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# Tiam1-IRSp53 Complex Formation Directs Specificity of Rac-Mediated Actin Cytoskeleton Regulation†

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**The exchange factor Tiam1 regulates multiple cellular functions by activating the Rac GTPase. Active Rac has various effects in cells, including alteration of actin cytoskeleton and gene expression, via binding to and modulating the activity of diverse effector proteins. How individual Rac effectors are selected for activation and regulated in response to upstream signals is not well understood. We find that Tiam1 contributes to both of these processes by binding to IRSp53, an adaptor protein that is an effector for both Rac and Cdc42. Tiam1 directs IRSp53 to Rac signaling by enhancing IRSp53 binding to both active Rac and the WAVE2 scaffold. Moreover, Tiam1 promotes IRSp53 localization to Rac-induced lamellipodia rather than Cdc42-induced filopodia. Finally, IRSp53 depletion from cells prevents Tiam1-dependent lamellipodia induced by Tiam1 overexpression or platelet-derived growth factor stimulation. These findings indicate that Tiam1 not only activates Rac but also contributes to Rac signaling specificity through binding to IRSp53.**

The Rac GTPase influences multiple cellular functions through participation in diverse signaling pathways, including those governing movements of the actin cytoskeleton, activation of transcription factors, and regulation of the NADPH oxidase complex (reviewed in reference 14). Active GTP-bound Rac mediates these various functions through the interaction of effector domain sequences with a number of downstream effector proteins and can thereby potentially activate numerous parallel downstream pathways (35). How signaling specificity is achieved in this system with myriad potential downstream outcomes is the focus of intense investigation. In particular, how Rac signals are specifically directed to the machinery regulating actin dynamics is not well understood.

Activation of the Rac GTPase occurs through the exchange of bound GDP for GTP, stimulated by one of multiple Rac guanine nucleotide exchange factors (Rac-GEFs) (2). Rac-GEFs all share homologous catalytic Dbl homology (DH) domains adjacent to pleckstrin homology (PH) domains but differ in tissue and cellular distribution and their regulation by upstream signals. Recent evidence suggests that Rac exchange factors may play a key role in determining signaling specificity downstream of Rac. Different Rac-GEFs activate downstream effector pathways differently despite stimulating similar levels of Rac-GTP (28, 37). This may be due to the fact that Rac-GEFs can select particular downstream effector proteins for activation by Rac, either through direct binding or through participation in scaffold protein complexes that organize components of a specific signaling pathway downstream of Rac. The former is exemplified by the Rac-GEF PIX/COOL, which binds directly to the Rac effector Pak (1, 18). The latter is exemplified by the ubiquitously expressed Rac-GEF Tiam1, which is implicated in numerous cellular events, including invasion, adhesion, axon formation, and apoptosis (reviewed in reference 19). In particular, we have shown that Tiam1 binds through its N-terminal regulatory domains to different scaffold proteins, IB2/JIP2 and spinophilin, leading to specific downstream activation of p38 and p70 S6 kinase, respectively, in distinct subcellular regions (5, 6). Here we show that Tiam1 can exemplify both mechanisms by binding to the adaptor protein IRSp53, which is part of another scaffold complex involving WAVE2. IRSp53 is implicated in two distinct pathways affecting actin cytoskeleton dynamics involving either Rac or Cdc42, through its ability to bind to either activated GTPase as well as to different scaffold proteins mediating actin polymerization (13, 16, 23). Tiam1 enhances the signaling specificity of IRSp53 toward Rac effects on actin by promoting the formation of complexes between IRSp53, activated Rac, and WAVE2 and by localizing IRSp53 to lamellipodia. Finally, IRSp53 is required for Tiam1-induced ruffling and both Tiam1 and IRSp53 are required for platelet-derived growth factor (PDGF)-induced ruffling. These findings lead us to conclude that interaction with IRSp53 enables Tiam1 to direct Rac effects specifically toward dynamics of the actin cytoskeleton.

## MATERIALS AND METHODS

**Yeast two-hybrid assay.** A partial IRSp53 cDNA clone was obtained using the yeast two-hybrid method with *Saccharomyces cerevisiae* (strain Y190). The bait plasmid was constructed by insertion of sequences encoding amino acids 431 to 670 of Tiam1 cDNA (spanning the N-terminal PH, coiled coil [CC], and Ex domains) in frame with the Gal4 DNA binding domain in the pAS-Cyh vector. Bait DNA was cotransfected into yeast, along with a commercial rat brain cDNA library cloned in frame with the Gal4 DNA activation domain in the pGAD10 vector (Clontech). DNAs from colonies growing under dual-selection conditions which also showed histidine auxotrophy and expressed β-galactosidase were retested as described above to confirm the yeast interaction and then sequenced.

**Plasmids.** Plasmids encoding cDNAs for full-length Tiam1, Tiam C-1199, ΔPCX-Tiam1, pEBG-Jnk, and Ral72L have been described previously (5, 6, 11).

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The plasmids encoding myc-IRSp53 and GFP-WAVE2 were kindly provided by A. Hall and S. B. Snapper, respectively. The plasmids encoding IRSp53 mutants with deletions of the N-terminal 179, 242, or 299 amino acids were engineered by PCR amplification using suitable primers followed by ligation into the pJ3Myc-vector prepared by BamHI/EcoRI digestion. For small interfering RNA (siRNA) reagents, sequences were derived from the public database based on homology as follows: for Tiam1 (murine and human), 5′-GAGACTCCTCCGTACAGTA-3′; for murine IRSp53, 5′-CTCGTACTCCAACACACTC-3′. RNA duplexes were synthesized by Dharmacon. The pSUPER and pSUPERIOR.retro.neo.gfp DNA vectors for intracellular siRNA synthesis were purchased from Oligo-Engine. DNA oligomers were designed based on the RNA duplex sequences and according to guidelines from OligoEngine, synthesized in the Tufts-NEMC Protein Synthesis Core Facility, and ligated into the vectors according to the manufacturer's instructions.

**Antibodies and immunoblotting.** Antibodies to Tiam1, green fluorescent protein (GFP; all from Santa Cruz), spinophilin (Upstate Biotechnology), and phospho-Jnk and phospho-Erk (Cell Signaling) were used according to the manufacturers' instructions. The anti-myc epitope antibody was generated by the Tufts-NEMC GRASP Center Antibody and Cell Culture Core Facility. To generate antibodies to IRSp53, a glutathione *S*-transferase (GST) fusion protein with the N-terminal 157 amino acids of IRSp53 was engineered with standard molecular techniques and used to immunize two rabbits (Cocalico Biologicals). The antisera from rabbit TF67 was affinity purified using GST-N157-IRSp53 coupled to an Affi-prep 10 column (Bio-Rad). Secondary antibodies, immunoblotting, and the chemiluminescence protocol for developing blots have been previously described (7).

**Cell culture and transfection.** HEK 293T cells were grown in Dulbecco's modified Eagle's medium containing 10% iron-supplemented bovine calf serum (HyClone) in an incubator with humidified air (5% $CO_2$) at 37°C. NIH 3T3 cells were grown as described above in Dulbecco's modified Eagle's medium containing 10% bovine calf serum (HyClone). Cells were plated in 60-mm plastic dishes, and transient transfections were performed using calcium phosphate precipitation (293T), Lipofectamine 2000 (NIH 3T3), or Oligofectamine (RNA interference [RNAi]) according to the manufacturer's instructions (Gibco BRL). For Lipofectamine and Oligofectamine transfections, cells were initially plated in antibiotic-free media. Cells were harvested 48 h after transfection. When indicated, cells were deprived of serum prior to harvest.

**Immunoprecipitations.** Transfected cells were washed with cold phosphate-buffered saline (PBS), harvested, pelleted, and lysed in buffer M (1% Triton X-100 in 20 mM Tris-HCl [pH 7.4], 125 mM NaCl, 1 mM $MgCl_2$) containing protease inhibitors (10 μg/ml aprotinin, 20 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (50 μM sodium fluoride and 100 μM sodium orthovanadate). Some cells were deprived of serum for 8 h, and a subset was treated with 20 ng/ml PDGF for 5 min prior to harvest. Rat brains were frozen, ground in liquid $N_2$, and then homogenized in buffer M using 10 strokes in a Dounce homogenizer. Brain or cell lysates were cleared of unbroken cells and debris by 10,000 × *g* centrifugation for 10 min. Cleared lysates were incubated with protein A-Sepharose beads (Pharmacia) and appropriate antibody (diluted according to the manufacturer's instructions) for 2 h at 4°C with constant agitation. Aliquots of cleared lysate were retained prior to immunoprecipitation for immunoblotting. After being washed two times with ice-cold PBS containing 1% Triton and one time with PBS, bound proteins were eluted in 4× Laemmli buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted.

**GTPase binding.** GST, GST-Rac, or GST-Cdc42 was expressed in *Escherichia coli*, extracted using phosphate-buffered saline containing 1% Triton X-100 and 5 mM dithiothreitol (DTT), immobilized on glutathione-agarose beads, and loaded with GTPγS in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 20 mM EDTA, 1 mM DTT, 1 mg/ml bovine serum albumin (BSA), 0.5 mM $MgCl_2$, and 0.2 mM GTPγS. Lysates of transfected cells were incubated with equivalent amounts of GST or GST-GTPase for 1 hour in Buffer M containing 1 mM DTT and 2 mM $MgCl_2$. After washing, bound proteins were eluted in 4× Laemmli buffer, resolved by SDS-PAGE, and immunoblotted.

**Immunofluorescence.** For immunofluorescence experiments, $2 \times 10^5$ cells were plated per 35-mm well containing coverslips coated with poly-L-lysine prior to transfection. Cells were deprived of serum for 8 h and, where indicated, stimulated with 20 ng/ml PDGF for 2 h prior to fixation in PBS containing 4% paraformaldehyde. Cells were blocked and permeabilized with PBS containing 1% BSA and 0.5% Triton X-100. Anti-rabbit Cy3 (Jackson Immunologicals), anti-mouse-Alexa Fluor 488 and phalloidin-Alexa Fluor 546 (Molecular Probes) were diluted in PBS containing 1% BSA according to the manufacturer's instructions. Cells were washed with PBS between each step; after the final washing, coverslips were mounted using 50% glycerol in PBS. The viability of all cells depicted and quantitated was assessed by Hoechst staining. Confocal images were obtained on a Leica TCS SP2 instrument through the Tufts-NEMC GRASP Center Imaging and Cell Analysis Core facility.

## RESULTS

**Tiam1 binds to IRSp53 in cells.** To identify additional Tiam1 binding scaffolds that might specifically connect Tiam1 signaling to the actin cytoskeleton, we used the N-terminal regulatory region containing the PH-CC-Ex domains of Tiam1 in a yeast two-hybrid screen. We identified a cDNA clone encoding amino acids 242 to 521 of IRSp53, a protein previously shown to bind active Rac-GTP and promote actin polymerization through binding to the WASP family protein WAVE2 (Fig. 1A) (23). To begin to test the physiological significance of this finding, we confirmed that Tiam1 binds to IRSp53 in mammalian cells. Endogenous Tiam1 was first immunoprecipitated from 293T cells transfected with myc epitope-tagged IRSp53. Immunoblots using anti-myc antibody detected significant IRSp53 in Tiam1 but not control immunoprecipitations (Fig. 1B). Similar results were obtained with endogenous proteins from rat brain extracts when Tiam1 immunoprecipitates were immunoblotted with antisera against IRSp53 (Fig. 1C) (see Fig. S1 in the supplemental material for assays of antibody specificity). As expected, the ΔPCX-Tiam1 mutant, which lacks the PH-CC-Ex domains used as bait in the two-hybrid screen, did not coprecipitate with IRSp53 (Fig. 1D). We also began to map the Tiam1 interaction region on IRSp53. Deletion of either the IRSp53 SH3 domain or sequences C-terminal to it did not prevent Tiam1 binding (not shown). In addition, deletion of the N-terminal 242 amino acids also did not prevent Tiam1 binding, consistent with the two-hybrid results (Fig. 1E). However, further deletion of the N-terminal 299 amino acids greatly reduced Tiam1 binding. This suggests that the same central region of IRSp53 may mediate binding to both Tiam1 and activated Cdc42.

**Tiam1/IRSp53 binding is regulated in cells.** To determine whether this interaction can be regulated in cells, we examined the effect of PDGF, a known stimulator of Rac-mediated membrane ruffling (26), on complex formation between Tiam1 and IRSp53 in NIH 3T3 cells (Fig. 2A). We found that PDGF stimulation enhanced complex formation between Tiam1 and IRSp53. Furthermore, PDGF-enhanced complex formation was abrogated in the presence of dominant-negative Ras 17N, consistent with reports that Tiam1 is a target of activated Ras (17). Additional support for this model was derived from experiments in 293T cells, where the interaction of Tiam1 and IRSp53 was enhanced in the presence of constitutively active Ras61L but not in the presence of dominant-negative Ras 17N (Fig. 2B). These results support the idea that Ras activation by growth factor enhances Tiam1 association with IRSp53.

**Tiam1 enhances IRSp53 interaction with both the WAVE2 scaffold protein and activated Rac.** The finding that Tiam1 interacts with IRSp53 is of particular interest because it explains how a specific Rac target that mediates effects on the actin cytoskeleton can be selected for activation by Rac. However, IRSp53 is involved in both Rac-mediated lamellipodium and Cdc42-mediated filopodium formation via its ability to bind to both active Rac and active Cdc42 as well as to either WAVE2 or Mena, two scaffolds associated with actin filament

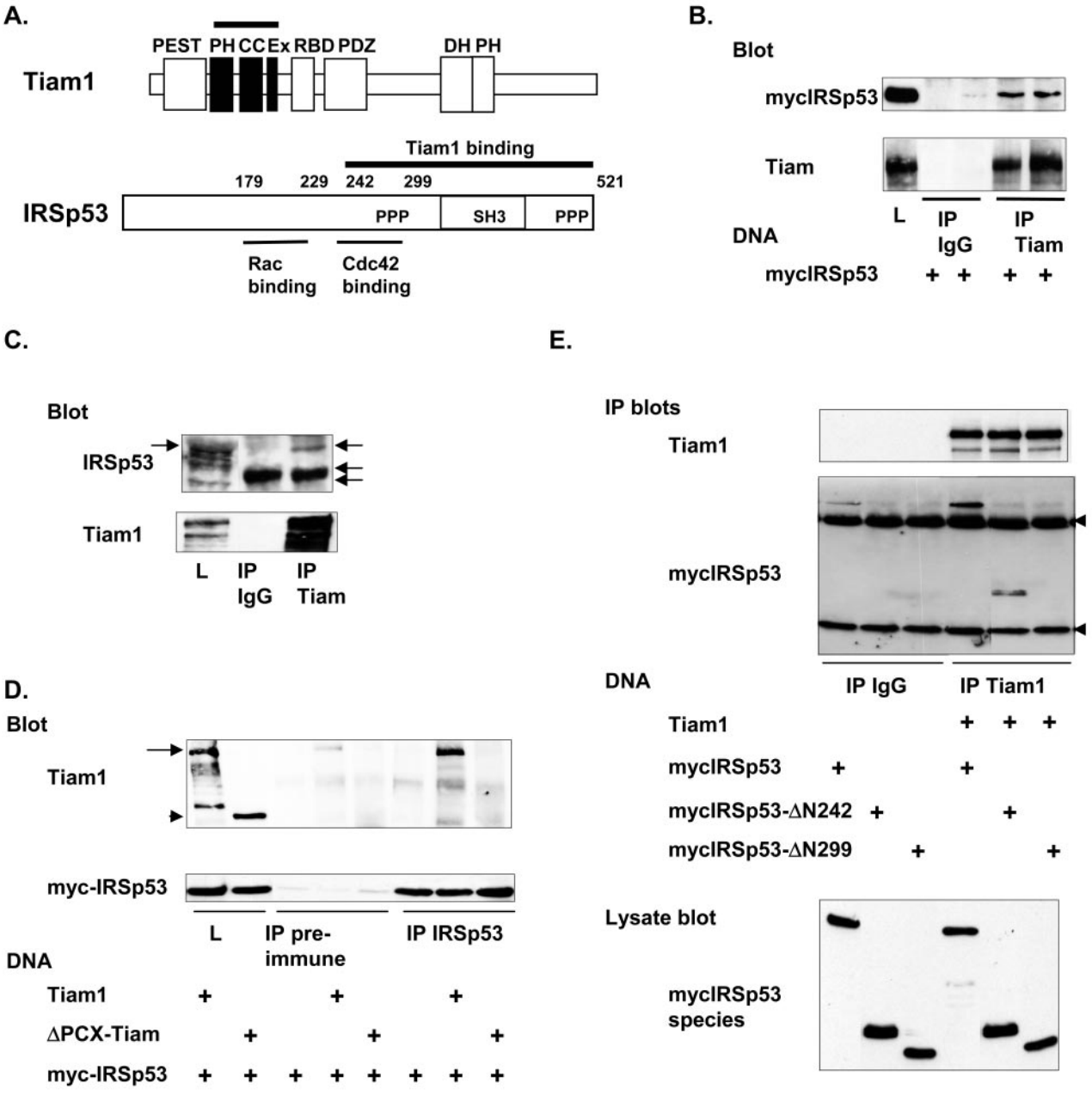


FIG. 1. Tiam1 interacts with IRSp53 in cells. (A) Domains of Tiam1 and IRSp53, with regions interacting in the yeast two-hybrid screen denoted by heavy black bars. PH, pleckstrin homology domain; CC, coiled coil domain; Ex, Ex region; RBD, Ras-binding domain; DH, Dbl homology domain; PPP, proline-rich regions. The PEST, PDZ, and SH3 domains are also indicated. Thin black bars indicate reported GTPase binding regions on IRSp53. (B) Endogenous Tiam1 associates with transfected myc-IRSp53 in HEK 293T cells. L denotes input lysates, which were precipitated with anti-rabbit immunoglobulin G (IgG) or anti-Tiam1 antibody, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted as indicated. IP, immunoprecipitation; +, transfected DNA. (C) Endogenous Tiam1 and IRSp53 associate in cells. Rat brain lysates were precipitated with anti-rabbit IgG or Tiam1 and processed as described for panel B. The single arrow indicates the position of IRSp53, and the double arrow indicates precipitating heavy chain. (D) Tiam1 interacts with IRSp53 via the PH-CC-Ex domains. Lysates of 293T cells transfected with myc-IRSp53 and Tiam1 species were precipitated with preimmune or anti-IRSp53 antibody and processed as described for panel B. ΔPCX-Tiam1 lacks the PH-CC-Ex region used as bait in the yeast two-hybrid screen. The arrow and arrowhead indicate full-length Tiam1 and ΔPCX-Tiam1, respectively. (E) IRSp53 interacts with Tiam1 sequences spanning the Cdc42-binding region. Tiam1 precipitates of lysates of 293T cells transfected with Tiam1 and myc-IRSp53 species, either wild type or lacking the N-terminal 242 or 299 amino acids, were processed as described for panel B. The arrowheads indicate precipitating heavy and light chains.

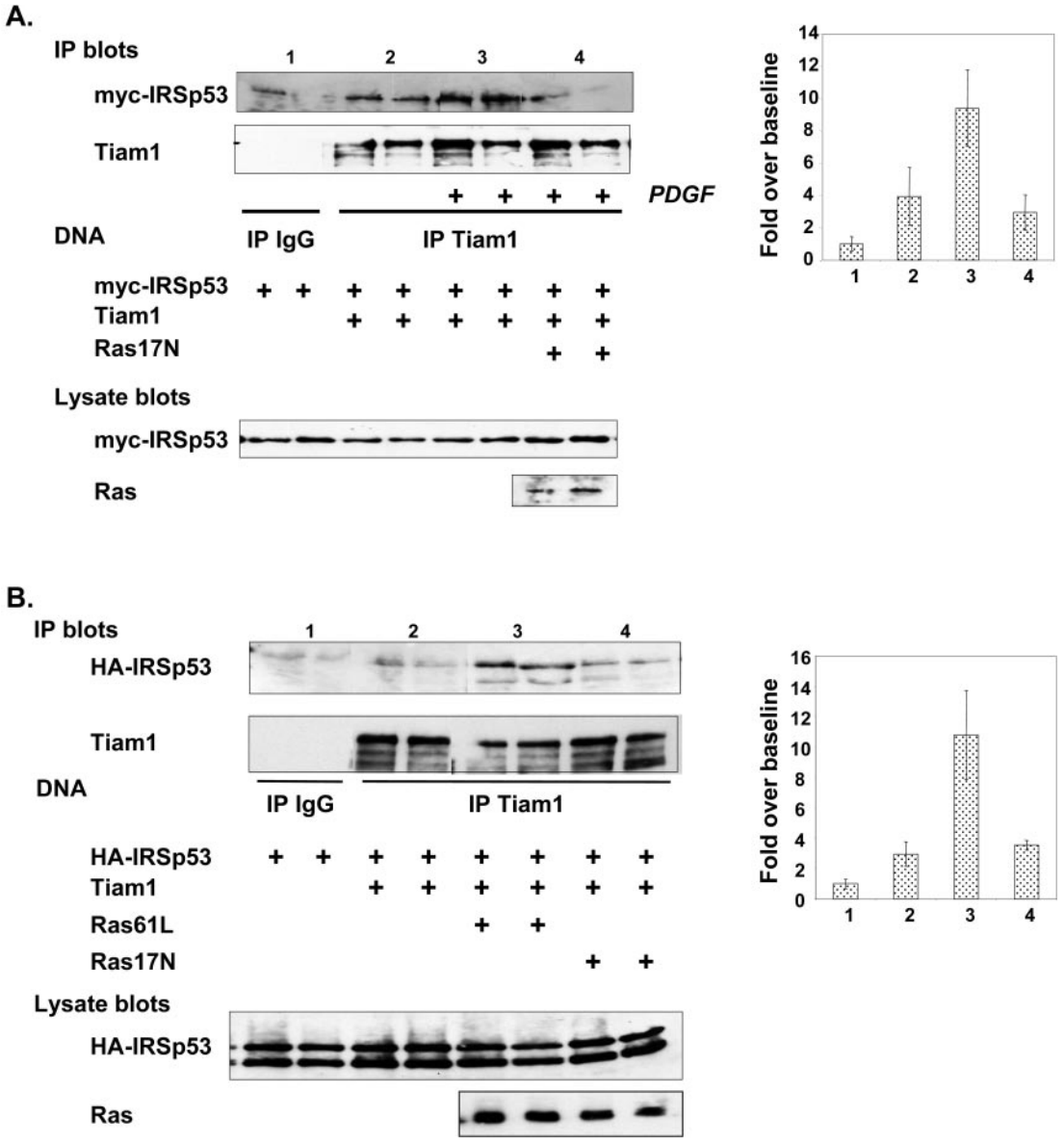


FIG. 2. Tiam1-IRSp53 interaction is regulated in cells. (A) PDGF stimulation enhances the interaction of IRSp53 and Tiam1 in NIH 3T3 cells. Cells transfected with myc-IRSp53 and Tiam1 in the absence or presence of dominant-negative Ras17N were deprived of serum for 8 h and some were stimulated with 20 ng/ml PDGF for 5 min prior to harvest, as indicated (+). For both panels A and B, cell lysates were precipitated with control or Tiam1 antibody and processed as described in the legend to Fig. 1B. IP, immunoprecipitation. (B) Ras activation enhances the interaction of Tiam1 with IRSp53. HEK 293T cells were transfected with IRSp53 and Tiam1, some in the presence of Ras61L or Ras17N as indicated, and serum deprived for 8 h before harvest. For panels A and B, representative experiments in duplicate are shown. Results from at least two independent experiments in duplicate were quantified using NIH Image software, numbers on graphs correspond to numbers on blots, and error bars represent standard deviations.

dynamics (16, 23). WAVE2 triggers Arp2/3-mediated actin polymerization in response to active Rac (34). Mena synergizes with IRSp53 to promote formation of filopodia dependent on active Cdc42 and is recruited with the Arp2/3 complex to actin polymerization sites in nerve growth cones (12, 16). Both WAVE2 and Mena are found in lamellipodia, with Mena at the tips of actin microspikes and WAVE2 along the lamellipodial edge (24). Mechanisms controlling the degree to which IRSp53 interacts with these two alternative actin regulatory pathways have not been described. We hypothesized that Tiam1 might direct IRSp53 preferentially toward WAVE2, consistent with its role as a Rac exchange factor. We found that transfected Tiam1 enhanced the association of WAVE2 with IRSp53 in 293T cells (Fig. 3A). Furthermore, while deletion of only the extreme N-terminal PEST sequences preserved this effect (Tiam-C1199), deletion of the IRSp53-interacting PH-CC-Ex region abolished it. We did not detect direct interaction between Tiam1 and WAVE2 (not shown). In addition, the

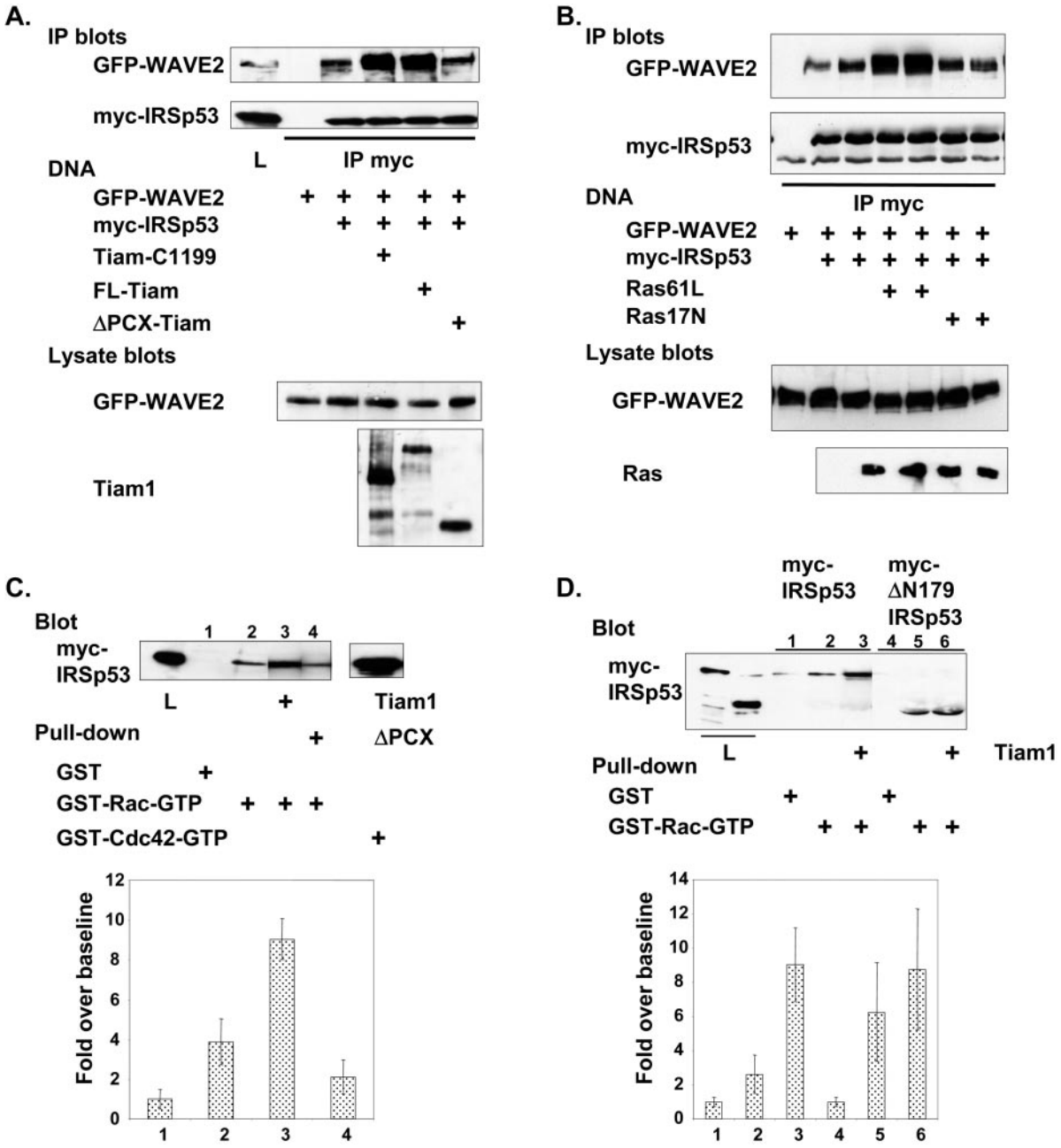


FIG. 3. Tiam1 enhances IRSp53 interaction with both WAVE2 and RacGTP. (A) Tiam1 enhances IRSp53 interaction with WAVE2. Cells were transfected with GFP-WAVE2 along with myc-IRSp53 and Tiam1 species as indicated. Tiam1-C1199 lacks the PEST sequences located at the extreme N terminus but retains the PH, CC, and Ex domains. myc immunoprecipitations (IPs) were resolved by SDS-PAGE, transferred, and immunoblotted with anti-GFP antibody to indicate coprecipitating WAVE2 or with anti-myc antibody to indicate precipitated IRSp53. (B) Activated Ras enhances IRSp53 interaction with WAVE2. Cells were transfected with WAVE2 and IRSp53, along with Ras61L or Ras17N. Cells were processed as described for panel A. (C) Tiam1 enhances IRSp53 interaction with Rac-GTP. Lysates of cells expressing equivalent levels of myc-IRSp53, some also expressing Tiam1 species as indicated, were incubated with equivalent amounts of immobilized GST, GST-Rac-GTP, or GST-Cdc42-GTP. Interacting IRSp53 was immunoblotted with myc antibody. L indicates input lysates containing full-length IRSp53; plus signs indicate the presence of coexpressed Tiam1 or ΔPCX-Tiam1 in the lysates. (D) Tiam1 may regulate IRSp53-Rac interaction through effects at the N terminus of IRSp53. Lysates of cells expressing equivalent levels of myc-IRSp53 or myc-IRSp53-ΔN179 were incubated with equivalent amounts of immobilized GST or GST-Rac-GTP. Interacting IRSp53 species were immunoblotted with myc antibody. L indicates input lysates containing full-length or mutant IRSp53 (first 2 lanes, respectively), plus signs indicate the presence of expressed Tiam1 in the lysates. For panels C and D, results were quantified as described in the legend to Fig. 2.

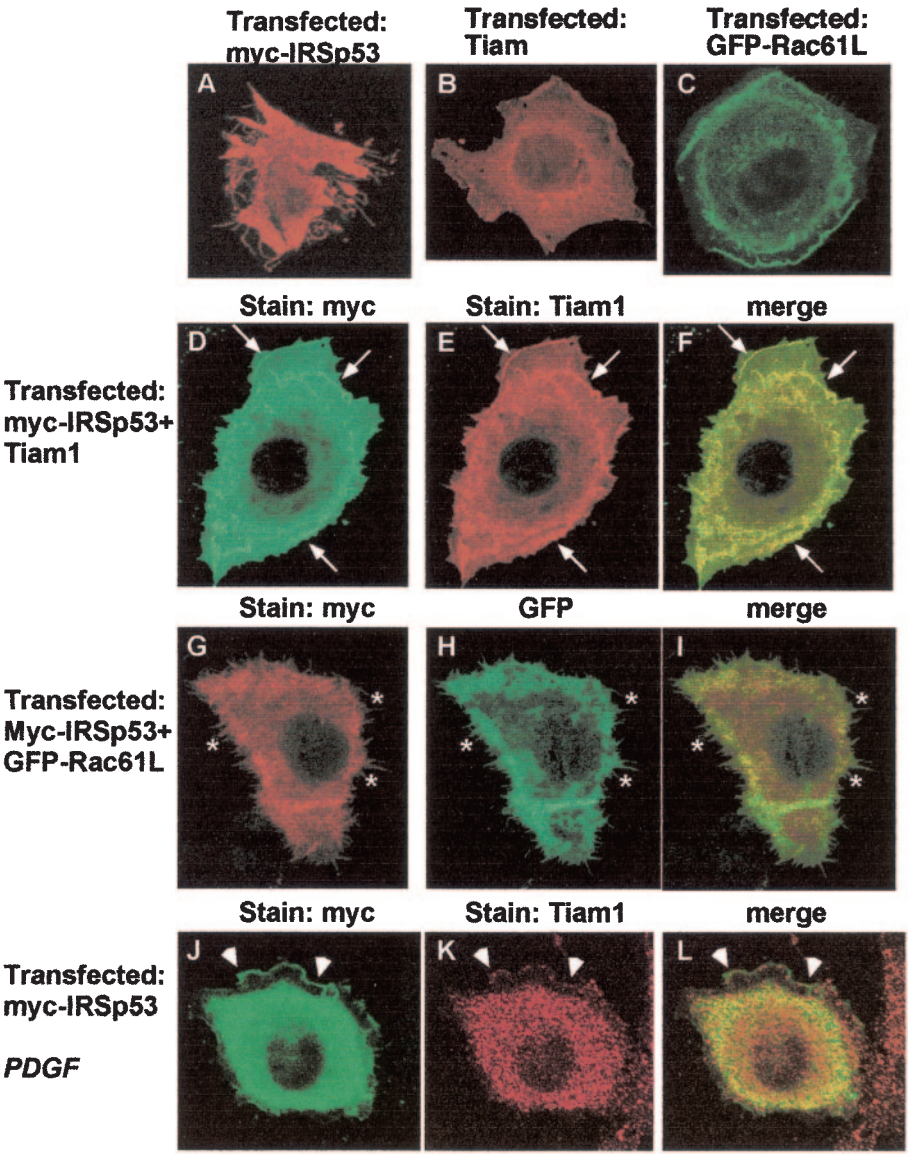


FIG. 4. Tiam1 interaction promotes IRSp53 localization at lamellipodia. (A) IRSp53 expression leads to filopodia in NIH 3T3 cells. Cells were transfected with myc-IRSp53 and visualized using anti-myc antibody and anti-mouse Alexa fluor 488 conjugate. Images in panels A through L were obtained using confocal fluorescence microscopy. (B) Tiam1 expression leads to lamellipodia and ruffling in NIH 3T3 cells. Cells were transfected with Tiam1 and visualized using anti-Tiam1 antibody and anti-rabbit Cy3 conjugate. (C) Expression of activated Rac leads to ruffling in NIH 3T3 cells. Cells were transfected with GFP-Rac61L and visualized by GFP fluorescence. (D to F) Tiam1 expression leads to IRSp53 localization along ruffles. Cells were transfected with both myc-IRSp53 and Tiam1, and coexpressing cells were visualized as described above. The same field is shown in panels D to F. (D) myc-IRSp53 staining (green); (E) Tiam1 staining (red); (F) overlay image (colocalization in yellow). Arrows indicate some of the ruffles present. (G to I) Activated Rac does not change the localization of IRSp53 in filopodia. Cells were transfected with both myc-IRSp53 and GFP-Rac61L, and coexpressing cells were visualized with anti-myc antibody and anti-mouse Cy3 conjugate or GFP fluorescence. The same field is shown in panels G to I. (G) myc-IRSp53 staining (red); (H) GFP-Rac61L staining (green); (I) overlay image. Asterisks indicate some of the filopodia present. (J to L) PDGF stimulation leads to colocalization of IRSp53 and endogenous Tiam1 along ruffles. Cells expressing levels of myc-IRSp53 sufficient for detection but insufficient to promote extensive filopodia were visualized with anti-myc (J) or anti-Tiam1 antibody (K) after stimulation with 20 ng/ml PDGF for 2 h prior to fixation. The same field is shown in panels J to L. (J) myc-IRSp53 staining (green); (K) Tiam1 staining (red); (L) overlay image. Arrowheads indicate ruffle edges.

presence of activated, but not dominant-negative, Ras also promoted the interaction of IRSp53 with WAVE2 (Fig. 3B), consistent with the previous finding that activated Ras enhanced IRSp53-Tiam1 binding. These findings suggest that signaling through Tiam1 enhances the interaction of IRSp53 with WAVE2, generating Rac-induced effects on the actin cytoskeleton. We could not directly test the converse hypothesis that Tiam1 interaction might negatively affect the interaction of IRSp53 with Mena, as we were unable to demonstrate binding between full-length IRSp53 and Mena in our system. This may reflect limiting amounts of available proteins, uncharacterized negative regulatory factors, or restricted subcel-

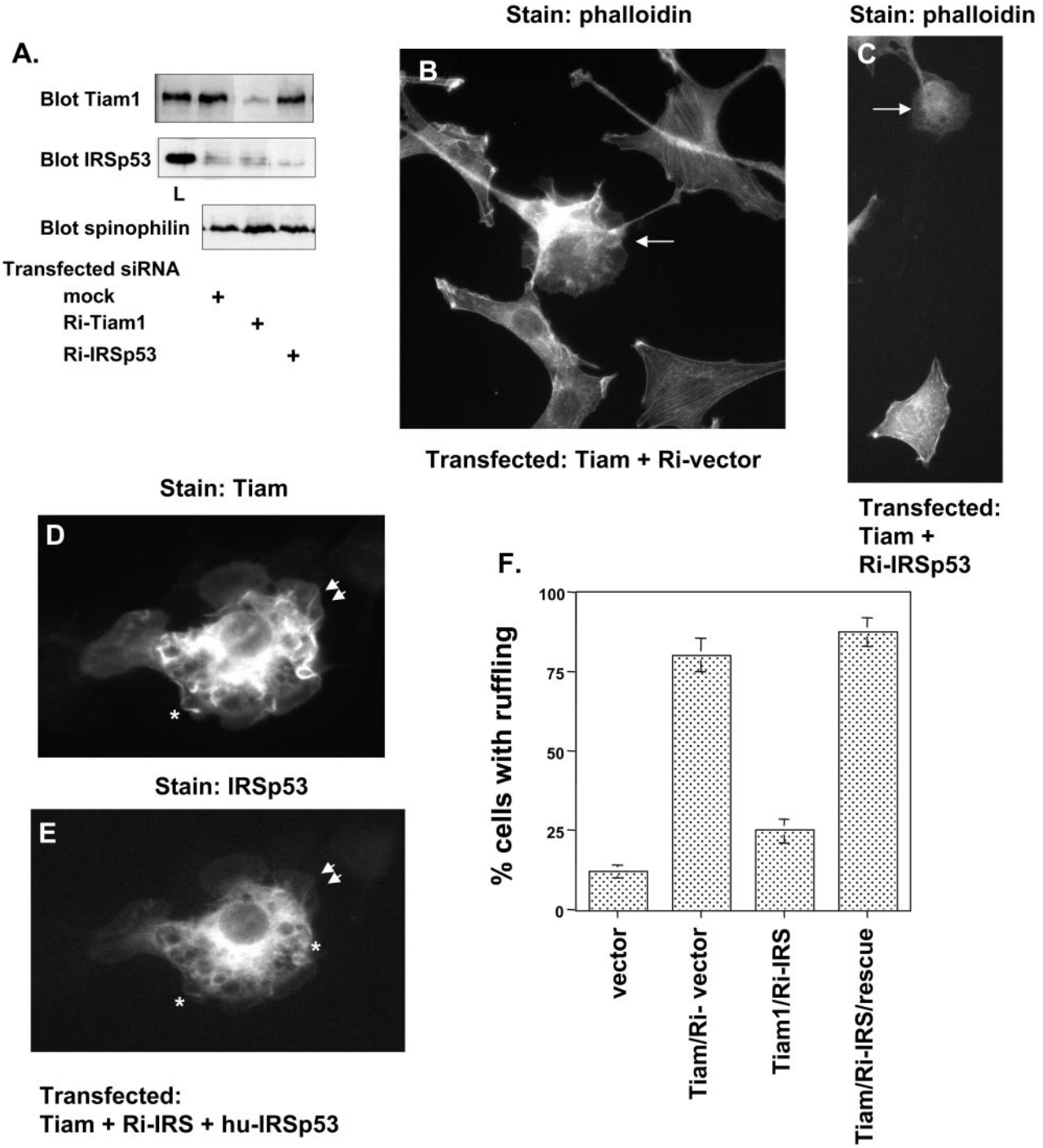


FIG. 5. IRSp53 is required for Tiam1-mediated effects on actin. (A) siRNA oligomer suppression of IRSp53 and Tiam1 expression. NIH 3T3 cells were transfected with RNA duplexes designed to suppress expression of Tiam1 or IRSp53 as indicated. Lysates of equivalent numbers of cells were assayed for protein expression by Western blotting. Lysates of 293T cells expressing Tiam1 or IRSp53, respectively, were also loaded as size controls (L). These oligomer sequences were then used to derive plasmid vectors, producing intracellular siRNA-like transcripts for the experiments shown in panels B to E. (B) Transfection of Tiam1 and Ri vector control leads to lamellipodia and ruffling in NIH 3T3 cells. Cells were stained with phalloidin-Alexa fluor 546 conjugate to visualize filamentous actin morphology. Transfected cells were visualized with anti-Tiam1 antibody. For fluorescence images, arrows indicate transfected cells. Images were obtained using standard fluorescence microscopy. (C) Inhibition of endogenous IRSp53 expression by RNAi inhibits Tiam1 effects on actin. NIH 3T3 cells were transfected with Tiam1 and Ri-IRSp53. (D, E) Expression of human IRSp53 can rescue the effect of suppressing endogenous IRSp53. Cells were transfected with Tiam1, Ri-IRSp53, and myc-IRSp53 (human) and stained for either Tiam1 (D) or myc (E). Asterisks and double arrowheads indicate ruffles and lamellipodia, respectively. (F) Quantification of ruffling cells from panels B ($n = 107$), C ($n = 200$), and D and E ($n = 161$). Error bars represent standard deviations.

lular localization of proteins in our in vivo system in contrast to the interaction between in vitro-generated Mena and IRSp53 species shown previously (16).

In addition to binding to more than one actin-modifying scaffold, IRSp53 also interacts with more than one activated GTPase. IRSp53 binds directly to Cdc42-GTP through a partial CRIB motif. IRSp53 also binds directly to Rac-GTP via sequences upstream of the Cdc42-binding site. However, Rac binding may be regulated, as it is enhanced when an inhibitory intramolecular interaction in IRSp53 is relieved through N-terminal deletion (22). Conditions promoting the association of IRSp53 with either GTPase have not been described. Thus, we investigated whether Tiam1 also specifically promotes Rac signaling through IRSp53 by promoting the association of IRSp53 with active Rac (Fig. 3C). We found that the interaction of immobilized GTP-loaded Rac with expressed IRSp53 was increased by coexpression of Tiam1, but not ΔPCX-Tiam1. Furthermore, we found that an IRSp53 deletion mutant lacking the previously reported inhibitory N-terminal 179 amino acids exhibited somewhat enhanced binding to Rac-GTP compared with full-length IRSp53 (22), but this binding was much less sensitive to the presence of Tiam1 (Fig. 3D).

As shown previously (16), binding of Cdc42-GTP to IRSp53 was considerably greater than that of Rac-GTP (Fig. 3C). This binding appears to be independent of Tiam1 binding, since in our in vitro system, overexpressed Tiam1 did not compete with Cdc42 for binding to IRSp53, despite the fact that the same region on IRSp53 mediates binding to both Tiam1 and Cdc42 (not shown).

**Tiam1 interaction promotes IRSp53 localization at lamellipodia.** We then asked whether Tiam1 might promote IRSp53 signaling to Rac and WAVE2 through changes in protein targeting and localization in addition to changes in binding affinity. In immunofluorescence studies of NIH 3T3 cells, transfection of IRSp53 led to cells with elaborate and/or complex filopodial networks in most (more than 75%) transfected cells (Fig. 4A), consistent with a Cdc42 phenotype, as previously reported (16). IRSp53 was found along filopodia as well as in the cytoplasm. In contrast, transfection of Tiam1 into NIH 3T3 cells led to flattened cells with the Rac phenotype of ruffles and lamellipodia, with Tiam1 present throughout the cytoplasm and at some membrane edges in most transfected cells (Fig. 4B), as shown previously (21). As expected, expression of activated Rac also produced significant cell ruffling (Fig. 4C). Examples of untransfected cells, with polyhedral morphology, are seen throughout Fig. 5.

Interestingly, when Tiam1 was expressed along with IRSp53 (Fig. 4D, E, and F) clear colocalization of the two proteins was observed by confocal microscopy in membranous structures consistent with Rac-induced ruffles in most coexpressing cells (confirmed by phalloidin staining) (not shown). Concomitantly, a dramatic decrease in filopodia was observed compared with cells expressing IRSp53 alone. Expression of ΔPCX-Tiam1, which does not bind to IRSp53, did not affect IRSp53 distribution or the extent of the formation of filopodia (see Fig. S2 in the supplemental material). Importantly, the shift from the filopodial to the ruffling phenotype was not due solely to Rac activation by Tiam1, since most cells coexpressing activated Rac with IRSp53 maintained a CDC42-like phenotype with numerous filopodia (Fig. 4G, H, and I).

These findings suggested that Tiam1 promotes a shift of IRSp53 from filopodial to lamellipodial structures in cells. We therefore examined whether this phenomenon occurs upon a natural cell stimulus such as exposure to PDGF, which induces Tiam1 membrane translocation and membrane ruffling in fibroblasts (4, 26). In contrast to the marked filopodial projections seen with IRSp53 overexpression (Fig. 4A), cells expressing levels of myc-IRSp53 just sufficient for detection exhibited only rare filopodia after serum deprivation, with endogenous Tiam1 present within the cytosol (not shown). In response to PDGF stimulation, these cells formed ruffles, with a fraction of the expressed IRSp53 present at the ruffle edge (Fig. 4J). A population of endogenous Tiam1 was also present at the ruffle edge in response to PDGF (Fig. 4K), largely colocalizing with IRSp53 (Fig. 4L).

**IRSp53 is necessary for Tiam1-induced effects on actin reorganization.** These results led us to hypothesize that the default function of IRSp53 is to promote a Cdc42 phenotype (filopodia), presumably through interactions with other scaffold proteins (such as Mena). Stimulation of Tiam1 interaction with IRSp53, either through forced Tiam1 overexpression or Ras activation by growth factor, enhances IRSp53 interaction with WAVE2 and Rac and leads to its redistribution, recruiting IRSp53 effects toward a Rac phenotype (ruffles).

To support the idea that IRSp53 is needed for Tiam1 effects on actin, we used RNAi-induced gene silencing (9). We developed RNA duplex oligomers leading to effective suppression of either Tiam1 or IRSp53 expression in NIH 3T3 cells, as shown in Fig. 5A. Suppression of one protein did not affect expression of the other, neither protein was affected by mock transfection, and the level of an unrelated protein (spinophilin) was constant throughout. Lysates of transfected 293T cells were used as standards for protein position in gels (Fig. 5A, lane L). To study the effect of IRSp53 depletion on Tiam1-mediated actin effects, we then prepared an analogous mammalian DNA plasmid vector that directs intracellular synthesis of siRNA-like transcripts (Ri-IRSp53) (3). Transfection of Tiam1 and the control Ri vector led to significant ruffling and lamellipodia in 80% of transfected cells, similar to what is observed with expression of Tiam1 alone (Fig. 5B). Transfected cells were identified using Tiam1-specific antibodies. In contrast, only 25% of cells expressing Tiam1 and Ri-IRSp53 demonstrated ruffling or lamellipodia (Fig. 5C).

We were able to rescue the effect of inhibiting endogenous IRSp53 expression by cotransfecting human IRSp53 along with Tiam1 and Ri-IRSp53, indicating specificity of the phenotype induced by depletion of endogenous IRSp53. Actin changes characteristic of Rac effects were restored in over 80% of cotransfected cells. A representative cell stained for either Tiam1 (Fig. 5D) or myc-IRSp53 is shown (Fig. 5E). Both proteins were again colocalized along ruffles and lamellipodia. A quantification of the results shown in Fig. 5B to E is shown in Fig. 5F.

We also tested the specificity of the IRSp53 siRNA for Tiam1/Rac-mediated actin effects. Expression of a constitutively active RalA allele (Ral72L) in cells leads to projection of fine actin-containing hair-like structures (see Fig. S3A in the supplemental material and reference 27). In contrast to the effect on Tiam1-induced ruffling, expression of Ri-IRSp53 had no effect on the actin changes induced by expression of acti-

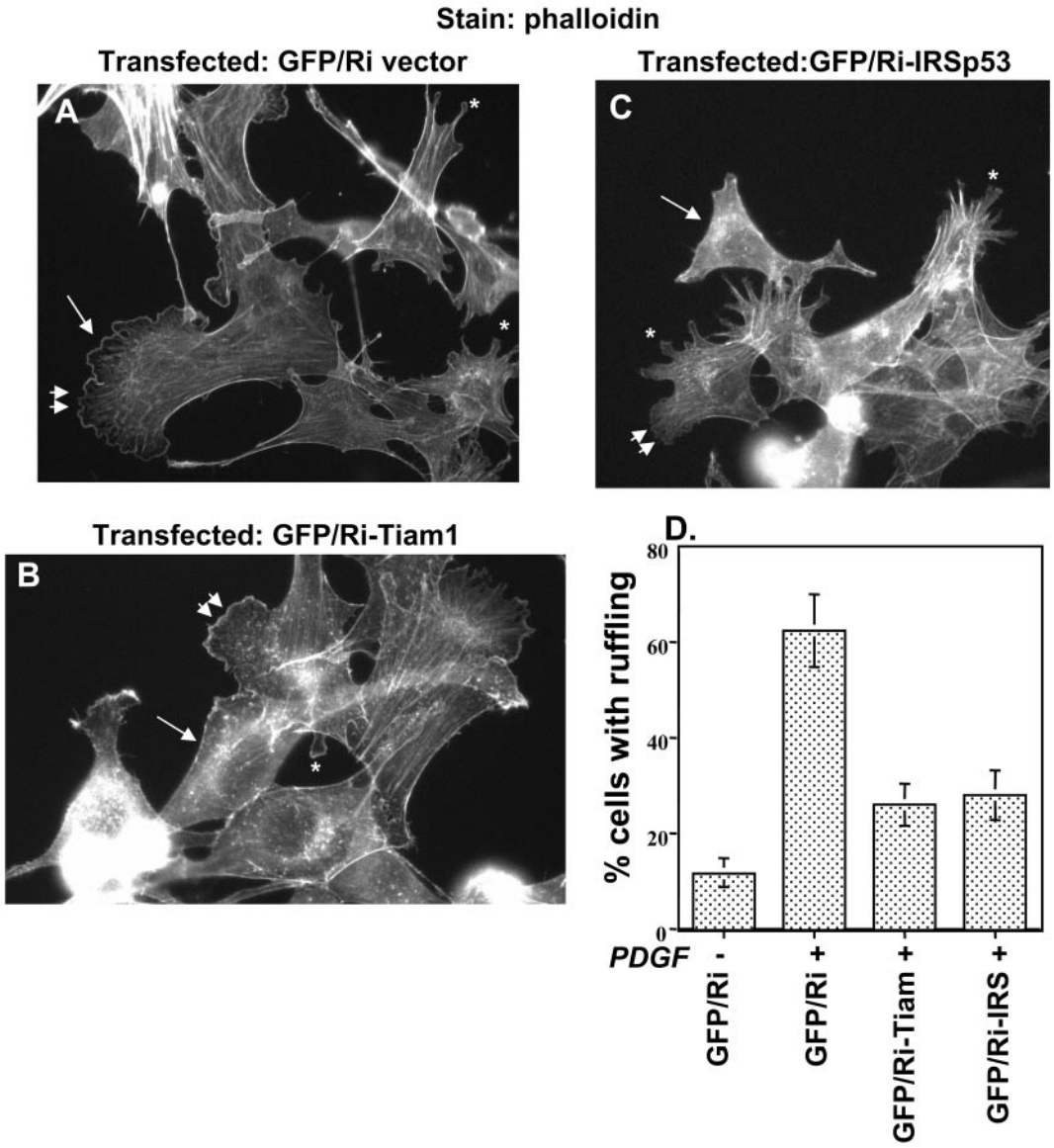


FIG. 6. Both IRSp53 and Tiam1 are required for PDGF-induced ruffling in NIH 3T3 cells. NIH 3T3 cells were stimulated with PDGF for 2 h prior to fixation and stained with phalloidin to visualize actin morphology. Cells were transfected with DNA-based siRNA-generating vectors that also express GFP to identify transfected cells (indicated by arrows). Cells were transfected with GFP/Ri vector alone (A), GFP/Ri-Tiam1 (B), or GFP/Ri-IRSp53 (C). Asterisks indicate cell extensions with small ruffles; double arrowheads indicate lamellipodia. (D) Quantification of ruffling cells from panels A ($n$ = 307), B ($n$ = 322), and C ($n$ = 984). Error bars represent standard deviations.

vated Ral in cells (see Fig. S3B in the supplemental material). In addition, inhibiting endogenous IRSp53 expression did not prevent Tiam1-induced Jnk phosphorylation in these cells (see Fig. S4 in the supplemental material), indicating that other Tiam1 signaling pathways remained intact. These results support the idea that endogenous IRSp53 is required specifically for Tiam1-induced ruffling. In contrast, suppression of endogenous Tiam1 had no effect on filopodia induced by IRSp53 overexpression (not shown).

We then studied the effect of IRSp53 or Tiam1 depletion on ligand-induced ruffling, using related DNA-based siRNA-generating vectors that also express GFP/Ri to identify transfected cells. In our system, PDGF stimulation increased the number of cells transfected with vector alone that exhibited small ruffles or lamellipodia to 63%, from a baseline of 12%, similar to the effect on untransfected cells (Fig. 6A). PDGF-induced ruffling was largely dependent on Tiam1, as only 26% of cells expressing GFP/Ri-Tiam1 exhibited small ruffles or lamellipodia upon stimulation (Fig. 6B). Consistent with the effect of IRSp53 depletion on Tiam1-induced ruffling (Fig. 5C), only 28% of GFP/Ri-IRSp53-expressing cells exhibited small ruffles or lamellipodia in response to PDGF (Fig. 6C). A quantification of results shown in Fig. 6A to C is shown in Fig. 6D. As a control, we also tested PDGF-induced Erk phosphorylation in cells that were similarly transfected and stimulated (see Fig. S5 in the supplemental material). In untransfected cells and cells

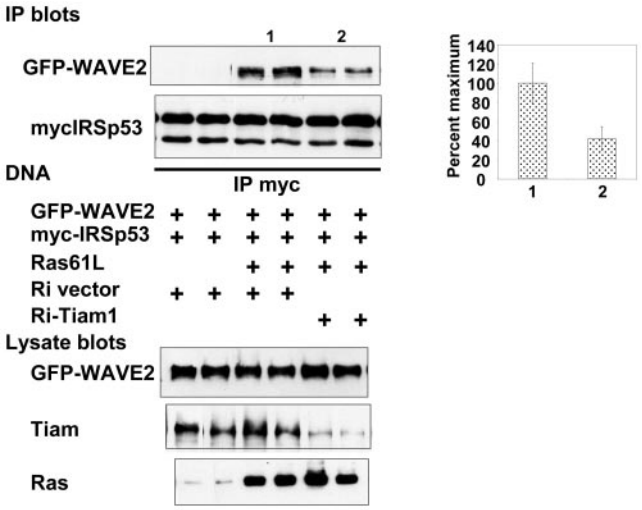


FIG. 7. Suppression of endogenous Tiam1 decreases Ras stimulation of IRSp53-WAVE2 binding. Cells were transfected with GFP-WAVE2, myc-IRSp53, and Ras61L along with Ri-Tiam1 or Ri vector alone, as indicated. Myc immunoprecipitations (IPs) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-GFP antibody to indicate coprecipitating WAVE2 or with anti-myc antibody to indicate precipitated IRSp53. Results were quantified as described in the legend to Fig. 2. +, transfected DNA.

expressing the GFP/Ri vector, PDGF stimulation led to increased perinuclear phospho-Erk staining (see Fig. S5 in the supplemental material; compare panels A and B). Similarly, cells expressing GFP/Ri-IRSp53 also exhibited increased perinuclear phospho-Erk staining with PDGF stimulation comparable to untransfected cells (see Fig. S5C and D in the supplemental material), indicating that PDGF signaling remained intact in these cells.

Finally, we used the DNA-based Ri-Tiam1 vector to examine the role of Tiam1 in Ras-induced IRSp53-WAVE complex formation (Fig. 7). In the presence of the Ri vector alone, active Ras significantly enhanced the coprecipitation of WAVE2 with IRSp53 in transfected 293T cells. Ras-stimulated complex formation was reduced by 60% when endogenous Tiam1 expression was suppressed by Ri-Tiam1. This supports the idea that Ras signaling enhances Tiam1-IRSp53 interaction, which then enhances IRSp53-WAVE interaction.

## DISCUSSION

Our results indicate that Tiam1 actively directs Rac signaling specifically to the actin cytoskeleton through binding to IRSp53. This is based on several lines of evidence. We have demonstrated a direct interaction between IRSp53 and the N-terminal protein interaction domains of Tiam1 in a yeast system and confirmed the interaction in vivo using both coprecipitation and colocalization experiments. Tiam1 also functionally alters IRSp53 function in cells by enhancing its interaction with the WAVE2 scaffold, a protein that mediates Rac signaling to the actin cytoskeleton. In fact, Tiam1 also increases the efficiency of Rac signaling down this pathway by enhancing the binding of activated Rac to its target IRSp53. The interactions of IRSp53 with both Tiam1 and WAVE2 are enhanced by signaling through Ras. Finally, siRNA experiments show that Tiam1 is required for Ras stimulation of IRSp53-WAVE2 binding as well as membrane ruffling induced by PDGF and that IRSp53 is required for membrane ruffling induced by both Tiam1 and PDGF. In contrast, IRSp53 does not participate in Tiam1 activation of another pathway downstream of Rac leading to Jnk activation.

While the ability of Tiam1 to produce Rac-dependent lamellipodia and ruffling has been well described previously (20, 21), the specific cellular components directing the actions of Tiam1-activated Rac toward the actin cytoskeleton have not been previously identified. We now propose a model for this pathway, whereby the interaction of Tiam1 with IRSp53 in turn promotes the interaction of IRSp53 with both activated Rac and WAVE2 (Fig. 8). WAVE proteins serve as scaffolds for the Arp2/3 complex and monomeric actin, assembling in tightly regulated protein complexes and acting as platforms for the formation of actin filaments (8, 15, 33). The addition of activated Rac to WAVE complexes triggers actin polymerization. Participation of Tiam1 in an IRSp53-WAVE2 complex generates a local pool of Rac-GTP at the complex, prompting Arp2/3-mediated assembly of actin monomers and consequent changes in actin filament morphology. In addition, we have shown that the interaction of Tiam1 with IRSp53 itself is stimulated by PDGF. This requires signaling through the Ras GTPase, consistent with previous data showing that activated Ras can signal to Rac through the binding of Ras-GTP to a Ras-binding region on Tiam1 (17). Furthermore, the Ras effect on the Tiam1-IRSp53 interaction appears to be specific to the interaction of Tiam1 with this particular scaffold complex, as activated Ras does not enhance Tiam1 interaction with spinophilin, a scaffold protein previously shown to couple Tiam1 to p70 S6 kinase activation (6; data not shown). The PDGF-stimulated interaction of Tiam1 with IRSp53 thereby directs Rac signals toward the machinery regulating dynamics of the actin cytoskeleton.

The interactions of IRSp53 itself are complex. IRSp53 interacts with multiple potential partners through its SH3 domain. IRSp53 can function as an effector for Cdc42-mediated effects on the actin cytoskeleton through interactions of its SH3 domain with different scaffold proteins such as Mena or Shank (16, 32) rather than with WAVE2. In addition, the IRSp53 SH3 domain can mediate interaction with Eps8 and may enhance Eps8/Abi/Sos-1 signaling to Rac (10, 30). Of note, growth factor stimulation has not been described for interactions of the IRSp53 SH3 domain, in contrast to the IRSp53-Tiam1 interaction. We now show that Tiam1 uses IRSp53 to generate Rac-mediated effects on the cytoskeleton by enhancing IRSp53 interaction with WAVE2. This implies not only that IRSp53 does affect Tiam1 signaling specificity but also that Tiam1 alters the specificity of IRSp53 effects. This hypothesis is consistent with our finding that expression of Tiam1 changes the morphology of cells coexpressing IRSp53 from a Cdc42-like phenotype to that of a Rac-like phenotype coincident with the movement of IRS-p53 from filopodia to lamellipodia (Fig. 4). Recently, GFP-IRSp53 was found to localize at the tips of both lamellipodia and filopodia (25), suggesting that multiple factors may regulate IRSp53 localization. Specific interaction with Tiam1 may account for IRSp53 localization at lamellipodia, where it participates in Rac sig-

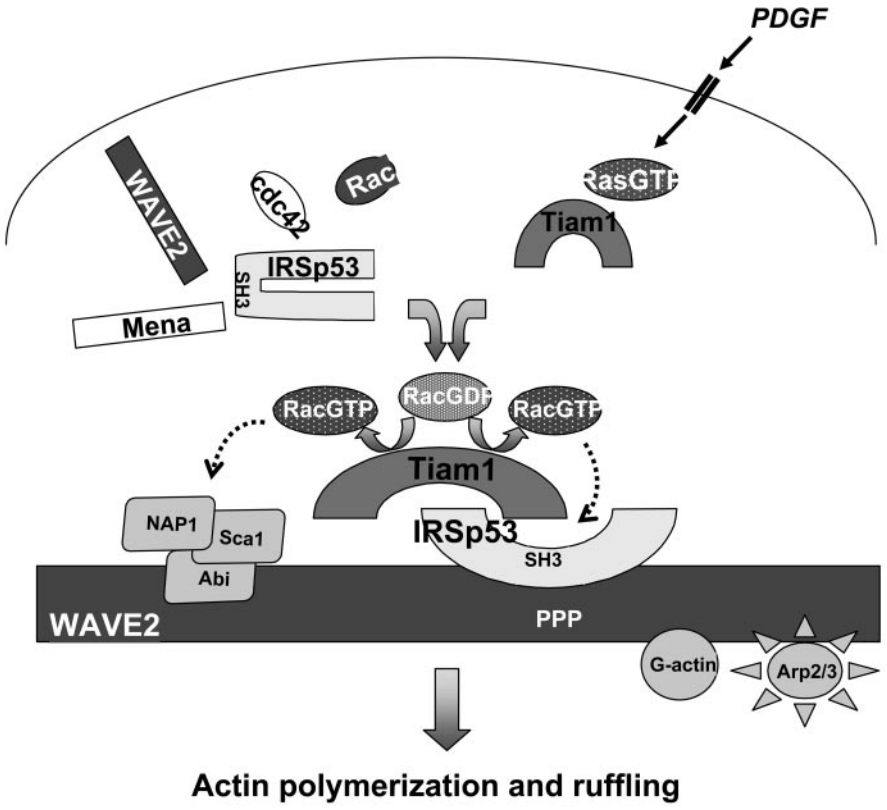


FIG. 8. Proposed model of Tiam1 and IRSp53 signaling. IRSp53 links Tiam1/Rac signaling to actin. IRSp53 has a role in multiple pathways involving actin dynamics through interaction with at least two scaffold proteins and two Rho family GTPases. Tiam1 directs IRSp53 to a pathway involving WAVE2 and Rac. Interaction of IRSp53 with Tiam1, stimulated by Ras activation, leads to preferential binding of IRSp53 to WAVE2 and Rac-GTP. WAVE2 assembles in a protein complex with multiple regulatory proteins, including Abi1 and Rac effector proteins Sra-1/Pir121 and Nap1 (Nck-associated protein). These proteins regulate WAVE function with respect to the Arp2/3 complex and actin polymerization, although their precise roles in terms of positive and negative regulation are still being elucidated. The generation of Rac-GTP at the complex by Tiam1 triggers Arp2/3-mediated polymerization of actin monomers and changes in actin morphology characteristic of Rac signaling.

naling to actin, while targeting by other factors may promote IRSp53 localization at filopodia where it participates in Cdc42 signaling. It is reasonable to hypothesize that the factors regulating the other interactions of the IRSp53 SH3 domain will play such a targeting role.

Tiam1 also appears to influence how IRSp53 interacts with specific Rho family members. IRSp53 was first noted to bind to Rac-GTP via amino acids near the N terminus and to link Rac-GTP to WAVE2-induced fibroblast ruffling (23). However, subsequent reports detected a distinct Cdc42-binding partial CRIB motif on the protein, which mediates neurite outgrowth and filopodia formation by IRSp53 (13, 16). While Cdc42-GTP binding appeared to be constitutive, Rac-GTP binding depended upon deletion of inhibitory amino acids at the N terminus of IRSp53 (22). Consistent with this model, we find that Tiam1 facilitates complex formation between Rac-GTP and IRSp53. The fact that we find this effect to be dependent on the presence of these same N-terminal sequences of IRSp53 suggests that Tiam1 binding relieves the inhibitory conformation in IRSp53 to promote Rac-GTP binding (Fig. 3D). This effect is specific for the IRSp53-Rac interaction, since Tiam1 does not affect the affinity of IRSp53 for Cdc42-GTP (not shown). Interestingly, WAVE2 can also relieve the intramolecular inhibitory conformation of IRSp53 and enhance the interaction of purified IRSp53 with Rac-GTP (22). Thus, the ability of Tiam1 to facilitate IRSp53 interaction with Rac could be a direct consequence of Tiam1 binding to IRSp53, the result of Tiam1-enhanced complex formation between IRSp53 and WAVE2, or the result of a trimolecular complex between Tiam1, IRSp53, and WAVE2. Nevertheless, the ability of Tiam1 to specifically increase the affinity of IRSp53 for activated Rac is a novel mechanism for directing downstream signaling specificity.

Why should IRSp53 have a role in both Cdc42- and Rac-mediated actin polymerization? Complex changes in actin are needed to make a lamellipodium at the correct place and time. Cdc42 signals are required for cell polarity and proper direction of cell movement. At the beginning of stimulated cell movement, IRSp53 may function in the formation of Cdc42-mediated actin microspikes that initiate formation of a motile edge (29). However, to form a leading lamellipodial edge requires actin bundles to move laterally out from the microspikes (24). The role of Tiam1 may be to shift the effects of IRSp53 toward these Rac-mediated actin changes in response to specific upstream signals (24). The involvement of IRSp53 in both processes could allow efficient and properly timed coordina-

tion of the signals needed for directed cell motility. Remarkably, IRSp53 may also be involved in the bundling of actin filaments as well as in polymerization of actin monomers, as it has also been shown to bind to the espin family of actin-bundling proteins through the SH3 domain (31). Furthermore, IRSp53 itself may bind to and bundle filamentous actin through N-terminal sequences, with this function potentially synergizing with its GTPase-dependent actin polymerization function in forming mature actin structures (36).

The experiments described in this paper are now the third example supporting our model that Tiam1 directs signaling to specific Rac effector pathways by interacting with specific scaffold proteins. We have previously shown that Tiam1 binding to the IB2 scaffold leads to activation of scaffold-associated p38, while Tiam1 binding to spinophilin leads to activation of p70 S6 kinase and decreases activation of another Rac effector, the Pak kinase (5, 6). Indeed, our finding here that Tiam1 enhances complex formation between IRSp53, Rac, and WAVE2 is reminiscent of our previous finding that Tiam1 enhances complex formation between the IB2/JIP2 scaffold and kinase components of the p38 map kinase cascade.

Moreover, the studies presented here and those of our previous papers show that exchange factor interaction with scaffolds may also generate signaling specificity by targeting activation of specific signaling cascades to particular sites in the cell. For example, we have found that the interactions of Tiam1 with IB/JIP proteins, spinophilin, and IRSp53 occur in the cytosol, at the plasma membrane, and at cytoskeletal structures, respectively. An interesting possibility remains that individual or specific combinations of Tiam1-scaffold interactions may be activated by particular upstream signals. The regulation of exchange factor function by scaffold proteins may allow for activation of either single Rac effectors or, more likely, complementing subsets of Rac effectors at multiple sites in the cell to elicit complex biological phenomena.

## ACKNOWLEDGMENTS

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# AUTHOR'S CORRECTION

## Tiam1-IRSp53 Complex Formation Directs Specificity of Rac-Mediated Actin Cytoskeleton Regulation

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Volume 25, no. 11, p. 4602–4614, 2005. Page 4604, Fig. 1E: The mutant shown in lanes 2 and 5 lacks the N-terminal 257 amino acids and should be labeled IRS p53-ΔN257, and the mutant shown in lanes 3 and 6 lacks the N-terminal 304 amino acids and should be labeled IRSp53-ΔN304. This does not affect our conclusions in any way.