Regulation of p70 S6 Kinase by Complex Formation between the Rac Guanine Nucleotide Exchange Factor (Rac-GEF) Tiam1 and the Scaffold Spinophilin*  

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Tiam1 is a ubiquitous guanine nucleotide exchange factor (GEF) that activates the Rac GTPase. We have shown previously that the N terminus of Tiam1 contributes to the signaling specificity of its downstream target Rac via association with IB2, a scaffold that promotes Rac activation of a p38 kinase cascade. Here we show that the N terminus of Tiam1 can influence Rac signaling specificity in a different way by interaction with spinophilin, a scaffold that binds to p70 S6 kinase, another protein regulated by Rac. In particular, part of spinophilin binding promotes the plasma membrane localization of Tiam1 and enhances the ability of Tiam1 to activate p70 S6 kinase. In contrast, spinophilin binding suppresses the ability of Tiam to activate Pak1, a different Rac effector. Finally, a mutant spinophilin that cannot bind to Tiam1 suppresses serum-induced p70 S6 kinase activation in cells, suggesting that a Tiam1/spinophilin complex contributes to p70 S6 kinase regulation by extracellular signals. These findings add to a growing body of evidence supporting the concept that some Rac-GEFs not only activate Rac GTPases but also participate in the selection of Rac effector proteins for activation. PIX/COOL is one example of this process because it binds directly to a specific Rac effector protein, Pak1 (15–17). Another example is the Rac-GEF Aedes aegypti Dscam, which binds to Drosophila Dscam. These findings add to a growing body of evidence supporting the concept that some Rac-GEFs not only activate Rac GTPases but also participate in the selection of Rac effector proteins for activation. PIX/COOL is one example of this process because it binds directly to a specific Rac effector protein, Pak1 (15–17). Another example is the Rac-GEF Aedes aegypti Dscam, which binds to Drosophila Dscam.  

The Rac GTPase participates in multiple cellular functions, including cell motility and adhesion, cell survival, proliferation and apoptosis, and generation of reactive oxygen species (1, 2). Activated Rac influences diverse signaling pathways, including those governing movements of the actin cytoskeleton, activation of transcription factors, and regulation of the NADPH oxidase complex. GTP-bound Rac mediates these multiple functions through interaction with a host of downstream effector proteins, including p65 Pak, phosphatidylinositol 3-kinase, IQ GAP, p67 phox, POR1, p70 S6 kinase, STAT3, POSH, and MLK3.  

Activation of the Rac GTPase occurs through the exchange of bound GDP for GTP, catalyzed by one of multiple Rac guanine nucleotide exchange factors (Rac-GEFs). Rac-GEFs all contain similar catalytic Dbl homology (DH) domains adjacent to pleckstrin homology (PH) domains but differ in their tissue distribution and activation by distinct upstream signals (3). Tiam1 is a ubiquitously expressed Rac-GEF. It promotes invasion in lymphocytes and fibroblasts, adhesion in epithelial cells, regulation of axon formation at neuronal growth cones, and induction of apoptosis in a human leukemia cell line (4–8). In a breast cancer cell line, ectopic Tiam1 potentiated β-catenin-dependent TCF/LEF1 transactivation and heregulin-induced invasiveness (9). Conversely, in a renal cell carcinoma line Tiam1 promoted adhesion and inhibited invasion through at least two distinct downstream pathways (10). A Tiam1 knockout mouse was relatively resistant to chemical induction of skin tumors but paradoxically more prone to malignant histologic progression of those tumors (11). In cell-based assays, the myriad effects of Tiam1 are dependent on its ability to activate Rac.  

The cellular signals that enhance Tiam1 activation of Rac are beginning to be defined. Tiam1 is phosphorylated on threonine via the actions of CamKII and protein kinase C in response to agonist stimulation, but only CaMKII-induced phosphorylation promotes a modest increase in the intrinsic GEF activity of the protein (10, 12). The binding of phosphatidylinositol 3-kinase lipid products similarly enhances exchange activity of Tiam1 (13). Dephosphorylation through protein phosphatase 1 (PP1) leads to the inhibition of Tiam1 exchange activity. Factors that govern Tiam1 targeting, rather than exchange activity, may also be important for determining the ultimate outcome of Tiam1 activation of Rac, but these mechanisms are poorly understood.  

Recent evidence suggests that some Rac-GEFs influence Rac GTPase signaling specificity in addition to promoting their activation. The first hint of this concept came from the observation that, although different Rac-GEFs stimulated similar levels of Rac-GTP in cells, they produced different cellular responses (14). One possible mechanism underlying this phenomenon is that individual Rac-GEFs participate in the selection of specific Rac effector proteins for activation. PIX/COOL is one example of this process because it binds directly to a specific Rac effector protein, Pak1 (15–17). Another example is Tiam1, which contains N-terminal domains that participate in protein-protein interactions that may mediate both the responses to extracellular signals and the selection of Rac targets. Near the N terminus of Tiam1 are an adjoining PH domain, the coiled-coil (CC) domain, and an undefined region of calmodulin-binding domain; N543, N-terminal 543 amino acids of spinophilin.
termed Ex. These domains function cooperatively in targeting Tiam1 to the plasma membrane and are required for Tiam1-induced membrane ruffling (18, 19). Ras-GRF1, a dual exchange factor for both Ras and Rac, possesses a similar cluster of domains at its N terminus that also have a role in protein targeting and calcium activation of the protein (20). We have recently found that Tiam1 and Ras-GRF1 bind to the scaffold protein IB2/JIP through these same N-terminal domains (21). This provides for a mechanism for Rac signaling specificity, because IB2/JIP2 is bound to the Rac effector, MLK3, and its downstream target kinases, MKK3 and p38 MAP kinase. In fact, binding of IB2/JIP to Tiam1 or Ras-GRF1 promotes activation of the p38 MAP kinase cascade.

Here we show that Tiam1 and Ras-GRF1 also interact through this same N-terminal domain cluster with a different scaffold protein, spinophilin (also known as neurabin II). A ubiquitously expressed protein, spinophilin binds PP1 and is thought to serve as a targeting device for the phosphatase (22). Spinophilin is also an actin-binding protein and has been shown to bind to a subset of actin filaments involved in Rac-mediated actin reorganization (23, 24). Importantly, spinophilin and its close relative, neurabin I (25), have also been shown to bind to p70 S6 kinase, another protein dependent upon Rac activity. S6 kinase plays a key role in cell growth through regulating the initiation of protein translation (26). We show that interaction with spinophilin leads to localization of Tiam1 at the plasma membrane. We also show that spinophilin enhances the activation of p70 S6 kinase by Tiam1. Importantly, the interaction of Tiam1 with spinophilin influences Rac GTPase signaling specificity by promoting the activation of p70 S6 kinase over other Rac effectors in cells.

MATERIALS AND METHODS

Yeast Two-hybrid Assay—A partial rat spinophilin cDNA clone was obtained using the yeast two-hybrid method in Saccharomyces cerevisiae (strain Y190). The bait plasmid was constructed by insertion of sequences encoding amino acids 431–670 of Tiam1 cDNA (spanning the N-terminal PH, CC, and Ex domains) in-frame with the Gal4 DNA-binding domain in the pAS-Cyh vector. Bait DNA was co-transfected into yeast along with a commercial rat brain cDNA library cloned in the pAS-Cyh vector. Bait DNA was co-transfected with his3 and leu2 genes in the parental yeast strain to allow selection on Trp- and Leu-. The transformed yeast clones were selected on Trp-. The transformants were plated on a Trp- medium containing 10% iron-supplemented bovine calf serum (Hyclone). Cells were harvested 48 h after transfection when colonies were visible with the untransformed control. When indicated, cells were deprived of serum for 16 h prior to the Trp- selection.

Immunoprecipitation—Transfected cells were harvested, washed with cold PBS, pelleted, and lysed in immunoprecipitation assay buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors (10 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (0.5 mM sodium fluoride and 100 μM sodium orthovanadate). 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 (Amersham Biosciences) and the appropriate antibody (diluted according to manufacturer’s instructions) for 2 h at 4 °C with constant agitation. Aliquots of cleared lysate were retained prior to immunoprecipitation for immunoblotting. After washing three times with ice-cold radioimmune precipitation assay buffer, bound proteins were eluted in 4× Laemmli buffer, resolved by SDS-PAGE, and immunoblotted. For detection of the interaction between endogenous proteins, cells were lysed in buffer containing 50 mM Tris, pH 8.0, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 15% glycerol, and the inhibitors described above.

Immunofluorescence—The day after transfection, Cost cells were replated onto coverslips coated with poly-l-lysine. Cells were deprived of serum for 16 h prior to fixation in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Cells were blocked and permeabilized with PBS containing 1% bovine serum albumin and 0.5% Triton X-100. Anti-Tiam antibody and anti-rabbit Cy3 conjugate were diluted 1:200 in PBS containing 1% bovine serum albumin and 0.5% Triton X-100 with PBS between each step; after the final washing, coverslips were mounted using 50% glycerol in PBS. Fluorescing proteins were visualized using a confocal fluorescence microscope.

Rac-GTP Pull-down Assay—Transfected cells were harvested, washed with cold PBS, pelleted, and lysed in buffer containing 0.5% Triton HCl, pH 7.5, 0.5% Nonidet P-40, 40 mM NaCl, 1% dithiothreitol containing 1% Nonidet P-40 and twice with buffer D without detergent. Bound proteins were eluted in 4× Laemmli buffer, resolved by SDS-PAGE, and immunoblotted.

S6 Kinase Activation—Transfected cells were washed with cold PBS, pelleted, and lysed in cell lysis buffer (10 mM KPO4/1 mM EDTA, pH 7.05, 5 mM EGTA, pH 7.2, 10 mM MgCl2, 50 mM β-glycerophosphate, pH 7.2, 0.5% Nonidet P-40, 0.1% Brij-35, and 0.1% deoxycholate, pH 7.2) containing protease and phosphatase inhibitors as above. Clear lysates containing equivalent 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 PAKI expressed as a GST fusion in Escherichia Coli, as described (27). After incubation, precipitates were washed once with buffer D (25 mM Tris-HCl, pH 7.5, 30 mM MgCl2, 40 mM NaCl, and 1 mM dithiothreitol) containing 1% Nonidet P-40 and twice with buffer D without detergent. Bound proteins were eluted in 4× Laemmli buffer, resolved by SDS-PAGE, and immunoblotted.

S6 Kinase Activation—Transfected cells were washed with cold PBS, pelleted, and lysed in cell lysis buffer (10 mM KPO4/1 mM EDTA, pH 7.05, 5 mM EGTA, pH 7.2, 10 mM MgCl2, 50 mM β-glycerophosphate, pH 7.2, 0.5% Nonidet P-40, 0.1% Brij-35, and 0.1% deoxycholate, pH 7.2) containing protease and phosphatase inhibitors as above. Clear lysates containing equivalent amounts of total protein were incubated for 30 min at 30 °C in buffer containing 20 mM Hepes, pH 7.2, 10 mM MgCl2, 100 μM bovine serum albumin, and 25 μM ATP with 5 μCi of [γ-32P]ATP and 1 μg of purified GST-S6 peptide per reaction, as described (28). Reactions were stopped with the addition of Laemmli buffer, resolved by SDS-PAGE, visualized with Coomassie stain, dried, and quantified on a PhosphorImager. All controls were deprived of serum for 16 h prior to harvest and re-stimulated where indicated.

Pak Activation—Transfected cells were washed with cold PBS, pelleted, and lysed in buffer B containing 50 mM Tris, pH 7.4, 1% Triton X-100, 1 mM MgCl2, and 125 mM NaCl containing protease and phosphatase inhibitors as above. MYC immunoprecipitates from cleared lysates were washed three times in ice-cold buffer A containing 1% Nonidet P-40, 0.5% NaDOC, 100 mM NaCl, 10 mM Tris pH 7.2, 1 mM EDTA, once in high salt buffer (1× at NaCl, 0.1% Nonidet P-40, 10 mM Tris, pH 7.2), and once in ice-cold ST buffer (150 mM NaCl, 50 mM Tris, 5 mM NaCl, 7.2). Kinase reactions proceeded for 10 min at 30 °C in buffer containing 20 mM Hepes, pH 7.2, 10 mM MgCl2, 100 μM bovine serum albumin, and 25 μM ATP with 5 μCi of [γ-32P]ATP and 1 μg of purified GST-S6 peptide per reaction, as described (28). Reactions were stopped with the addition of Laemmli buffer, resolved by SDS-PAGE, visualized with Coomassie stain, dried, and quantified on a PhosphorImager. All controls were deprived of serum for 16 h prior to harvest and re-stimulated where indicated.

JNK Activation—Transfected cells were processed as for Pak assays except that the expressed JNK was immobilized on S-hexyl-glutathione beads, and kinase reactions included 50 μg/ml GST-Jun as substrate.
**RESULTS**

Tiam1 Interacts with Spinophilin—The N-terminal PH, CC, and Ex domains of Tiam1 function to localize and regulate the exchange factor properly in cells (19). We performed a yeast two-hybrid screen to identify proteins that might regulate the exchange factor through interaction with these domains. A cDNA identified this way included DNA sequences spanning amino acids 444–817 of spinophilin (also called Neurabin II), a scaffold protein known to bind actin, PP1, and p70 S6 kinase (S6K1). The domain structures of both Tiam1 and spinophilin are depicted in Fig. 1, A and B, with the interacting Tiam1 and spinophilin regions defined by the two-hybrid screen as noted by the heavy black bars.

To confirm that Tiam1 can bind to spinophilin in cells, endogenous Tiam1 from COS7 cells was immunoprecipitated, and then the sample was immunoblotted with anti-spinophilin antibodies. As a control, non-immune IgG was used instead of Tiam1 antibodies. Fig 2A shows spinophilin in the experimental but not control immunoprecipitation, indicating that at least a fraction of the two proteins do in fact interact in cells.

To map the binding sites between Tiam1 and spinophilin, Tiam1 was transiently transfected along with epitope tagged spinophilin, and then spinophilin was immunoprecipitated from cell lysates. The presence of Tiam1 in the immune complex was detected by immunoblotting (Fig. 2B). Lanes 1–3 in the left panels demonstrate expression of the transfected proteins, while lanes 4–6 reflect immunoblots of precipitated proteins. As expected, Tiam1 was clearly present in a complex with spinophilin at levels greater than those found in control experiments when spinophilin was not co-transfected (compare Fig. 2B, lanes 4 and 5, top panel). As an additional control experiment, ΔPCX-Tiam1, a mutant lacking the spinophilin binding region, was substituted as the Tiam1 species. This mutant associated with spinophilin (Fig. 2B, lane 6) at levels no greater than the background level observed in lane 4. The background band seen in lanes 4 and 6 represents nonspecific binding to the protein A-Sepharose, as it is also present on complexes produced with control antibody alone (data not shown). These findings were confirmed by performing the reverse experiments (see Fig. 2B, right panels), where Tiam1 was immunoprecipitated, and complex formation was assayed by immunoblotting for spinophilin (see Fig. 2B, lanes 7–12). As expected, transfected spinophilin was present in a complex with immunoprecipitated Tiam1 (compare lanes 10 and 11) but not with immunoprecipitated ΔPCX-Tiam1 (lanes 12).

Based on the results of the two-hybrid assay, Tiam1 interacts with sequences toward the C terminus of spinophilin. We used a convenient BamHI site to engineer a mutant spinophilin containing only the N-terminal 543 amino acids and lacking the entire coiled-coil domain as well as C-terminal sequences in the PDZ domain (GFP-spino-N543), shown diagrammatically in Fig. 1C. This mutant does not interact with Tiam1 in similar co-transfection experiments (Fig. 2C, compare lane 8 with lane 6). Fig. 2C, lanes 1–4 demonstrate relatively equivalent expressions of both spinophilin species, whereas lanes 6 and 8 show the presence of spinophilin but not spinophilin-N543 in Tiam1 immunoprecipitates. Fig. 2C, lanes 5 and 7 show negligible signals in the absence of expressed Tiam1. When we attempted to localize the binding domain of spinophilin in more detail by testing the C-terminal coiled coil or the PDZ domain individually, no binding activity was observed (data not shown), suggesting that the binding site spans the two domains or that proper folding of the binding domain requires surrounding amino acids.

Spinophilin Binding Targets Tiam1 to the Plasma Membrane—We used immunofluorescence to investigate the cellular localization of spinophilin and Tiam1. We compared the cellular distribution of spinophilin expressed as a GFP fusion protein with endogenous Tiam1 in COS7 cells. We found that GFP-spinophilin localized primarily to the plasma membrane, whereas GFP alone was distributed throughout the cell (compare Fig 2D, left column, top two panels). In contrast, Tiam1 exhibited punctate staining throughout the cytoplasm (Fig. 2D, center column, top panel). Interestingly, a fraction of endogenous Tiam became localized at the plasma membrane when spinophilin was transfected into cells (center column, second panel from the top). Overlay images confirmed that Tiam1 and spinophilin co-localized at the plasma membrane (Fig. 2D, compare top panel, right column with the second panel from the top, right column). Importantly, while the C terminally truncated spinophilin GFP-spino-N543, which cannot bind to Tiam, maintained its plasma membrane localization (left column, bottom panel), it failed to re-localize endogenous Tiam1 to the plasma membrane in cells (center column, bottom panel). The inability of GFP-spino-N543 to interact with Tiam1 was confirmed by the lack of co-localizing signals in the overlay image (right column, bottom panel). These findings not only confirm that spinophilin can interact with endogenous Tiam1, they show that spinophilin regulates the distribution of Tiam1 in cells.

Tiam Binding Is Associated with the Ability of Spinophilin to Activate S6 Kinase in Cells—To test whether the interaction between spinophilin and endogenous Tiam1 has biologic significance, we investigated p70 S6 kinase activity, because it is known to be stimulated by spinophilin or Rac, the downstream target of Tiam1. First, spinophilin or control vector was transfected into NIH3T3 cells along with FLAG-p70 S6 kinase.
Tiam1/Spinophilin Binding Affects Rac Signaling Specificity

A
Blot Spinophilin

B
Blot Tiam
Blot Spinophilin

C
Blot Spinophilin

D
GFP
GFP-Spinophilin
GFP-Spinophilin N543

E
Blot Spinophilin
Blot FLAG

S6 Kinase Activity (Fold activation)

FLAG-S6K1  +  +  +  +  +  +  +  +
Spinophilin  +  +  +  +  +  +  +
Spinophilin N543  +  +  +  +

S6K1
Then, p70S6 kinase was immunoprecipitated, and its activity was assayed in vitro. Consistent with previous reports, we found that transfection of spinophilin activated p70 S6K1 ~2-fold above background (25) (Fig. 2E, top right panel, compare lanes 1 and 2). When we tested spino-N543, the mutant spinophilin that cannot bind to Tiam1 or relocalize it in cells (see Fig. 2, C and D) but retains the ability to bind to p70 S6 kinase in cells (Fig. 2E, left panels). In contrast to wild-type spinophilin, spino-N543 failed to significantly alter p70 S6 kinase activity in cells (Fig. 2E, top right panel lane 3). These findings show that the ability of spinophilin to activate p70 S6 kinase correlates with its ability to bind to and redistribute endogenous Tiam1 in cells.

The Exchange Factor Ras-GRF1 Also Interacts with Spinophilin—The Ras and Rac exchange factor, Ras-GRF1, has an N-terminal domain structure that is similar to that of Tiam1 (shown diagrammatically in Fig. 1D). Like Tiam1, the localization and regulation of Ras-GRF1 also relies upon its N terminus, which contains the PH, CC, and calmodulin binding (IQ) domains. We have recently reported that both Ras-GRF1 and Tiam1 bind to members of the IB/JIP scaffold family through their analogous domains (21). We therefore tested Ras-GRF1 for the ability to bind spinophilin in transiently transfected cells (Fig. 3). Transfected spinophilin was present in a complex when Ras-GRF1 was also expressed and immunoprecipitated (compare Fig. 3, lanes 7 and 6). To further define this interaction, we took advantage of previously described deletion mutants of Ras-GRF1, which contain serial fusions of the N-terminal regulatory domains of the exchange factor to its C-terminal catalytic domain (Fig. 1, E–G) (20). The PH domain fused to the catalytic domain (P-Cat) did not co-precipitate transfected spinophilin (Fig. 3, lane 8). When both the PH and CC domains were included, there was a slight interaction with expressed spinophilin above background (Fig. 3, lane 9). Full interaction with spinophilin required the presence of the PH, CC, and IQ domains (Fig. 3, lane 10). This is reminiscent of our previous experiments, wherein all three N-terminal domains were required for proper activation and localization of Ras-GRF1 (20). Similar results have been reported for Tiam1, in that all three analogous N-terminal domains were required for membrane localization and full activity of the exchange factor (18). Our results suggest that interactions of spinophilin with the N-terminal domains of these GEFs are involved in the regulation of both Ras-GRF1 and Tiam1 functions.

**Spinophilin Enhances Tiam1-mediated p70 S6 Kinase Activation**—We showed previously that interaction of Tiam1 with other scaffolds influences the signaling specificity of the Tiam1 target Rac (21). For example, expression of IB2/JIP2, a scaffold for components of the p38 MAP kinase cascade including the Rac target MLK3, enhanced the ability of Tiam1 to activate scaffold-bound p38. Here we investigated the effect of spinophilin on the ability of Tiam1 to activate the spinophilin-binding protein p70 S6 kinase (Fig. 4A). As reported previously (25) and noted above in Fig. 1, the expression of spinophilin alone activated S6K1 activity ~2-fold above background (compare Fig. 4A, lane 1 with lane 2). Ectopic Tiam1 expression activated S6K1 ~4-fold (Fig. 4A, lane 3). This activity occurred through the Rac-activating ability of Tiam1, as Tiam1 activation of S6K1 was abrogated in the presence of dominant negative Rac (Fig. 4A, lane 4). The concurrent expression of spinophilin with Tiam1 led to a synergistic enhancement of S6K1 activity (~9-fold; Fig. 4A, lane 5), which was clearly greater than the sum of the effects of spinophilin or Tiam1 alone. Importantly, this enhancement was not seen in the presence of spinophilin N543, which cannot bind Tiam1 (Fig. 4A, lane 6). Moreover, enhanced S6K1 activation was also suppressed by the expression of dominant negative Rac, although this effect was only partial, suggesting that the binding between the N terminus of Tiam1 and spinophilin was capable of partial S6K1 activation (Fig. 4A, lane 7).

We then assessed whether the Tiam1/spinophilin interaction was important for stimulation of p70 S6K1 by extra-cellular signals. S6K1 is activated by multiple stimuli, including mitogens, insulin, and serum (26). We therefore determined whether the C-terminal spinophilin 543-amino acid mutant, which still retains the ability to bind S6K1, could interrupt serum-induced activation of S6K1 (Fig. 4B). Serum stimulation led to approximately a 3-fold activation of transfected S6K1.

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**Fig. 2. Tiam1 and spinophilin interact in cells.** A, endogenous Tiam1 binds to endogenous spinophilin in cells. Immunoprecipitates using control IgG (lane 2) or anti-Tiam1 antibodies (lane 3) were immunoblotted with anti-spinophilin antibodies. Lane 1 contains 5% of total lysate. B, Tiam1 binds to spinophilin through the N-terminal PH/CC/Ex region of the exchange factor. Tiam1 or ΔPCX-Tiam1 was cotransfected into COS-7 cells along with GFP-tagged spinophilin as indicated (+1). C, cell lysates (lanes 1–5) or immunoprecipitates of spinophilin (lanes 6–10) were immunoblotted with antibodies to Tiam1 (lanes 1–6, upper panel; 7–12, lower panel) or GFP (lanes 1–6, lower panel; 7–12, upper panel). The N-terminal PH/CC/Ex region of the exchange factor is required for the binding to spinophilin. D, expression of spinophilin in wild-type Tiam1 led to a 3-fold increase in p70 S6 kinase activity. E, expression of spinophilin in Tiam1 or ΔPCX-Tiam1 led to a synergistic enhancement of p70 S6 kinase activity (~9-fold). F, expression of spinophilin in wild-type Tiam1 led to a synergistic enhancement of p70 S6 kinase activity (~9-fold). G, expression of spinophilin in wild-type Tiam1 led to a synergistic enhancement of p70 S6 kinase activity (~9-fold). H, expression of spinophilin in wild-type Tiam1 led to a synergistic enhancement of p70 S6 kinase activity (~9-fold).
CamKII activity, and this correlates with the presence of the exchange factor at the plasma membrane (12, 29). As interaction with spinophilin leads to Tiam1 localization at the plasma membrane, and expression of spinophilin enhances Tiam1 activation of p70 S6K1, we asked whether ectopic expression of spinophilin could affect Tiam1 exchange activity in vivo. Tiam1 activity was assessed by its ability to increase the amount of Rac-GTP in cells, assayed by affinity purification with the Rac-binding domain of GST-PAK1 (Fig. 6A). The expression of spinophilin did not lead to further activation of Rac (Fig. 6B, lane 4 to lanes 3 and 2). However, the co-transfection of spinophilin did not lead to further activation of Rac (Fig. 6B, lane 6 to lanes 5 and 7). Moreover, expression of spinophilin in the absence of ectopic Tiam1 did not activate Rac (data not shown). Thus, rather than enhancing the intrinsic GEF activity of Tiam1, it is likely that spinophilin increases Tiam1 activation of p70S6K1 by targetting activated Rac to S6K1.

**Spinophilin Suppresses Tiam1-mediated Pak1 Activation**—To test the specificity of the spinophilin effect on Tiam1 mediated activation of S6K1, a different Rac target, Pak kinase, was substituted for S6K1. Consistent with previous reports, expression of wild-type Rac or Tiam1 led to a modest activation of Pak1 (Fig. 6A, compare lanes 1, 2, and 4), whereas expression of Tiam1 along with Rac led to enhanced activation (−5-fold; lane 6). The expression of spinophilin did not affect the activation of Pak by Rac (Fig. 6A, compare lane 3 with lane 2), but significantly diminished the activation of Pak by Tiam1 (compare lane 5 with lane 4). Spinophilin also partially diminished the synergistic activation of Pak by Rac and Tiam1 (Fig. 6A, lane 7). The effect of spinophilin was specific for Tiam1-mediated Rac effects. Spinophilin did not affect the ability of constitutively activated Rac61L to stimulate Pak (Fig. 6B, left panels) or JNK (Fig. 6B, right panels) activities. These findings are consistent with the idea that spinophilin targets Tiam1 action toward a specific Rac target, p70 S6 kinase, at the expense of other potential Rac effectors.

**Ras-GRF1 Does Not Enhance p70 S6 Kinase Activity**—Finally, as the Ras and Rac exchange factor Ras-GRF1 also interacts with spinophilin, we asked whether Ras-GRF1 had analogous effects on S6 Kinase activation (Fig. 7). In contrast to our results with Tiam1, we found that the presence of Ras-GRF1 did not enhance the activation of S6K1 beyond that seen.
with spinophilin (Fig. 7, compare lanes 2 and 3 and contrast with Fig. 4A, lanes 2 and 3). Furthermore, the co-expression of spinophilin and RasGRF1 did not lead to enhanced S6K1 activation (Fig. 7, lane 4).

**DISCUSSION**

Recognition of the importance of scaffold proteins in signal transduction has been steadily emerging (for review, see Ref. 30). Scaffold proteins organize groups of proteins with related functions in time and space within the confines of a cell. In this way, they have the potential to congregate proteins that act on the same signaling pathway, facilitating the transmission of an upstream signal through a complex of molecules at a specific intracellular site. Although scaffold proteins can induce efficient signal transduction, they also limit signal amplification by promoting stoichiometric association of relevant molecules. Scaffold proteins can also serve to direct signaling specificity. By organizing a complex of proteins, a scaffold protein can facilitate the transmission of an upstream signal to a particular downstream pathway out of a set of possible outcomes.

An example of this type of scaffold function was revealed by our recent observation that the Rac exchange factors Tiam1 and Ras-GRF1 bind to members of the IB/JIP scaffold family (21). The binding of Tiam1 enhances complex formation of the IB2/JIP2 scaffold with members of a particular MAP kinase cascade, i.e. MLK3, MKK3, and p38. Tiam1 and Ras-GRF1 also activate scaffold-associated p38 through the activation of Rac. Thus, the interaction of these proteins with IB2/JIP2 recruits activated Rac to a complex containing MLK3, one of the many downstream effector proteins of Rac. In this way Tiam1/IB2 binding provides a means for specifying Rac signaling specificity.

Here we report that Tiam1 can influence Rac signaling specificity differently, such that it favors p70-S6K1 activation. The regulation of S6K1 is complex, requiring serial phosphorylation of multiple serine/threonine sites and involving inputs from multiple pathways, including mTOR, phosphatidylinositol...
Spinophilin also binds to actin, which mediates yet another downstream signaling pathway emanating from the Rac GTPase (23, 24). In fact, Tiam1 (5, 7), spinophilin (23), and Rac (1) are all known to modulate the actin cytoskeleton. Whether a complex between Tiam1 and spinophilin yields a unique rearrangement of actin in cells is presently under investigation. In addition, spinophilin binds to PP1 (22) and targets it to specific substrates in cells. PP1 has been suggested to be a negative regulator of Tiam1 activity in cells (12). However, we did not detect suppression of Tiam1 activation of Rac when spinophilin was transfected along with Tiam1. This could be due to the fact that only a fraction of spinophilin contains PP1, because PP1 binding to neurabin I competes with S6K1 binding (32). Finally, spinophilin has also been shown to bind to the D2 dopamine and α2-adrenergic receptors (33, 34) and the tumor suppressor ARF (35) as well as to the trans-Golgi network membrane protein, TGN38 (36). Whether Tiam-mediated Rac activation affects the functions of these proteins remains to be determined.

Ras-GRF1 can bind spinophilin through its N-terminal region, which is analogous to that found in Tiam1. However, we found that overexpressed Ras-GRF1 did not activate S6K1, either alone or in the presence of spinophilin. This is despite the fact that we recently showed that Ras-GRF1 can enhance Rac-mediated p38 activation through interaction with the scaffold IQGAP1 (21). This difference between Ras-GRF1 and Tiam1 may represent unique ways that the two related proteins interact with spinophilin, because spinophilin binding to Ras-GRF1 is significantly weaker than to Tiam1. However, it may also reflect the fact that Rac activation by Ras-GRF1 is not detectable under basal conditions. Its activity requires activation by specific Src family kinases (37, 38). Thus, Ras-GRF1 may activate S6K1 under stimulation conditions not present in our system.

The results reported in this paper add support to the model that some Rac GEFs do more than just activate Rac GTPases. By binding to specific scaffolds, these Rac-GEFs may deliver activated Rac to specific signaling complexes containing distinct sets of Rac effector proteins (see Fig. 8). Mechanisms that determine which scaffolds these Rac-GEFs bind to under different physiological conditions remain to be determined.

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