determining signaling specificity between Rac1 and Rac2.

The seemingly contradictory effects of TAT-RhoA on migration and actin polymerization might be explained by observations that RhoA is involved in cell contraction, which is necessary for migration, but that does not require actin polymerization (35, 36). Also Cdc42 has been implicated in SDF-1-induced chemotaxis (37), although we found that the Cdc42 C terminus did not interfere to a large extent with chemotaxis in our assays. A major difference of the current approach with earlier published work is that the effects we describe are induced almost instantaneously because of the fact that the peptides enter the cells very rapidly and with a close to 100% efficiency. Conclusions from studies using transfected T-cell lines that were cultured for several days might be based on selected cell populations, possibly causing different findings.

The use of biotinylated versions of the peptides allowed us to determine the differential retention and localization to cellular compartments of the various C termini. This localization was most likely because of protein-protein, rather than to protein-lipid, associations. Using Coomassie stainings of SDS-PAGE gels we indeed found that the C-terminal peptides, in particular of Rac1, associate to a range of cellular proteins, even under stringent conditions, i.e. in the presence of 0.5 M NaCl, 0.1% SDS. We tested a series of proteins that were considered candidate binding partners for the Rac1 C terminus. Neither RhoGDI nor PAK did bind to the Rac1 C terminus, possibly because of lack of prenylation and the fact that the CRIB domain of PAK associates to the effector loop of Rac1. Also, activation of PAK was not blocked by transduction with the Rac1 C-terminal peptide. This is in line with data that showed PAK activation to be dependent on the Rac effector domain and independent from the induction of cytoskeletal changes (38) and indicates that the proper localization of Rac1 (which is disturbed by the transduced C terminus) is not absolutely required for growth factor-induced PAK activation.

We could confirm the interaction of the Rac1 C terminus with PIP5K (22) and also show its specificity, as the C termini of Rac2 or Cdc42 did not associate efficiently with PIP5K. In line with earlier data on the Rac-PIP5K interaction (28), we found that a peptide in which basic residues, including Lys-186 were replaced by alanines, no longer bound PIP5K. This peptide showed PAK activation to be dependent on the Rac effector domain and independent from the induction of cytoskeletal changes (38) and indicates that the proper localization of Rac1 (which is disturbed by the transduced C terminus) is not absolutely required for growth factor-induced PAK activation.

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We could confirm the interaction of the Rac1 C terminus with PIP5K (22) and also show its specificity, as the C termini of Rac2 or Cdc42 did not associate efficiently with PIP5K. In line with earlier data on the Rac-PIP5K interaction (28), we found that a peptide in which basic residues, including Lys-186 were replaced by alanines, no longer bound PIP5K. This peptide showed only a partial inhibition of HL60 migration and no longer blocked growth factor-induced membrane ruffling. PIP5K is involved in the control of actin polymerization (39) and in the formation of lipid rafts and membrane polarization, which is required for ruffling and cell migration. Our data suggest a causal relationship between the fact that the Rac1 RKR-AAA peptide did not associate to PIP5K and did not block actin polymerization, ruffling, or chemotaxis. However, other proteins may also associate with Rac1 through this binding.
allow interactions of endogenous, activated Rac with a subset of previously bound partners, and because our data show that PIP5K and Crk. The GTPase effector loop has been implicated in signaling toward the actin cytoskeleton. Cell migration was shown to be controlled by the Rac-Crk interaction for Rac-mediated signal. The binding of Rac to adapter proteins may also play an important role in the receptor-mediated recruitment of Rac1 to the plasma membrane. Most SH3 domain-containing adapter proteins, such as Nck and Grb2 to the Rac1 C-terminal peptide, whereas other SH3 domain-containing proteins, e.g., Eps8, did not associate to the peptide. Thus the proline-rich region in the Rac1 C terminus represents a previously unrecognized, second C-terminal motif that mediates specific protein-protein interactions.

The role of the Rac-Crk interaction for Rac-mediated signaling was further analyzed by replacing the three consecutive prolines to alanines in the C-terminal peptide sequence. This Rac1 PPP-AAA peptide still localized to the plasma membrane in HL60 cells, associated to PIP5K, and blocked actin polymerization and membrane ruffling, suggesting that the proline-rich domain in the Rac1 C terminus, in contrast to the PIP5K binding RKR sequence, is not an absolute requirement for Rac signaling toward the actin cytoskeleton. Cell migration was blocked as efficiently by the Rac1 PPP-AAA peptide as with the wild type sequence. This suggests that proteins, interacting with the second, polybasic binding motif in the Rac1 C terminus, are essential for cell motility. Conversely, proteins that interact with the proline-rich motif also contribute to cell migration, albeit that the inhibition obtained with the RKR-AAA peptide was less pronounced.

The binding of Rac to adapter proteins may also play an important role in the receptor-mediated recruitment of Rac1 to the plasma membrane. Most SH3 domain-containing adapter proteins also have SH2 domains that mediate the binding to tyrosine-phosphorylated receptors (43). In addition, Crk is known to be part of a larger complex, including p130Cas, which was shown recently (44) to be required for proper Rac activation and cell migration. In agreement with this, we also found p130Cas to associate with the Rac1 C terminus (not shown). Because Crk can also bind Rho-GTPase exchange factors, such as SOS and Dock180 (25), Crk binding may provide an important means for Rac to reach its exchange factor. However, because we found that the Rac1 C terminus does not interfere with Rac GTP loading, Rac activation may be promoted by, but is not solely dependent on, Crk binding (44).

The present data show that the C-terminal domain of Rac shows discrete intracellular targeting and can act as a selective inhibitor because of its association with effector proteins, e.g., PIP5K and Crk. The GTPase effector loop has been implicated previously in target binding, and because our data show that the C-terminal peptide is not able to compete with the CRIB domain-effector loop interaction, this peptide will therefore still allow interactions of endogenous, activated Rac with a subset of effectors to occur. However, our data show that the C-terminal domain is crucial for proper localization, e.g., for Rac1 in membrane ruffles. This suggests that the effects of the isolated C terminus are because of dislocalization of Rac in a specific fashion, which is caused by interference with effector interactions that occur at specific sites in the cell, such as lipid rafts.

In conclusion, the current results reveal the unique and non-redundant role of the C terminus of Rho-like GTPases in the control of signaling toward cell-cell adhesion and migration (Fig. 8). Moreover, we have defined two adjacent protein-protein binding motifs that are important for Rac1-mediated control of the actin cytoskeleton and for cell-cell adhesion and migration. Although this work has focused on Rho-like GTPases, there is little doubt that the principle described here will apply to many, if not all, members of the Ras superfamily of small GTPases.

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