Scaffold Proteins IRSp53 and Spinophilin Regulate Localized Rac Activation by T-lymphocyte Invasion and Metastasis Protein 1 (TIAM1)*

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Soumitra Rajagopal, Yuxin Ji, Kun Xu, Yuhuan Li, Kathleen Wicks, Jiewei Liu, Ka-Wing Wong, Ira M. Herman, Ralph R. Isberg, and Rachel J. Buchsbaum

From the Molecular Oncology Research Institute, Tufts Medical Center, the Department of Medicine, Tufts University School of Medicine, and the Departments of Molecular Biology and Microbiology and Cellular and Molecular Physiology, Tufts University Sackler School of Graduate Biomedical Sciences, Boston, Massachusetts 02111, the Providence Portland Medical Center, Portland, Oregon 97213, and the Boston University School of Medicine, Boston, Massachusetts 02118

The Rac exchange factor Tiam1 is involved in diverse cell functions and signaling pathways through multiple protein interactions, raising the question of how signaling and functional specificity are achieved. We have shown that Tiam1 interacts with different scaffold proteins activate different Rac-dependent pathways by recruiting specific Rac effector proteins, and reasoned that there must be regulatory mechanisms governing each interaction. Fibroblasts express at least two Tiam1-interacting proteins, insulin receptor substrate protein 53 kDa (IRSp53) and spinophilin. We used fluorescent resonance energy transfer (FRET) to measure localized Rac activation associated with IRSp53 and spinophilin complexes in individual fibroblasts to test this hypothesis. Pervanadate or platelet-derived growth factor induced localized Rac activation dependent on Tiam1 and IRSp53. Forskolin or epinephrine induced localized Rac activation dependent on Tiam1 and spinophilin. In spinophilin-deficient cells, Tiam1 co-localized with IRSp53 in response to pervanadate or platelet-derived growth factor. In IRSp53-deficient cells, Tiam1 co-localized with spinophilin in response to forskolin or epinephrine. Total cellular levels of activated Rac were affected only in cells with exogenous Tiam1, and were primarily increased in the membrane fraction. Downstream effects of Rac activation were also stimulus and scaffold-specific. Cell ruffling, spreading, and cell adhesion were dependent on IRSp53, but not spinophilin. Epinephrine decreased IRSp53-dependent adhesion and increased cell migration in a Rac and spinophilin-dependent fashion. These results support the idea that Tiam1 interactions with different scaffold proteins couple distinct upstream signals to localized Rac activation and specific downstream pathways, and suggest that manipulating Tiam1-scaffold interactions can modulate Rac-dependent cellular behaviors.

The Rac GTPase influences a diverse set of cellular functions in cells (reviewed in Ref. 1). Like other members of the Ras family of monomeric GTPases, Rac becomes activated through exchange of bound GDP for GTP, undergoing a conformational change that allows it to bind to a large number of downstream target proteins (2). The wide range of potential Rac effector pathways raises the question of how cells maintain signaling specificity in pathways involving Rac and other Ras superfamily members. Activation of Rac is catalyzed by a number of guanine-nucleotide exchange factors (3). Tiam1 is a ubiquitous exchange factor for the Rac GTPase that also participates in multiple signaling pathways, leading to effects on a variety of cell behaviors (4).

A major factor in the involvement of Tiam1 in multiple signaling pathways in cells is its ability to interact with a number of different proteins, including an isoform of the hyaluronic acid receptor CD44, cytoskeletal protein ankyrin, JNK interacting protein/islet-brain (JIP/IB) proteins, spinophilin, IRSp53, the Par3 component of the Par3-Par6-αPKC polarity complex, sub-units of the N-methyl-D-aspartate receptor, the brain-derived neurotrophic factor receptor TrkB, and the Arp2/3 complex, active Ras, active Rap1, and the tumor suppressor Nm23H1 (5–17). The physiologic significance has been established for some but not all of these interactions. Many of these interactions, including CD44, ankyrin, JIP/IB proteins, spinophilin, IRSp53, and Par3 are mediated through the same relatively limited N-terminal region containing a pleckstrin homology domain immediately upstream of a coiled-coil region. There is no commonly identified Tiam1-binding motif on the different Tiam1 interactors and how all these interactions with the same region of Tiam1 are regulated is not known.

We have previously found that some of these proteins, including JIP2/IB2, spinophilin, and IRSp53, serve as scaffold proteins for Rac signaling, in that they form complexes with both the Rac exchange factor as well as with specific Rac effector proteins (7–9). Our results have led us to propose that interactions of exchange factor with such scaffold proteins govern...
Tiam1 and Rac signaling specificity by directing the effects of Tiam1-activated Rac to specific downstream effector pathways. This mechanism would be particularly important in cells or cellular compartments where more than one Tiam1 interactor is expressed. Some Tiam1-interacting proteins have restricted tissue or cell type expression, such as JIP/IB proteins, N-methyl-D-aspartate receptor, and TrkB in neuronal cells, CD44v3 in breast cancer cells, and Par3 in epithelial cells. However, spinophilin and IRSp53 are ubiquitously expressed proteins that are both present at the plasma membranes of cells and are both major Tiam1 interacting proteins present in fibroblasts (reviewed in Refs. 18 and 19). Rac signaling is a critical component in the biology of human tumors, in both the transformed cells as well as the cells of the tumor microenvironment. Fibroblasts are the most abundant cell type within the tumor microenvironment.

In this study we have looked at the role of Tiam1-interacting scaffold proteins in mediating input signals from upstream sources such as extracellular stimuli. By examining activation of localized pools of Rac in individual cells we have found that an individual scaffold protein facilitates Tiam1-dependent Rac activation in response to a limited set of upstream signals. This is then tied to activation of a specific downstream Rac effector pathway relevant to protein complexes mediated by the specific scaffold protein. This allows us to conclude that the presence of multiple Tiam1-interacting scaffold proteins allows for finely controlled Rac activation in response to a range of stimuli, leading to a range of specifically regulated cellular responses.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**NIH3T3 cells were passaged in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (HyClone) along with penicillin and streptomycin in a humidified 37 °C, 5% CO2 incubator. The day prior to transfection, cells were plated in antibiotic-free medium onto 12-mm coverslips coated with either 10 μg/ml of poly-L-lysine or 100 μg/ml of fibronectin (Sigma) in 24-well culture plates at 5 × 10⁴ cells/well. Cells were cotransfected with the pmCFP-Rac1 species (WT, V12, N17, or C40, 2 μg/well) and pmYFP-PBD using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Eighteen h after transfection cells were deprived of serum for 5 h, and then some were stimulated as indicated with 200 μM pervanadate for 10 min, 10 ng/ml of PDGF for 10 min, 50 μM of forskolin for 15 min, 10 μmol of epinephrine for 15 min, or 200 mmol of H₂O₂ for 30 min, respectively. Some cells were treated with 100 μmol of the Rac inhibitor NSC23761 (Calbiochem) for 16 h before analysis. At the end of the stimulation, cells were washed three times in phosphate-buffered saline and fixed with fixative solution (3% paraformaldehyde, 0.2 M 1-lysine, 0.1 M NaH₂PO₄, 40% sucrose, 21.4 mg of sodium periodate). Coverslips were mounted on glass slides in the presence of Antifade (Bio-Rad). For replating experiments, cells plated on glass coverslips were trypsinized briefly at room temperature until completely rounded, and then serum-containing medium was added to the wells to neutralize the trypsin. Morphology was monitored over time under light microscopy, and the numbers of cells exhibiting rounded or spread morphology were counted at serial time points after replating.

**Generation of Cell Lines with Stable Suppression of Protein Expression—**NIH3T3 cell lines with stable expression of short hairpin RNAs targeting Tiam1, Spinophilin, and IRSp53 were generated using the pSuperior retro.neo retroviral shRNA expression vector (OligoEngine). Silencing hairpins were cloned into the pSuperior vector using double-stranded oligomers synthesized in the Tufts DNA Core Facility (Tiam1) or OligoEngine (Spinophilin and IRSp53). Hairpins were derived from the following template sequences: Tiam1, 5'-GAGACTCTCCGTAACAGTAAT; Spinophilin, 5'-AAAGCTGTGCA-CAAGTTT; and IRSp53, 5'-CTCGTACTCCACACACTC. A control line was generated using the pSuperior retro.neo vector itself. For virus production murine amphotrophic 293 cells were transfected with 30 μg of DNA each of the appropriate retroviral construct along with Ecopac viral packaging cDNA using standard calcium phosphate precipitation. Cultured supernatants containing virus were collected after 48 h and filtered. Recipient NIH3T3 cells were plated overnight at a cell density of 8 × 10⁴/ml in 6-cm plates, infected with the filtered viral supernatants in the presence of Polybrene solution (8 μg/ml, Sigma), and infected with a second round of fresh viral supernatants 24 h later. Infected NIH3T3 cells were selected for 7 days in growth medium containing 100 μg/ml of gentamycin (Invitrogen). Single colony isolates were then tested for loss of protein by immunoblot or real time PCR analysis.

**Plasmids—**DNA constructs of pmCFP-Rac1WT, pmCFP-Rac1V12, pmCFP-Rac1N17, pmCFP-Rac1C40, and pmYFP-PBD were generated as described (20). Briefly, using PCR standard molecular cloning techniques, human cDNAs from plasmids pCGT-Rac1, pCGT-Rac1V12, and pCGT-Rac1N17 were fused into the multicloning site downstream of the 3' end of CFP in pmECFP to generate the respective cDNAs in pmEYFP. The Rac1Y40C was created by site-directed mutagenesis of pmCFP-Rac1WT. Human cDNA sequence corresponding to the Cdc42-Rac binding domain from pcmVM-Pak1 was inserted in the multicloning site at the 3' end of YFP in pmEYFP to generate the pmYFP-PBD plasmid.

To generate shRNA-resistant plasmids for rescue of gene silencing, full-length cDNAs were cloned into pmCherry (Clontech) or pRSET-B-mCherry (21) to generate fluorescent fusion proteins with far-red emission spectra that do not overlap with CFP and YFP. Overlap PCR was used to generate full-length cDNAs with silent mutations as follows: Tiam1*, 5'-GAACTCCACACATGCGATTTGAGTTTTAGTTGGTCTACATAAATG; Spinophilin*, 5'-AAGCTATGACATATAATCT; and (human) IRSp53*, 5'-CTC-CTACTCCACACACTC.

**Real Time PCR—**Gene silencing of IRSp53 in NIH3T3 cells was serially monitored by real time PCR analysis. Total RNA and first strand cDNA synthesis were carried out using the TRiZol™ and SuperScript™ (Invitrogen) protocols, respectively. PCR were performed as duplicates in 15-μl volumes containing cDNA, SYBR® Green PCR Mastermix (Applied Biosystems), and specific primers as follows: sense 5'-gTgTGcAgCTgAgCTAgAa; antisense 5'-gTgATAgTgTAgCTAgAa. Cycling conditions were as follows: 94 °C for 2 min followed by 45 amplification cycles (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s).
for 90 s). Data analysis was done using a Opticon™ 2 Continuous Fluorescence Detector (MJ Research). Fold-knockdown for cDNA was normalized to β-actin cDNA in the sample (actin primers 5'-TggATTCCTgTggCATCCATgAAAC and 5'-TAAAACgCAgCTCCTgTAAgAgCTC). Equation 1, as described (22) in Equation 1,

\[ I_{sensitized\ FRET} = I_{FRET} - (0.27 \times I_{CFP}) - (0.22 \times I_{YFP}) \]  

where \( I_{FRET} \), \( I_{CFP} \), and \( I_{YFP} \) are the mean intensities of each region of interest in the FRET, CFP, and YFP images, respectively, and correction norms for bleed-through were derived by analyzing cells expressing CFP or YFP only in the appropriate channels (0.27 for CFP and 0.22 for YFP). For derivation of normalized FRET, sensitized FRET values were normalized to the square root of the (CFP-YFP) product to control for molecular concentration and cell thickness within regions of interest, as described (23). Normalized FRET ratios are calculated as mean normalized FRET (periphery) divided by mean normalized FRET (interior).

Affinity Precipitation for Detection of Rac Activity—Affinity precipitation for active Rac species was performed as previously described (8). Briefly, cleared lysates of cells transiently expressing equal amounts of total protein were incubated for 30 min at 4 °C with S-hexyl-glutathione-agarose beads loaded with the p21-binding domain of PAK1 expressed as a glutathione S-transferase fusion in Escherichia coli (24). Precipitates were washed, and bound proteins were eluted in 4x Laemmli buffer, resolved by SDS-PAGE, and immunoblotted. Equal amounts of cleared lysate were also resolved by SDS-PAGE and immunoblotted in parallel to verify equivalent Rac expression.

Cell Fractionation—Cell fractionation was performed as previously described (25). Briefly, cells were lysed by sonication in buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl₂, 200 mM NaCl, 1 mM dithiothreitol, along with protease inhibitors (10 μg/ml of aprotinin, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (50 μM sodium fluoride and 100 μM sodium orthovanadate). Lysates cleared of nuclear debris and non-broken cells by low-speed centrifugation then underwent high-speed centrifugation (100,000 × g for 30 min). After recovery of the soluble (cytosolic) fraction, pellets were resuspended in the same buffer supplemented with 2% Nonidet P-40 and 10% glycerol to release membrane fractions. Equivalent protein loading was verified by the Bio-Rad protein assay according to the manufacturer’s instructions.

Immunofluorescence—For Tiam1-scaffold protein localization experiments, 1.5 × 10⁵ cells were plated onto 12-mm glass coverslips coated with 100 μg/ml of fibronectin in 24-well culture plates. Cells were serum starved for 5 h and some were stimulated with pervanadate, PDGF, forskolin, or epinephrine as indicated prior to fixation in phosphate-buffered saline containing 4% paraformaldehyde. Cells were permeabilized and blocked with phosphate-buffered saline containing 1% bovine serum albumin and 0.5% Triton X-100, and incubated with antibodies as previously described (9). For detection of actin localization, cells were replated on uncoated glass coverslips prior to fixation and permeabilized with buffer containing 0.1% Triton X-100. Phalloidin Alexa Fluor 488 (both from Molecular Probes), and polyclonal anti-Tiam1 antibody (Santa Cruz) were diluted per the manufacturer’s instructions. Anti-β-actin IgG was affinity purified and diluted in phosphate-buffered saline prior to use as previously published (26, 27). Coverslips were mounted using Antifade (Bio-Rad). Cells were observed under ×63 magnification using an Axioplasm Zeiss microscope, and images were obtained using a Retiga 2000R imaging camera.

Antibodies and Immunoblotting—Antibodies to Tiam1, spinophilin, IRSp53, β-actin, and myc epitope, secondary antibodies, immunoblotting, and chemiluminescence protocols have been described previously (8, 9, 25, 27).

Transwell Migration Assays—Cultured cells were deprived of serum overnight, trypsinized, and plated at a density of 1 × 10⁵ cells/ml (5 × 10⁴ cells/basket) in the upper basket of transwell chambers with a filter pore size of 8 μm (Costar). Cells were incubated for 4–5 h at 37 °C and allowed to migrate toward the lower chambers containing either Dulbecco’s modified Eagle’s medium alone or supplemented with 25% filter-sterilized conditioned NIH3T3. Non-migrated cells were removed using a cotton swab. Migrated cells were fixed and stained using the Protocol 3®HEMA Stain kit (Fisher). Filters were cut out and mounted on glass slides under coverslips using Immersion oil (Resolve®, Richard Allen Scientific). Cell migration was determined by counting migrated cells in nine random fields using a Zeiss microscope and ×16 objective. Where indicated, epinephrine was added at a concentration of 10 μmol 1 h after placing cells in the upper basket.

RESULTS

We have previously found that Tiam1 interaction with different scaffold proteins leads to activation of specific pathways downstream of Rac. Tiam1 is known to interact with a number of different proteins and many of these interactions are mediated through the same N-terminal region of the protein, which contains a pleckstrin homology domain immediately followed by a coiled-coil domain (28). This raises the question of how signaling specificity is achieved. We reasoned that there must be regulatory mechanisms governing the specific conditions under which each interaction occurs. In particular, whereas
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A. Raf/GTPase-activating protein (GAP) signaling. The binding of activated, GTP-bound Rac, to the G12/13-binding domain of AP-2 can be visualized by FRET between Rac and PBD when Rac and PBD are each expressed as fusion proteins with suitable donor and acceptor fluorophores (in this case CFP and YFP, respectively) (20). Measuring Rac-FRET by this method allows for detection of activation of small populations of Rac molecules at a specific place and time in an individual cell.

Detection of RacFRET in Cells—We first determined that we could detect FRET signals reliably using established controls with known effects on Rac signaling. We found that the FRET signals were too weak to consistently measure using confocal imaging, so signals were recorded using an inverted microscope as previously reported (20).

Little to no increase in non-nuclear FRET signals was seen in cells expressing wild-type Rac fused with CFP (CFP-RacWT) along with YFP-PBD (Fig. 1, A, lane 1, and B, top panels). Cells expressing the dominant active Rac mutant, CFP-RacV12, which resists hydrolysis of bound GTP, and YFP-PBD exhibited localized areas of increased FRET at or near the periphery of the cell, in comparison to cells expressing wild-type CFP-Rac (Fig. 1, A, compare lane 1 to lane 2, and B, second row panels). In addition, an NIH3T3 line stably expressing the constitutively active C1199 mutant of Tiam1 also exhibited elevated FRET when transfected with CFP-Rac and YFP-PBD, in comparison to parental cells with endogenous wild-type Tiam1 (Fig. 1, A, compare lane 1 to lane 3, and B, third row panels). No increase in RacFRET was seen in cells expressing the dominant-negative CFP-RacN17, which binds GDP preferentially over GTP (Fig. 1, A, compare lane 1 to lane 4, and B, fourth row panels). In addition, no increase in RacFRET was detected with the effector mutant CFP-RacC40, which does not bind to PBD, indicating that FRET signals seen with wild-type Rac and PBD represent specific binding between Rac and PBD and not nonspecific dimerization of fluorophore moieties (Fig. 1, A, lane 5, and B, fifth row panels). We have previously shown that photobleaching of YFP-PBD leads to increases in CFP emission in cells co-transfected with CFP-RacWT and YFP-PBD, or in cells co-transfected with CFP-RacV12 and YFP-PBD, but not in cells co-transfected with either CFP-RacN17 or CFP-RacC40 and YFP-PBD (20), further supporting the conclusion that we are detecting specific binding of Rac-GTP to YFP-PBD by this method.

Pervanadate Treatment Leads to RacFRET in Cells—We then determined that we could detect Rac activation using this method in response to an upstream stimulus. Published reports and our own work (not shown) suggest that treatment of cells with the tyrosine phosphatase inhibitor pervanadate leads to phosphorylation of Tiam1 on tyrosine and activation of pathways downstream of Rac (33). We found that in NIH3T3 cells expressing CFP-Rac and YFP-PBD, localized areas of increased...
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RacFRET could be seen at the cell periphery within minutes after treatment with pervanadate, peaking at 10 min and largely disappearing after 20 min (Fig. 2, A, compare lanes 1 and 2, B, compare second row with first row FRET panels). Treatment of the cells with a cell-permeable Rac inhibitor designed to interfere with Rac activation by exchange factors (34) did not affect basal levels of Rac activation but largely blocked an increase in RacFRET upon pervanadate treatment (Fig. 2, A, lanes 3 and 4, B, third and fourth row panels).

**Tiam1 Is Required for Localized Activation of Rac by Pervanadate**—To determine the role of Tiam1 on stimulated Rac activation in cells, we developed an NIH3T3 cell line with stable suppression of Tiam1 expression, using retroviral delivery of short hairpin DNAs. To study the role of Tiam1-interacting proteins in stimulated Rac activation in cells, we also developed NIH3T3 lines with stable suppression of either the scaffold protein spinophilin or the scaffold adaptor protein IRSp53, which are both expressed in these cells. Protein expression levels of Tiam1 and spinophilin were verified and quantified by immunoblot (supplemental Fig. S1) and monitored serially over time. Tiam1 levels were suppressed to 10–15%, and spinophilin levels were suppressed to 40%, compared with control cells. Due to the presence of multiple contaminating bands on the immunoblot, IRSp53 expression was monitored serially using real time PCR, with IRSp53 suppression ranging from 25 to 40% normalized to actin controls. Tiam1 silencing did not affect levels of spinophilin or IRSp53, and conversely silencing of either spinophilin or IRSp53 did not affect the level of Tiam1 (not shown).

We then determined the effect of silencing Tiam1 expression on pervanadate-induced Rac activation in cells. Pervanadate treatment led to an increase in RacFRET at the cell periphery of control retrovirus-infected cells expressing wild-type levels of Tiam1 (Fig. 3, A, compare lane 1 with lane 2, and B, compare top right and left panels), consistent with the effect seen in parental cells in Fig. 2. Cells with Tiam1 suppression showed baseline levels of Rac activation, but no enhancement of Rac activation in response to pervanadate (Fig. 3, A, lanes 3 and 4, and B, middle panels). Cells with Tiam1 suppression were also assayed after transient expression of an engineered Tiam1 carrying silent mutations leading to resistance to shRNA-mediated protein suppression (Tiam*). In these cells baseline levels of Rac activation were not affected (Fig. 3, A, lane 5, and B, bottom left panel), but Rac activation in response to pervanadate was restored (Fig. 3, A, lane 6, and B, bottom right panel), indicating specificity of the silencing hairpin for Tiam1-mediated Rac activation.

The overall level of active Rac in cells is often assessed using affinity precipitation by the Pak PBD expressed as a glutathione S-transferase fusion protein and immobilized on glutathione-agarose. This method gives a measure of the overall amount of GTP-bound Rac across a population of cells. We used this assay to determine whether the effects of pervanadate treatment on Rac activation were generalized throughout a population of cells (Fig. 4A). We found significantly more active Rac in positive control cells expressing the constitutively active C1199 mutant of Tiam1 (Fig. 4A, lanes 7 and 8) compared with untransfected cells (lanes 1 and 2) or unstimulated Rac-transfected cells (lanes 3 and 4). However, in contrast to the results obtained using FRET, we were unable to detect a significant increase in overall Rac activation upon treatment with pervanadate using this method in these cells with endogenous Tiam1 levels (Fig. 4A and supplemental Fig. S2A, lanes 3 and 4). These results support the idea that pervanadate leads to activation of only a subpopulation of Rac molecules through localized action of Tiam1. However, in cells overexpressing exogenous Tiam1, pervanadate treatment led to increased levels of activated Rac in pooled cell lysates (supplemental Fig. S2B, lanes 3 and 4), confirming the validity of our pulldown assays.

The localization and curvilinear morphology of the localized increases in RacFRET signals suggested Rac activation at plasma membranes, consistent with prior published reports (35, 36). We performed affinity precipitation on cytosolic and membrane fractions of cell lysates under conditions of quiescence or pervanadate treatment. Increased levels of active Rac are seen only in the membrane fraction of pervanadate-treated cells (Fig. 4B, lanes 7 and 8) compared with the cytosolic fractions (lanes 1–4) or unstimulated membrane fractions (lanes 5 and 6), suggesting that the increases in RacFRET that we detected represent localized activation of Rac at plasma membranes.
Tiam1 and IRSp53 Are Required for Localized Activation of Rac by Pervanadate—We next asked whether we could use the RacFRET assay to determine the role of different Tiam1-interacting scaffold protein complexes in transmitting specific upstream signals to localized effects on Rac. As before, control cells showed increases in RacFRET at the plasma membrane in response to pervanadate, whereas cells with Tiam1 silencing showed no increase in RacFRET upon pervanadate treatment (Fig. 5A, lanes 1–2 and 3–4, respectively, and supplemental Fig. S3, first and second row panels). Similarly, cells with IRSp53 silencing retained baseline levels of Rac activation but showed no increase in RacFRET upon pervanadate treatment (Fig. 5A, lanes 5 and 6, and supplemental Fig. S3, third row panels). Response to pervanadate was restored by transient expression of shRNA-resistant IRSp53 (IRSp53*) in these cells (Fig. 5A, lane 8, and supplemental Fig. S3, fourth row panels). In contrast, cells with spinophilin silencing exhibited Rac activation similar to control cells, with increased Rac activation upon pervanadate treatment (Fig. 5A, lane 10, and supplemental Fig. S3, bottom panels).
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Tiam1 and IRSp53 Are Required for Localized Activation of Rac by PDGF—These results suggest that both Tiam1 and IRSp53, but not spinophilin, are involved in pervanadate-mediated Rac activation. We have previously found that PDGF stimulation leads to membrane ruffling in a manner that is dependent on both Tiam1 and IRSp53 (9). We therefore tested whether PDGF stimulation could also lead to an increase in Rac activation using our RacFRET system. We found that PDGF treatment led to Rac activation in a time course similar to that of pervanadate, which again was inhibited after treatment with the Rac inhibitor (not shown). We then tested the effect of PDGF stimulation on cells with suppressed levels of Tiam1 or Tiam1-interacting scaffold proteins (Fig. 5B and supplemental Fig. S4). Similar to the results seen with pervanadate treatment, cells with suppression of Tiam1 (lanes 3 and 4) or IRSp53 (lanes 5 and 6) retained baseline levels of Rac activation but did not exhibit enhanced RacFRET with PDGF stimulation. Expression of shRNA-resistant IRSp53 in these cells restored response to PDGF without affecting baseline levels of Rac activation (lanes 7 and 8). Cells with suppressed spinophilin expression (lanes 9 and 10) again showed Rac activation similar to control cells. This is consistent with the idea that stimulation with either pervanadate or PDGF, but not spinophilin, leads to localized Rac activation through effects on Tiam1 and IRSp53.

Tiam1 and Spinophilin Are Required for Localized Activation of Rac by Forskolin—Spinophilin is an F-actin-binding protein also known to bind protein phosphatase 1 and p70 S6 kinase. We have previously shown that spinophilin and Tiam1 co-localize at the plasma membrane of cells and that spinophilin enhances Tiam1-mediated activation of p70 S6 kinase in a Rac-dependent manner (8). The p70 S6 kinase is a key protein in the regulation of translation of mRNAs containing a 5′ oligopyrimidine sequence and is a downstream target of mammalian target of rapamycin, responding to multiple upstream inputs, including insulin, growth factors, nutrients, and mitogens (37). We therefore sought to determine whether spinophilin plays a role in Rac activation in response to specific stimuli, analogous to the role of IRSp53 above. Treatment of our cells with insulin in doses ranging from 10 to 1000 nM did not yield significant RacFRET (data not shown). However, we were able to detect RacFRET upon treatment of cells with forskolin, a cell-permeable diterpenoid that activates adenylate cyclase, leading to elevated intracellular cAMP levels. Pre-treatment of the cells with the Rac inhibitor blocked forskolin-induced RacFRET, similar to the results seen with pervanadate and PDGF (not shown). We also tested whether forskolin treatment leads to generalized Rac activation in cells using the pulldown assay described in Fig. 4A. Similar to the results seen with pervanadate in Fig. 4A, forskolin treatment did not increase the amount of activated Rac detected in lysates of stimulated cells with endogenous Tiam1 levels (supplemental Fig. S2A, lanes 7 and 8).

We then assessed Rac activation upon forskolin treatment of the cell lines described above. Forskolin treatment consistently led to increased localized RacFRET on the order of 1.5–2-fold over baseline in control cells with intact Tiam1 levels (Fig. 6A, lanes 1 and 2). In contrast, in cells with Tiam1 suppression (Fig. 6A, lanes 3 and 4), no increase in RacFRET was observed upon forskolin treatment (lane 4). In cells with IRSp53 suppression

![Image](https://via.placeholder.com/150)
transient expression of shRNA-resistant spinophilin (spino*) in these cells restored a significant fraction of the Rac activation in response to forskolin treatment (Fig. 6A, lane 10). Supplemental Fig. S5 depicts corresponding sensitized FRET images for these cells and conditions.

Tiam1 and Spinophilin Are Required for Localized Activation of Rac by Epinephrine—We then went on to test the effect of epinephrine, a physiologic activator of cAMP through β2-adrenergic receptors. Epinephrine treatment also led to increased localized RacFRET levels (Fig. 6B, compare lanes 1 and 2). This increase was blocked in cells with Tiam1 suppression (Fig. 6B, lane 4), but largely preserved in cells with IRSp53 suppression (Fig. 6B, lane 6). Similar to the results seen with forskolin treatment, cells with spinophilin suppression exhibited decreased RacFRET compared with control cells upon epinephrine stimulation (Fig. 6B, compare lane 2 with lane 8). As before, expression of shRNA-resistant spinophilin in these cells largely restored the response to epinephrine (Fig. 6B, lanes 9 and 10). Supplemental Fig. S6 depicts corresponding sensitized FRET images for these cells and conditions. These results suggest that localized activation of Rac upon forskolin or epinephrine stimulation requires Tiam1 and spinophilin, but not IRSp53.

Tiam1 Is Not Involved in H₂O₂-stimulated Rac Activation in Cells—The above results support the idea that Tiam1 is involved in Rac activation in response to more than one upstream stimulus. Although multiple upstream stimuli are known to activate Rac, various proteins function as Rac exchange factors, and Tiam1 would not be expected to be involved in every upstream pathway leading to Rac activation. We therefore tested whether suppression of Tiam1 affected the activation of Rac by oxidative stress, as this mechanism of Rac activation has not been reported to involve Tiam1. Exposure of cells to hydrogen peroxide led to increased localized RacFRET (Fig. 6C, lanes 1 and 2, and supplemental Fig. S7), which was preserved in cells with suppressed Tiam1 levels (Fig. 6C, compare lanes 2 and 4). Suppression of IRSp53 or spinophilin expression also did not affect hydrogen peroxide-induced increases in RacFRET (Fig. 6, lanes 5–6 and 7–8, respectively; and sFRET images shown in supplemental Fig. S7). These data suggest that Rac activators other than Tiam1 mediate the generation of RacGTP in response to oxidative stress.

As noted above, our FRET results suggest that IRSp53 is involved in Tiam1 activation of Rac in response to pervanadate and PDGF, whereas spinophilin is involved in Tiam1 activation of Rac in response to forskolin and epinephrine. We used cells with endogenous levels of Tiam1 to assess the effects of scaffold suppression in order not to swamp any specific signaling pathway with excess protein. As noted above and shown in supplemental Fig. S2A, we were unable to detect the small pools of activated Rac in these cells using the Rac affinity precipitation assay in response to any of these stimuli. In cells overexpressing exogenous Tiam1, each of these stimuli led to increased levels of activated Rac in pooled cell lysates (supplemental Fig. S2B), confirming the validity of our pull-down assays. Thus the use of the FRET technique enabled us to determine stimulus-specific signaling at physiologic levels of Tiam1.

Tiam1 Colocalizes with Scaffold Proteins in Response to Specific Stimuli—To confirm the results of our FRET experiments we examined the localization of Tiam1 and each scaffold protein in response to specific stimulation. To isolate the specific pathways involved, we did this analysis in fibroblast lines with specific suppression of each scaffold protein while expressing the other scaffold as a GFP fusion protein. Thus in cells with IRSp53 suppression we were able to examine how each stimulus affected the co-localization of Tiam1 and expressed spinophilin; conversely in cells with spinophilin suppression we were able to look at how each stimulus affected the co-localization of Tiam1 and expressed IRSp53. We again examined endogenous Tiam1 in these assays, as we have found it difficult to detect shifts of small subpopulations of molecules in cells with overexpressed Tiam1.

In resting shIRSp53 cells expressing GFP-spinophilin, there is a population of spinophilin at the plasma membrane (Fig. 7A), whereas most of the Tiam1 is in the cytosol in a perinuclear location (Fig. 7B). After PDGF stimulation a similar pattern was seen for both proteins (Fig. 7, D and E), with no real co-localization at the membrane (Fig. 7F). We found similar results after stimulation with pervanadate, with Tiam1 remaining in a perinuclear cytosolic location after stimulation (supplemental Fig. S8, panels D–F). This is consistent with our FRET data showing that IRSp53 suppression prevented Tiam1-dependent Rac activation in response to either pervanadate or PDGF.

In contrast, in shIRSp53 cells expressing GFP-spinophilin, a subpopulation of Tiam1 was seen at the plasma membrane, co-localizing with spinophilin, in response to epinephrine (arrows in Fig. 7, H and I). Similar findings were seen after forskolin stimulation (supplemental Fig. S8, panels H and I). This is also consistent with our conclusions from the FRET data that spinophilin may be involved in Tiam1 activation of Rac in response to epinephrine or forskolin.

In resting shSpinO cells expressing GFP-IRSp53, the IRSp53 has a diffuse cytosolic localization (Fig. 7J) and the Tiam1 is again largely perinuclear (Fig. 7K), similar to resting shIRSp53 cells (Fig. 7B). Upon PDGF stimulation, a population of IRSp53 shifts to the plasma membrane (Fig. 7M), and Tiam1 is found throughout the cytosol with a subpopulation at the membrane (Fig. 7N), colocalizing with IRSp53 at the plasma membrane (Fig. 7O). Similar findings were seen with pervanadate stimulation (supplemental Fig. S8, panels M–O). In contrast, epinephrine or forskolin stimulation did not lead to a localization shift for either IRSp53 (Fig. 7P and supplemental Figs. S8P) or Tiam1 (Fig. 7Q and supplemental Fig. S8Q). These findings again complement our FRET data showing that IRSp53 is involved in Tiam1-dependent Rac activation in response to PDGF or pervanadate, whereas spinophilin is involved in Tiam1-mediated Rac activation in response to epinephrine or forskolin.
fraction of cells exhibiting lamellipodia that are rich in isoactin localization and spreading studies, we used both anti-spinophilin and transient expression of GFP-spinophilin (panels A–I) or stable suppression of spinophilin and transient expression of GFP-IRSp53 (panels J–R). Cells were unstimulated (panels A–C and J–I), or stimulated with PDGF (panels D–F and M–O), or epinephrine (G–I and P–R). Arrows indicate examples of Tiam1 localization at plasma membranes (panels H and N) and co-localization with scaffold (I and O).

Tiam1-dependent Changes in Cell Spreading and Actin Require IRSp53, but Not Spinophilin—We have previously found that transiently induced RNA interference-mediated suppression of either Tiam1 or IRSp53 inhibits the formation of actin-based ruffles and lamellipodia in response to PDGF (9). Our FRET results suggest that either PDGF or pervanadate treatment induces Tiam1- and IRSp53-dependent Rac activation. We next determined whether stable suppression of Tiam1 or IRSp53 would block induced Rac-dependent actin changes in cells undergoing replating after trypsinization. For in vitro isoactin localization and spreading studies, we used both antibody to β-actin, the actin isoform that is preferentially localized to plasma membranes associated with regions of cell motility and observed during cell spreading (e.g. filopods, pseudopods, and fan lamellae), and phalloidin, which binds to filamentous (F)-actin, so that β-actin and γ-actin isoforms could be readily visualized (27, 38). Control cells exhibited rapid reattachment of spread cells for each cell line at selected time points. Representative images of all cells at 4 h after spreading are shown in Supplemental Fig. S9. In contrast, cells with suppressed IRSp53 expression (panels J–L). In contrast, cells with suppressed spinophilin expression exhibited rapid replating and lamellipodia formation (panels M–O), similar to control cells. Panel P shows the quantification of these results. Expanded images of lamellipodia and rounded cell morphology are shown in supplemental Fig. S9.

We also followed the cells over time for kinetics of adhesion and spreading after replating. Panel Q shows the quantification of spread cells for each cell line at selected time points. Representative images of all cells at 4 h after spreading are shown in
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Increased migration significantly (compare lane 4 with lane 2). Suppression of spinophilin did not seem to affect migratory ability at all when compared with control cells (lanes 5 and 6), whereas suppression of IRSp53 also led to significantly increased migration (compare lane 8 with lane 2).

Increased cell migration reflects decreased cell adhesion. These results suggest that suppression of a specific Tiam1-scaffold protein pathway involving IRSp53 can affect cell adhesion and migration in these cells. We therefore asked whether using specific stimulation to direct Tiam1 signaling down a different scaffold protein-directed pathway (spinophilin) would mimic the effects of IRSp53 suppression to some extent. Control cells were induced to migrate toward conditioned medium (Fig. 9B, lanes 1 and 2) and were then treated with epinephrine to induce Tiam1 signaling in association with spinophilin rather than in association with IRSp53. Epinephrine treatment led to a reproducible increase in migration over conditioned medium alone (lane 3). The enhanced migration triggered by epinephrine was Rac-dependent, as cells treated with the Rac inhibitor retained directed migration toward conditioned medium (compare lane 4 with lane 5), but lost the response to epinephrine (lane 6). Epinephrine-enhanced migration was also dependent on an intact spinophilin pathway, as cells with spinophilin silencing also lost the epinephrine response while retaining directed migration toward conditioned medium (lanes 7–9).

DISCUSSION

Our results indicate that Tiam1 induces localized activation of subcellular pools of Rac molecules in response to multiple upstream signals, leading to targeted activation of specific downstream pathways, through interactions with different scaffold-mediated protein complexes. This is supported by several observations. We have been able to measure Rac activation in response to different cell stimuli through detection of FRET from activated Rac to a known Rac target. Of note, we could not detect global activation of Rac in pooled cell lysates using affinity precipitation by an immobilized version of the same Rac target under the same stimulus conditions in cells with endogenous levels of Tiam1. We used exogenous Rac species for both the FRET and the affinity precipitation “pulldown” assays, in the former as a requirement of the technique and in the latter because endogenous levels of Rac in these cells are low and very difficult to detect with available antibodies. Of note, we have previously found that the levels of CFP-Rac expressed in the cells chosen for FRET analysis are equivalent to the levels of endogenous Rac (22), confirming the utility of this technique in assessing specific signaling involving subpopulations of cells under physiologic conditions. Using the FRET technique allowed us to detect activated Rac levels and then normalize for the Rac expression level and changes in localized Rac recruitment due to intracellular protein localization shifts or cellular shape changes. This enabled us to derive a measure of Rac activation per molecule of Rac in designated subcellular regions of individual cells. In contrast, the negative results of affinity precipitation experiments under similar conditions suggest that affinity precipitation is not sufficiently sensitive to discriminate a small pool of activated Rac in comparison to the total Rac population in pooled cell lysates. However, affinity precipitat-
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tion on fractionated cell lysates of cells with Tiam1 overexpression showed the pool of activated Rac to be largely in the membrane fraction, confirming our FRET-based findings of localized Rac activation.

Several different stimuli induced Rac activation in a Tiam1-dependent fashion, as Rac was blunted in cells treated with Tiam1 silencing and restored in cells re-expressing Tiam1 from a Tiam1 shRNA “rescue” plasmid (Fig. 3 and not shown). The FRET technique revealed stimuli not previously known to involve Tiam1. Although pervanadate and PDGF have already been shown to induce Tiam1 activation in other systems, forskolin and epinephrine were not known to do so. Equally important, the fact that we did not detect Tiam1 involvement in Rac activation by exposure to oxidative stress, linked recently to the NADPH oxidase complex and the exchange factor Vav but not Tiam1 (41), validates this approach to determining the dynamics of specific Rac activation on a subcellular level.

We also found that these stimuli lead to Tiam1-dependent Rac activation dependent on the presence of specific scaffold proteins known to interact directly with Tiam1. Furthermore, we found co-localization between Tiam1 and either IRSp53 or spinophilin in response to specific stimuli consistent with results from our FRET experiments. Based on our prior work, interaction between Tiam1 and an individual scaffold protein complex allows for Tiam1 to induce targeted Rac-dependent activation of a specific Rac effector pathway (Arp2/3-mediated actin remodeling in the case of IRSp53-WAVE2 complex, p70 S6 kinase activation in the case of the spinophilin complex). Determining the physiologic role of a scaffold protein in specific signaling pathways can be difficult as overexpression of such proteins can lead to artifactual sequestration of interacting endogenous species. We therefore established a system designed to test the effect of suppressing individual scaffold proteins on stimulated Rac activation. Of note, complete silencing of individual scaffold proteins was not needed to reveal stimulus-specific Rac activation, suggesting that partial suppression was enough to shift the balance between the pools of scaffold protein molecules available to Tiam1.

In addition, we found that both Tiam1 and IRSp53, but not spinophilin, influence isoactin dynamics, cell shape, and spreading. In particular the defect introduced by Tiam1 or IRSp53 suppression largely affected the cortical positioning of β-actin at mobile cell membranes and at the cytoskeletal-plasma membrane interface (Fig. 8). This suggests that IRSp53, but not spinophilin, may be specifically involved in signaling through the β-actin network, indicating a direction for future study. We were also able to alter the migratory phenotype of control cells by using a specific signal (epinephrine) to direct signaling to an alternate scaffold protein (Fig. 9). In our system, suppression of either Tiam1 or IRSp53, but not spinophilin, led to decreased cell adhesion and enhanced cell migration (Fig. 9A), suggesting that the “default” Tiam1 pathway under basal conditions in these cells in terms of adhesion/migration is through IRSp53. Shifting Tiam1 signaling toward spinophilin and away from IRSp53 through stimulation with epinephrine led to enhanced cell migration, mimicking the phenotype seen with IRSp53 silencing. This was dependent on both the ability to activate Rac and the presence of the Tiam1-interacting scaffold relevant to the epinephrine stimulus (i.e. spinophilin). Together with the results of our FRET and replating experiments, this indicates that by interacting with specific scaffold protein complexes, Tiam1 allows for discrimination of upstream signals while also targeting individual downstream effector pathways for activation. Taken together with our previous work, this suggests that in addition to actually catalyzing the activation of Rac itself, Tiam1 plays an active role in determining the circumstances in which Rac is activated and the consequent effects of that activation. Although our work focused on Tiam1, there are increasing examples in the literature of other exchange factors participating in scaffold protein complexes and it is reasonable to predict that this might be a general property for many guanine-nucleotide exchange factors.

Scaffold proteins such as IRSp53 and spinophilin possess no intrinsic catalytic activity, so it is not immediately intuitive to expect that their silencing would affect activation of Rac, as opposed to localization or sequestration of Rac. This suggests that interaction of the scaffold protein with Tiam1 could be required for activation of Tiam1 itself. It may be that the combination of a specific upstream stimulus and interaction with a scaffold protein leads to localized activation of Tiam1, which then generates localized activation of Rac in a targeted fashion. Little is known about the molecular steps involved in the activation of Tiam1. As is the case for many Rho family exchange factors, deletion of terminal amino acids (in this case, N1–342) leads to a constitutively active protein, consisting of the C-terminal 1199 amino acids. This suggests that relief of an inhibitory intramolecular interaction involving the N terminus may be involved in enabling Tiam1 activation. It may be that a specific stimulus and interaction with a particular scaffold protein combine to relieve the autoinhibited state, which we are beginning to investigate.

Of note, our results do not distinguish between different possible steps leading to the end result of localized Rac activation. Those Rac molecules that are activated in association with a specific scaffold in response to a specific stimulus may be immediately locally available to the exchange factor, or may be recruited from other complexes. Recent work indicates that interactions between RhoGD1 and cytoplasmic Rac lead to spatial restrictions on downstream interactions between active Rac and effector molecules (42). Furthermore, exchange factors including Tiam1 may participate in dissociating cytoplasmic Rac from complexes with RhoGD1 (43). Whether Tiam1-interacting scaffold proteins also participate in this process remains to be studied.

This work builds on our prior findings to answer the question of how cells can achieve signaling specificity through pathways involving monomeric GTPases, many of which receive multiple inputs and have multiple potential downstream effector proteins. We have found that for the Rac GTPase, the ubiquitous exchange factor Tiam1 is a key factor in translating multiple different upstream stimuli into specific downstream effects through generation of localized pools of activated Rac in association with different scaffold protein complexes. Tiam1 therefore acts as a master regulator at the hub of a network of path-
ways that intersect at the step of Rac activation. Our findings here suggest that it may be possible to modulate Rac-dependent cellular behaviors by shifting the signals traversing this network through manipulation of Tiam1 association with different scaffolds.

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