Trp\(^{56}\) of Rac1 Specifies Interaction with a Subset of Guanine Nucleotide Exchange Factors*  

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Yuan Gao‡, Jinchuan Xing‡, Michel Streuli§, Thomas L. Leto¶, and Yi Zheng¶  
From the ‡Department of Molecular Sciences, University of Tennessee Health Science Center, Memphis, Tennessee 38163, the §Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, and ¶Laboratory of Host Defenses, NIAID, National Institutes of Health, Bethesda, Maryland 20892  

Signaling specificity of Rho GTPase pathways is achieved in part by selective interaction between members of the Dbl family guanine nucleotide exchange factors (GEFs) and their Rho GTPase substrates. For example, Trio, GEF-H1, and Tiam1 are a subset of GEFs that specifically activate Rac1 but not the closely related Cdc42. The Rac1 specificity of these GEFs appears to be governed by Rac1-GEF binding interaction. To understand the detailed mechanism underlying the GEF specificity issue, we have analyzed a panel of chimeras between Rac1 and Cdc42 and examined a series of point mutants of Rac1 made at the switch I, switch II, and \(\beta_1/\beta_2\) regions for their ability to interact with and to be activated by the GEFs. The results reveal that Rac1 residues of both the switch I and switch II regions are involved in GEF docking and GEF-mediated nucleotide disruption, because mutation of Asp\(^{38}\), Asn\(^{39}\), Gln\(^{61}\), Tyr\(^{64}\), or Arg\(^{66}/\text{Leu}\(^{67}\) into Ala results in the loss of GEF binding, whereas mutation at Tyr\(^{32}\), Asp\(^{65}\), or Leu\(^{79}/\text{Ser}\(^{71}\) leads to the loss of GEF catalysis while retaining the binding capability. The region between amino acids 53–72 of Rac1 is required for specific recognition and activation by the GEFs, and Trp\(^{56}\) in \(\beta_3\) appears to be the critical determinant. Introduction of Trp\(^{56}\) to Cdc42 renders it fully responsive to the Rac-specific GEF \textit{in vitro} and in cells. Further, a polypeptide derived from the \(\beta_3\) region of Rac1 including the Trp\(^{56}\) residue serves as a specific inhibitor for Rac1 interaction with the GEFs. Taken together, these results indicate that Trp\(^{56}\) is the necessary and sufficient determinant of Rac1 for discrimination by the subset of Rac1-specific GEFs and suggest that a compound mimicking Trp\(^{56}\) action could be explored as an interfering reagent specifically targeting Rac1 activation.

Rac1 and Cdc42 are members of the Rho subfamily of the Ras superfamily small GTP-binding proteins (1). They are close family members, sharing over 70% sequence identity. Among a broad spectrum of cellular functions, Rac1 is known to be involved in mediating membrane ruffling and lamellipodia formation elicited by growth factors and cytokines (1, 2). Cdc42, on the other hand, appears to mediate the formation of filopodia and actin microspikes in multiple cell settings. It is well established that they can differentially respond to upstream stimulants and may cause diverse physiological effects in cell migration, adhesion, growth, and apoptosis (1, 2). As in the case for Ras, the cycling between the active, GTP-bound and the inactive, GDP-bound states of these Rho family proteins is tightly regulated in cells. The guanine nucleotide exchange factors (GEFs)\(^3\) accelerate the release of GDP from the small G-proteins, thereby facilitating GTP binding and G-protein activation; the GTPase-activating proteins catalyze the conversion of the GTP-bound form of the G-proteins to the GDP-bound form by increasing their intrinsic rates of GTP hydrolysis, and GDP dissociation inhibitors might serve to stabilize the G-proteins at either state and to target them to specific intracellular locations (1, 2).  

Signaling specificity of these Rho GTPases is controlled at multiple levels in the complex signal transduction chains. One of the signaling divergent points that might contribute to the specification of signal flows is at the small G-protein activation step (3). A large family of Dbl-like GEFs specific for Rho protein activation has emerged over the past decade (4, 5). Among the rapidly expanding Dbl family GEFs that share the structural arrangement of two tandemly located motifs of the Dbl homology (DH) and pleckstrin homology (PH) domains, the Dbl oncprotein is the prototype member that was shown to interact with and catalyze the GDP/GTP exchange of many Rho proteins, including RhoA and Cdc42. The DH/PH domains have been demonstrated to represent the minimum structural module required for GEF activity and biological functions (6). The T-cell invasion and metastasis gene product Tiam1 of the Dbl family was shown to be an active GEF for Rac1 and may influence the invasive capacity of T cells in a Rac1-dependent manner (7). Trio is a large multifunctional domain molecule containing two DH/PH domain modules, with the amino-terminal module (TrioN) displaying the Rac1- and RhoG-specific GEF activity and the carboxyl-terminal module exhibiting RhoA specific GEF activity (8). Recent biological studies in \textit{Drosophila} and mouse have implicated Trio as a key component of the intracellular signaling pathway that regulates axon guidance and cell migration in the nervous system with the TrioN module playing an important role in the process (9–12). Another Dbl family member, GEF-H1, was identified as a microtubule-associated protein that is capable of stimulating the guanine nucleotide exchange of Rac1 but is inactive toward Cdc42 (13). Therefore, among the Dbl family members, TrioN, GEF-H1, and Tiam1 constitute a subset of GEFs that share the
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The mechanism for such exquisite substrate selectivity by the GEFs is not known. Previous mutagenesis studies of the Rho family GTPase-GEF pairs of RhoA-Lbc and Cdc42-Cdc24 have revealed that multiple sites of the Rho GTPases are involved in the regulation by GEFs, contributing to GEF binding or GEF catalysis (14). Specific recognition of Lbc by RhoA or Cdc42 by Cdc24 is achieved at least in part through the unique residues Lys<sup>27</sup> of RhoA and Gln<sup>116</sup> of Cdc24, respectively. These results raise the possibility that activation of each Rho family G-protein by a specific GEF may engage in a distinct mechanism. Recently published x-ray crystal structure of Rac1 in complex with the DH/PH domains of Tiam1 (15) provides further insights into how Rac1 interacts with a GEF and what structural determinants might be involved in GEF substrate discrimination. The complex structure suggests an “induced fit” model in which Tiam1 first interacts with the conformationally rigid portions of Rac1 (β/β region and residues 65–74 of switch II) to provide sufficient binding energy, followed by an alteration of the conformations in switch I and the remainder of switch II resulting in destabilization of the bound nucleotide. The residues of Rac1 in the β/β region that would lose surface exposure upon complex formation with Tiam1 and are variable from those in the corresponding positions of Cdc42 are proposed as possible determinants that specify Tiam1 interaction in the activation reaction.

In the present work, we have attempted to determine the exact determinants of Rac1 that specify the GEF interaction. First, we found that the Rac1 specificity of three GEFs, TrioN, GEF-H1, and Tiam1, is governed by Rac1-GEF binding interaction. Second, by using an extensive collection of point mutants generated at switch regions of Rac1, we have examined the contribution of the switch I and switch II of Rac1 to the GEF interaction and catalysis and concluded that both switches are important for the physical association with the GEFs and the efficient exchange of nucleotides catalyzed by the GEFs. Third, based on the results of the GEF binding and GEF-responsive profiles of a set of Cdc42/Rac1 chimera mutants and point mutants made in the β/β region of Rac1, we pinpointed Trp<sup>56</sup> of Rac1 as the necessary and sufficient determinant for the GEFs to discriminate against Cdc42. By introducing Trp<sup>56</sup> into Cdc42, we were able to produce a Cdc42 mutant that is sensitive to Rac-specific GEF stimulation in vitro and in cells. Finally, we were successful in designing a polypeptide derived from the β region of Rac1 including Trp<sup>56</sup> that serves as a specific inhibitor for Rac1 interaction with the GEFs. Overall, these studies provide a mechanistic basis for substrate discrimination by Rac1-specific GEFs and initiate an effort for exploring interfering reagents specifically targeting Rac1 activation that might constitute a worthy therapeutic target site.

**Experimental Procedures**

Cdc42/Rac1 Chimera Generation and Site-directed Mutagenesis—Cdc42/Rac1 chimeric cDNAs were produced as previously described (16) or by the polymerase chain reaction (PCR) method using the Pfu polymerase (Stratagene), which generates blunt-ended DNA fragments in PCRs followed by forced insertion of the ligation product of the blunt-ended fragments into the pGEX-KG vector (14). Site-directed mutants described in this work were generated by the PCR-based second extension amplification technique using the Pfu polymerase with internal primers that contained the desired mutations (14). cDNAs of mutant/chimera forms of Rac1 and Cdc42 were subcloned into the BamHI and EcoRI sites of pGEX-KG vector to express as a glutathione S-transferase (GST) fusion protein in Escherichia coli DH5α strain. The sequences of mutated Rac1 and Cdc42 or Cdc42/Rac1 chimeras were confirmed by automated sequencing prior to protein expression. All point mutations used are based on single-letter amino acid designations.

**CDNA Cloning of Human Tiam1 DH/PH Domains**—Based on a Blast search in the human expressed sequence tag data base using the published Tiam1 sequences (17), two clones encoding the human Tiam1 DH/PH sequences were identified; the first one, with GenBank<sup>TM</sup> accession number AA233606, contains Tiam1 amino acids 1060–1259, while the second one, with accession number AA721939, includes residues 1088–1435. The two cDNAs were cloned from human liver cDNA, and were used as template to piece together an intact Tiam1 DH/PH domain module by the PCR method. To obtain the missing N-terminal sequences of the DH domain of Tiam1, an N-primer with a BglII cleavage site was designed with the sequence 5′-GGGAAGATCTGAGAACAATCTCGGAGATGTCGCGAAGGTGTTCTACGGCTCCACAGTTATGGGA-3′ and a C-primer containing an EcoRI digestion site 5′-GGAAATTACCTCCTGTTAGGAGGCTCCTGGATACATGTA-3′ were used to perform a second round PCR. The two PCR products were digested by BglII and EcoRI, respectively, and were ligated into a modified pET15b vector at the BamHI and EcoRI sites, creating a Tiam1 DH/PH clone (containing residues 1033–1406) with an N-terminal His<sub>6</sub> tag.

**Recombinant Protein Production**—Recombinant TrioN (residues 1225–1537 containing the N-terminal DH/PH module), GEF-H1 (residues 226–643 containing the DH/PH module), Tiam1 (residues 1033–1606, DH/PH module), and PAK1 p21-binding domain (PBD) domain (residues 51–135) were expressed in E. coli BL21(DE3) strain as N-terminal His<sub>6</sub>-tagged fusion proteins by using the pET expression system (Novagen). Rac1, Cdc42, and Cdc42/Rac1 chimeras and their mutants were expressed in E. coli DH5α strain as GST fusions using the pGEX-KG vector (14). The N-terminal tagged GST or His<sub>6</sub> fusion proteins were purified by glutathione- or Ni<sup>2+</sup>-agarose affinity chromatography (14, 18). GST-GTPases on glutathione beads were eluted off bound guanine nucleotides by washing three times with a buffer containing 50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, and 1 mM dithiothreitol. 0.5 mM GTP-S or GDP was added to the G-proteins after a 10-min incubation together with 12 mM MgCl<sub>2</sub> to generate the GTP·S- or GDP-bound small G-proteins.

**In Vitro Guanine Nucleotide Exchange Assay**—The [γ<sup>32</sup>P]GDP/GTP exchanges of Rac1, Cdc42, and different Cdc42/Rac1 chimeras were measured at 25 °C by a filter binding assay as described previously (19). The exchange reactions were carried out in a buffer containing 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, and 500 μM GTP in the presence or absence of the indicated amount of purified GEFs. The reactions were terminated at the 5-min time point by nitrocellulose filtration, and the amounts of [γ<sup>32</sup>P]GDP remaining bound to the Rho GTPases were normalized as the percentage of [γ<sup>32</sup>P]GDP bound at time 0.

Fluorescence measurement of mant-GDP/GTP exchange was carried out using an LS 50B Luminescent Spectrometer (PerkinElmer Life Sciences) in an exchange buffer including 200 mM concentrations of respective GTPases and 200 mM GEF as described before (18). The mant-GDP fluorescence changes during the exchange reactions were monitored with an excitation wavelength of 360 nm and the emission wavelength at 440 nm. All measurements were performed at 25 °C in the exchange buffer containing 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 0.3 mM mant-GTP, 0.5 mM mant-GDP, and 0.2 μM mant-GTP·S·G protein. The exchange assays of each mutant/chimera were carried out at least three times.

**Binding Interaction Assay**—2 μg of GST-tagged, nucleotide-free small GTPases and 0.5 μg of His<sub>6</sub>-Tiam1, His<sub>6</sub>-GEF-H1 or 1 μg of His<sub>6</sub>-Tiam1 were added to 200 μl of incubation buffer (20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 200 μg/ml bovine serum albumin, 1% Triton X-100, and 1 mM MgCl<sub>2</sub>) containing 10 μl of glutathione-agarose suspension. After incubation for 1 h at 4 °C under constant agitation, the glutathione beads were washed twice in the incubation buffer, boiled in Laemmli sample buffer, and analyzed by immunoblotting with anti-His monoclonal antibodies similar to that described (6, 13). In peptide inhibition assays, different amounts of chimeric forms of Rac1 or Cdc42 were preincubated with the GST-GEFs in the incubation buffer for 10 min prior to mixing with the GST-GEF and glutathione-agarose beads. Each set of binding experiments was carried out three times independently.

**Effecter Binding Assay**—The Myc-tagged full-length PAK1 cloned in pCMV6 vector (20) was transfected into COS-7 cells by using Lipo- fectAMINE reagent (Life Technologies, Inc.). After 48 h, cells were lysed in a buffer containing 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 2 mM
out. For the His6-PAK1 PBD pull-down assay, cell lysates expressing the His6-GST fusions were loaded with mant-GDP was incubated at 25 °C in an exchange buffer containing 100 mM NaCl, 0.3 mM MgCl$_2$, 50 mM Tris-HCl (pH 7.6), and 0.5 mM GTP in the absence (dashed line) or presence (solid line) of 200 nM TrioN (A), GEF-H1 (B), or Tiam1 (C). In the case of Tiam1-catalyzed reactions, 1 μM phosphatidylinositol-4,5-bisphosphate (4, 5) was also included. The fluorescence changes of bound mant-GDP were monitored at the indicated time period with an excitation wavelength at 360 nm and emission wavelength at 440 nm. Lower panels, 0.5 μg of His$_6$-tagged TrioN (A), GEF-H1 (B), or Tiam1 (C) was incubated with GST alone or nucleotide-free, GST-fused Cdc42 or Rac1 (0.5 μg) immobilized on glutathione-agarose beads in a binding buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% Triton X-100, and 0.5 mg/ml bovine serum albumin. The bead-bound proteins were washed, separated by electrophoresis, and immunoblotted by using anti-His antibodies.

MgCl$_2$, 1 mM dithiothreitol, 1% Triton X-100, and the protease inhibitor mixtures (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were cleared by brief centrifugation and incubated for 1 h at 4 °C with 2 μg of different GST-GTPases that were preloaded with GTP-γS and immobilized on glutathione-agarose beads. The GST beads were washed twice in the wash buffer and subjected to analysis by immunoblotting with anti-Myc monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The polypeptide W56 was added to 250 μM in the incubation buffer where it is indicated.

**Cell Culture, Transfection, and Effector Pull-down Assay**—Wild type Rac1, Cdc42, and their mutant cDNAs were subcloned into pCEFL-GST vector at the BamHI and EcoRI sites to be expressed as GST-tagged proteins. TrioN cDNA encoding the DH/PH domain was cloned into the pMX-IRES-GFP vector, which expresses the TrioN and the green fluorescent protein (GFP) as a bicistronic mRNA (21). Retroviral packaging and infection were carried out according to described methods (21). NIH 3T3 fibroblasts were infected with the retroviruses, and cells were harvested 72 h postinfection. GFP-positive cells were sorted by fluorescence-activated cell sorting and were used for analysis immediately after sorting. For transient expression, the NIH 3T3 GFP/TrioN cells were seeded in 6-cm dishes at a density of 5 × 10$^5$ cells in Dulbecco’s modified Eagle’s medium, supplemented with 10% calf serum. The next day, different pCEFL vectors were transfected into the cells by using LipofectAMINE Plus (Life Technologies) following the manufacturer’s instructions. In the following day, transfected cells were harvested and replated onto glass coverslips. 12 h prior to fixation, serum was withdrawn from the medium.

To create cell lines stably expressing the GST fusion proteins, 80% confluent NIH 3T3 GFP/TrioN cells in 10-cm dishes were transfected with different pCEFL vectors. 48 h post-transfection, cells were selected on growth medium supplemented with 400 μg/ml G418. ~3 weeks later, cell clones were pooled, and the expression of the GST fusions was checked by anti-GST immunoblotting of the cell lysates. The cells were serum-starved for another 12 h with serum-free Dulbecco’s modified Eagle’s medium before the effector PKA1 pull-down assay was carried out. For the His$_6$-PAK1 PBD pull-down assay, cell lysates expressing comparable levels of various GST fusions were incubated with Ni$^{2+}$-agarose-immobilized His$_6$-PAK1 PBD domain (~1 μg each) purified from E. coli for 30 min as described (21). The Ni$^{2+}$-agarose co-precipitates were washed twice in the wash buffer and analyzed by immunoblotting with anti-GST monoclonal antibody.

**Immunofluorescence**—Cells grown on coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline for 15 min, washed, and permeabilized with 0.1% Triton X-100 for 30 min (21). After washes, the fixed cells were blocked with phosphate-buffered saline containing 2% bovine serum albumin for 1 h. The cells were then incubated with monoclonal anti-GST antibody in phosphate-buffered saline containing 0.1% bovine serum albumin for 90 min, followed by incubation with tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG antibody. The coverslips with cells were mounted onto slides in VectaMount and viewed under a Zeiss fluorescence microscope.

**Molecular Modeling**—Simulation of the amino acid substitutions in the structural coordinates of the Rac1-Tiam1 complex was made using Insight II software (Molecular Simulations Inc.). After the substitution was introduced, energy minimizations of the resulting structure were carried out by applying the Biopolymer and Discover modules of Insight II, respectively.

**RESULTS**

**Rac1 Specificity of GEFs Is Governed by Rac1-GEF Binding Interaction**—Unlike the Dbl family GEFs Bcr and Ect2, which are capable of indiscriminately activating the two closely related Rho GTPases Rac1 and Cdc42 (22, 23), a subset of Dbl family members, including TrioN, GEF-H1, and Tiam1, appears to be specifically involved in Rac1, but not Cdc42, activation. Fig. 1A shows that purified TrioN protein acts as a Rac1-specific GEF by stimulating the rate of mant-GDP dissociation from Rac1 while displaying only marginal activity in accelerating the time courses of mant-GDP dissociation from Cdc42 under similar conditions. Similarly, GEF-H1 and Tiam1 also favor Rac1 over Cdc42 as a GEF reaction substrate (Fig. 1, B and C, upper panels). To determine whether the observed Rac1 specificity of the GEFs is reflected in their direct binding interaction, a complex formation assay was performed by using immobilized GST-Rac1 or GST-Cdc42 as a probe to pull down the respective GEFs. As shown in Fig. 1A (lower panel), TrioN selectively binds to GST-Rac1 without detectable affinity toward GST-Cdc42 or GST, indicating that substrate binding discrimination may be the governing factor in determining the

**Fig. 1.** Rac1 binding interaction with GEFs governs the specificity of the nucleotide exchange reaction. Upper panels, 200 nM Rac1 (red line) or Cdc42 (blue line) loaded with mant-GDP was incubated at 25 °C in an exchange buffer containing 100 mM NaCl, 0.3 mM MgCl$_2$, 50 mM Tris-HCl (pH 7.6), and 0.5 mM GTP in the absence (dashed line) or presence (solid line) of 200 nM TrioN (A), GEF-H1 (B), or Tiam1 (C). In the case of Tiam1-catalyzed reactions, 1 μM phosphatidylinositol-4,5-bisphosphate (4, 5) was also included. The fluorescence changes of bound mant-GDP were monitored at the indicated time period with an excitation wavelength at 360 nm and emission wavelength at 440 nm. Lower panels, 0.5 μg of His$_6$-tagged TrioN (A), GEF-H1 (B), or Tiam1 (C) was incubated with GST alone or nucleotide-free, GST-fused Cdc42 or Rac1 (0.5 μg) immobilized on glutathione-agarose beads in a binding buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% Triton X-100, and 0.5 mg/ml bovine serum albumin. The bead-bound proteins were washed, separated by electrophoresis, and immunoblotted by using anti-His antibodies.
GEF reaction specificity. Similar binding results were obtained in the case of GEF-H1 or Tiam1 interaction with Rac1 and Cdc42 (Figs. 1, B and C, lower panels). Of the three GEFs, GEF-H1 appears to be the most potent activator of nucleotide exchange on Rac1, whereas Tiam1 is the weakest. As described previously (17), we found that human Tiam1 requires the phosphoinositol lipid phosphatidylinositol (4,5)-bisphosphate for activation of its GEF activity, which differs from that found for the constitutively active murine Tiam1. Overall, TrioN, GEF-H1, and Tiam1 are able to discriminate Rac1 from Cdc42 as a substrate in their respective GEF reactions based on their binding preferences.

Both Switch I and Switch II Regions of Rac1 Are Essential for GEF Binding and Catalysis—Structural complexes and mutagenesis data obtained between Ras, ADP-ribosylation factor, and Ran and their respective GEFs have shown that the switch regions of the small GTPases play important roles in the GEF binding interaction and in the subsequent nucleotide dissociation (24–27). Switch II, in particular, has been proposed to contribute to the substrate docking, while switch I is thought to be involved in the subsequent step of stimulation of GDP dissociation. This feature is probably conserved in Rho GTPase-GEF interaction, although it may differ in details. Previous structural studies have shown that both switch I and II regions of Rac1 appear to be in broad contact with the GEF domain of Tiam1 (15), while the mutagenesis data has shown that many switch II residues of RhoA and Cdc42 do not seem to be required for the functional interaction with their respective GEFs, Lbc and Cdc24 (14). To further investigate the contribution of Rac1 switch regions to the GEF reaction, we have generated a panel of point mutants at both switch I (E31A, Y32A, I33A, T35A, D38A, and N39A) and switch II (A59G, Q61L, Y64A, D65A, R66A/L67A, L70A/S71A, and Q74D) positions at which most of the mutated residues are highly conserved among Rho family GTPases. The abilities of the mutants to bind to and to be activated by the GEFs, TrioN, GEF-H1, and Tiam1 were analyzed. Under similar conditions, the switch I mutants E31A, I33A, and T35A maintained both the binding activity and GEF responsiveness to TrioN and GEF-H1, Y32A retained the binding but lost the GEF sensitivity, and D38A and N39A were inactive in either binding or catalysis toward TrioN or GEF-H1 (Figs. 2, A and B). Similar results were also obtained for Tiam1 binding and catalysis (data not shown). These results indicate that the switch I
residues are probably involved in both aspects of the GEF reactions, the GEF binding (residues Asp$^{38}$ and Asn$^{39}$) and the nucleotide exchange catalysis (residue Tyr$^{32}$).

When the switch II mutants of Rac1 were examined in the similar assays, we found that A59G, D65A, L70A/S71A, and Q74D remained capable of binding to TrioN and GEF-H1, whereas Q61L, Y64A, and R66A/L67A lost binding activity to the GEFs (Fig. 2C). In the GDP/GTP exchange reactions, Q61L, Y64A, D65A, R66A/L67A, and L70A/S71A failed to undergo nucleotide exchange when incubated with TrioN or GEF-H1 (Fig. 2D). Tiam1 acted similarly to TrioN or GEF-H1 in both aspects (data not shown). Like the switch I situation, we conclude that switch II of Rac1 is also important for both the physical association with the GEFs (residues Gln$^{61}$, Tyr$^{64}$, and Arg$^{66}$/Leu$^{67}$) and the efficient exchange of nucleotides catalyzed by the GEFs (residues Asp$^{65}$ and Leu$^{70}$/Ser$^{71}$).

**Fig. 3.** Determination of the region of amino acids in Rac1 responsible for GEF specificity. A, alignment of the Rac1 and Cdc42 amino acid sequences. Conserved residues between Rac1 and Cdc42 are marked by asterisks, and switch I, switch II, and secondary structures are also indicated. B, schematic representation of the Cdc42/Rac1 chimeras used in this study. The primary sequences of Rac1 and Cdc42 are represented as dark and blank rectangles, respectively. The amino acid numbers at the chimera junctions are indicated at the top. C, interaction of TrioN or GEF-H1 with Cdc42, Rac1, and the Cdc42/Rac1 chimeras. The amount of His$_6$-tagged GEF co-precipitated with the immobilized GST fusion G-proteins was revealed by anti-His Western blotting. D, [H]GDP/GTP exchange reactions of the indicated wild type or chimeric GTPases were carried out with or without the catalysis of TrioN or GEF-H1 in 5 min. The amount of bound [H]GDP remaining was normalized to that of the zero time points.

**The Region of Amino Acids in Rac1 That Specifies Sensitivity to the GEFs—**Rac1 and Cdc42 share over 70% sequence identity throughout their sequences (Fig. 3A). Since the switch residues targeted in the above experiments are mostly conserved in Rac1 and Cdc42, they cannot provide the structural cues for Rac1 selectivity over Cdc42 by the GEFs. To localize the region of Rac1 that specifies interaction with the GEFs, we utilized a panel of chimeras that were made by swapping corresponding residues of Rac1 with those of Cdc42 (Fig. 3B). In the case where the structural determinant(s) of Rac1 specifying GEF interaction was present in a chimera molecule containing the region, we expect that the chimera would be capable of
recognizing the GEF. On the other hand, the replacement of that region by sequences from Cdc42 would result in the elimination of the interaction of the chimeric GTPase with the GEF. As shown in Fig. 3, we found that the Cdc42/Rac1 chimeras containing amino acids 53–70 of Rac1 (chimeras A, C, E, and F) could associate with TrioN and GEF-H1 regardless of the presence of other Cdc42 regions in the molecules. Conversely, the Cdc42/Rac1 chimeras in which the residue 53–70 region was replaced by the corresponding area of Cdc42 (chimeras B and D) lost the ability to bind to the GEFs (Fig. 3C). Similar results were also obtained for Tiam1 binding (data not shown). These observations and the fact that chimeras C and D, which differ only in the sequences of the 53–70 region, behaved oppositely in the GEF binding profiles indicate that the Rac1-specifying determinant(s) of the GEF action is located between residues 53 and 70 of Rac1.

To determine whether the area of residues 53–70 of Rac1 that specifies the physical interaction with the GEFs is also sufficient for specifying the GDP/GTP exchange catalyzed by the GEFs, the same set of Cdc42/Rac1 chimeras were tested in the exchange assays. As expected, only the Cdc42/Rac1 chimeras containing the 53–70 residues from Rac1 (chimeras A, C, E, and F) experienced GDP/GTP exchange after incubation with TrioN or GEF-H1 (Fig. 3D). In contrast, the Cdc42/Rac1 proteins lacking the region of residues 53–70 of Rac1 (chimeras B and D) were inactive in the exchange reac-

FIG. 4. Involvement of the Rac1 unique β3/β4, residues in the GEF interaction and responsiveness. A, locations of the Rac1 unique Ser41, Asn43, Asn52, and Trp56 residues in the tertiary structure of the Rac1-Tiam1 complex. Possible polar interactions less than 3.5 Å are indicated as green (intermolecular) or red (intramolecular) dashed lines. B, interaction of TrioN, GEF-H1, and Tiam1 with the Rac1 β3/β4 point mutants generated by swapping the corresponding residue of Rac1 with that of Cdc42. The binding assays were carried out similarly as in the previous figures. C, the responsiveness of the Rac1 mutants to TrioN, GEF-H1, and Tiam1 catalysis in the mant-GDP/GTP exchange reactions compared with that of wild-type Rac1 or Cdc42.
Fig. 5. Identification of Trp$^{56}$ of Rac1 as the necessary and sufficient determinant specifying GEF recognition. A, tertiary environment of Trp$^{56}$ in the binding pocket formed between Rac1 and Tiam1. B, energy minimization prediction of a structural model of the interaction between Rac1 and Tiam1. C, Western blot analysis showing the interaction between Rac1, Rac1W56F, Cdc42, Cdc42F56W, His-TrioN, His-GEF-H1, and His-Tiam1. D, quantification of the relative fluorescence intensity for different constructs. E, time course of relative fluorescence intensity for different conditions.
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**Introduction**—To determine whether Trp<sup>56</sup> constitutes the key residue in GEF activation of Rac1 in cells, the Rac1W56F and Cdc42F56W mutants as well as wild type Rac1 and Cdc42 were transfected into NIH 3T3 fibroblasts that express the Rac1-specific DH/PH domains of Trio, TrioN. The GFP was co-expressed in the cell line as a marker for TrioN expression as a bicistronic mRNA. As shown in Fig. 6A, TrioN caused the NIH 3T3 cells to flatten and to produce membrane ruffles, presumably by activation of endogenous Rac1. While Rac1 localized mostly along the membrane ruffles and in cytoplasm, Cdc42 was primarily found perinuclearly in the Golgi-like organelle. Expression of wild type GST-Rac1 in these cells further enhanced membrane ruffles stimulated by TrioN, whereas expression of GST-Cdc42 in the TrioN cells did not produce additional phenotype. Rac1W56F, which is unable to interact with TrioN in vitro, could not increase the ruffling effect further and was located mainly around the perinuclear/cyttoplasmic region. In contrast, the morphology of Cdc42F56W-expressing cells was different from all of the above described cells; in addition to membrane ruffles found at the cell leading edges, prolonged membrane extensions and microspikes were also clearly visible (Fig. 6A). Since the Rac1W56F and Cdc42F56W mutants do not appear to affect effector interaction, as shown in Fig. 7D in a complex formation assay with the Cdc42 and Rac1 effector PAK1, these morphological changes induced by Rac1W56F and Cdc42F56W strongly support a role of Trp<sup>56</sup> in specifying interaction with, and being a sensitivity cue of, TrioN in cells.

To further confirm that Rac1W56F is unresponsive to TrioN whereas Cdc42F56W can be activated by TrioN in cells, the amount of activated GST-Rac1/Cdc42 in the TrioN-expressing cells was assayed by using the PBD of PAK1, which specifically complexes with the GTP-bound GTPases (20). Fig. 6B shows that the Ni<sup>2+</sup>-agarose-immobilized His<sub>6</sub>-PAK1 pulls down significantly more GST-Rac1-GTP or GST-Cdc42F56W-GTP from the cell lysates than GST-Cdc42-GTP or GST-Rac1W56F-GTP. These results indicate that Cdc42F56W also cannot be activated in response to TrioN in cells.

**Specific Inhibition of Rac1-GEF Interaction by a Rac1-derived Polypeptide Containing Trp<sup>56</sup>**—Given the important role of Trp<sup>56</sup> of Rac1 in GEF discrimination, we wondered whether a Rac1-derived peptide targeting the Trp<sup>56</sup> site could serve as an effective inhibitor for Rac1 activation by the Rac1-specific GEFs. We synthesized two polypeptides of 16 amino acids in length encompassing Trp<sup>56</sup>, one including Trp<sup>56</sup> and the surrounding residues in Rac1 (W56); residues 45–60) and the other containing the same sequences except that Trp<sup>56</sup> was changed to Phe<sup>56</sup> (F56; Fig. 7A). The two peptides were tested for their ability to compete with wild type Rac1 in GEF binding assays. As shown in Fig. 7B, binding of TrioN, GEF-H1, or Tiam1 to Rac1 was significantly inhibited by the peptide W56 in a dose-dependent manner, such that at above 200 μM peptide concentration the GEF binding to Rac1 became undetectable. In contrast, the peptide F56 did not have any detectable effect on Rac1 binding to any of the three GEFs (Fig. 7C). Moreover, the inhibitory effect of the W56 peptide on Rac1 interaction with the GEFs appears to be specific, since the peptide did not interfere with the effector, PAK1, binding interaction with Rac1 (Fig. 7D). These results provide further proof that Trp<sup>56</sup> is a critical determinant for Rac1 interaction with the GEFs and suggest that a chemical compound mimicking Trp<sup>56</sup> action.

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*Rac1 Trp<sup>56</sup> Specifies Interaction with a Subset of GEFs* by guest on July 25, 2018http://www.jbc.org/Downloaded from
FIG. 6. Reversal of TrioN specificity by Cdc42F56W mutation in cells. A, the GFP/TrioN stable expressing NIH 3T3 cells were generated by retroviral induction of parental NIH 3T3 cells using recombinant retrovirus produced from pMX-IRES-GFP vector, which expresses GFP and the TrioN cDNA as bicistronic mRNAs. The GFP/TrioN cells were transfected with pCEFL vectors expressing GST-tagged Rac1, Rac1W56F, Cdc42, and Cdc42F56W, respectively. 34 h post-transfection, the serum-starved cells were fixed and stained by anti-GST monoclonal antibody followed by tetramethylrhodamine isothiocyanate-conjugated anti-mouse IgG. The expression of GFP/TrioN, GST, or respective GTPases as well as cell morphologies were visualized by GFP fluorescence or anti-GST immunofluorescence. B, the activation states of ectopically expressed GST-Rac1, GST-Cdc42, and their mutants in GFP/TrioN-expressing cells were detected by the His$_6$-PAK1(PBD) pull-down assay. Cell lysates containing a similar amount of GST fusions were incubated with Ni$_{2+}$-agarose immobilized His$_6$-PAK1(PBD), and the co-precipitates were subjected to anti-GST Western blotting to reveal the amount of GTP-bound GST-GTPases.
could be explored as an interfering reagent specifically targeting Rac1 activation.

**DISCUSSION**

Although much has been learned about the activation mechanisms of members of the Ras superfamily by their respective GEFs, mostly through x-ray crystallography and mutagenesis studies (15, 24–27), the details relating to signaling specificity at this critical step of many signal transduction chains, activation of Rho GTPases in particular, remain poorly understood. In the present study, building on the structural information of the complex of a pair of Rho GTPase-GEF, Rac1-Tiam1, we have identified the structural cue, Trp56 of Rac1, that specifies the interaction with and responsiveness to a subset of the Dbl family GEFs (i.e. TrioN, GEF-H1, and Tiam1). The results obtained from both *in vitro* biochemical and *in vivo* cell biological experiments provide a mechanistic basis for substrate discrimination by Rac1-specific GEFs. Furthermore, our peptide inhibition studies suggest that Trp⁵⁶ of Rac1 constitutes an attractive target site in designing Rac1-specific inhibitors that are effective in interfering with Rac1 activation.

The current biochemical model portrays that Dbl family GEFs function immediately upstream of Rho GTPases in transmitting the intracellular signal (5). Stimulation of growth factor receptors, cytokine receptors, cell-to-cell or extracellular matrix-to-cell adhesion receptors, or the G-protein-coupled serpentine receptors can all initiate intracellular signals that lead to Rho GTPase activation, a process that probably involves one or multiple Dbl family members. The active Rho species interact in turn with a large array of effector targets that further relay the signals to downstream components, resulting in diverse cell biological responses including actin cytoskeleton reorganization, transcriptional activation of genes, stimulation of DNA synthesis, endocytic or exocytic membrane trafficking, and regulation of translation (1, 2). Thus, the GEF-Rho protein interphase represents a key step in the complex signal transduction chains of diverse biological events controlled by Rho family GTPases.

**Fig. 7.** Inhibition of the GEF interaction with Rac1 by a polypeptide containing Trp⁵⁶. A, amino acid sequences of polypeptides W56 and F56. The amino acid numbering is in respect to Rac1 sequences. B, peptide W56 inhibits Rac1 interaction with TrioN, GEF-H1, and Tiam1. Increasing concentrations of the polypeptide (100–200 μM) were included in the Rac1-GEF binding assay buffer. C, peptide F56 has no effect on Rac1 interaction with its GEFs. Similar binding conditions were employed as in B. D, effect of W56 polypeptide on the effector PAK1 interaction with Rac1 or Cdc42. COS-7 cell lysates expressing Myc-PAK1 were incubated with glutathione-agarose immobilized GST-fused Cdc42, Rac1, or Rac1 mutants for 1 h at 4 °C. Bound proteins were washed, separated by electrophoresis, and immunoblotted by using anti-Myc antibody. The GTPases were preloaded with GTPγS, and the *lanes underlined by +W56* indicate the inclusion of the W56 peptide (250 μM) in the binding buffer.
model system have established that whereas the DH domain is responsible for the GEF catalytic activity, the adjacent PH domain is involved in intracellular targeting and/or regulation of the DH domain (6, 28). This appears to be a generalized principle among Dbl family GEFs, since similar conclusions have been drawn in other cases of Dbl-related molecules including Lbc, Lfc, FGD1, and Dbs (29–32). In this study, we employed three Rac1-specific DH/PH modules derived from Trio, GEF-H1, and Tiam1, respectively. Their biochemical behaviors conform to this principle such that they all retain the GEF activity toward the GTPase substrate, Rac1, and therefore the results obtained from the constitutively active GEF modules should easily be extrapolated to the full-length GEF situations.

The three-dimensional structures of three distinct members of Dbl family, TrioN, Sos1, and βPIX, show that these DH domains fold into highly homologous α-helical bundles (33–35). A surface created by conserved region 1, conserved region 3, and a part of αD of the DH domain as well as the DH-PH junction site is involved in the formation of a Rho GTPase interactive pocket (21, 34). Recent determination of the structure of the DH-PH module of Tiam1 bound to Rac1 provides a framework for further delineating the structural determinants of the Rho proteins involved in GEF coupling (15). It appears that the interaction with Tiam1 has altered the conformation of the sequences in and immediately flanking switch 1 and switch 2 regions of Rac1, two stretches of amino acid residues that display the most conformational changes in response to nucleotide binding. Not unlike the cases of other GEF-G complexes, Tiam1 disturbed the native coordination of Rac1 for a Mg\(^{2+}\) cofactor. In particular, Ala59 has moved within 2 Å of the Mg\(^{2+}\) binding site to disrupt effective Mg\(^{2+}\) chelation. This in part explains the previously observed lowered Mg\(^{2+}\)-binding affinity to Rho proteins when a GEF is present (18). In addition, a large portion of β2/β3 and switch 2 regions of Rac1 engages in extensive surface contact with Tiam1 residues, providing docking sites for GEF recognition and stabilizing the complex formation.

Adding to these structural observations, we demonstrate here by mutational analysis that the intermolecular interactions between Rac1 and its GEFs are highly complex. Rac1-specific activation by GEFs depends on the discriminative binding recognition between Rac1 and GEF and involves both switch I and switch II regions as well as the β2/β3 strands. The mutagenesis data in the switch I and II residues are in large part consistent with the structural predictions but provide further insight. In the structure of Rac1 and Tiam1 complex (15), Glu1047 of Tiam1 mediates a series of interactions with switch I, including hydrogen bonding with the hydroxyl group of Tyr32 and the amide nitrogen of Thr35. These interactions support the movement of Thr35 of Rac1 and thus may affect its ability to bind Mg\(^{2+}\). In agreement with this, mutation in Tyr32 abolishes the exchange activity of the GEFs. But mutant Rac1T35A remains sensitive to the GEFs, similar to the Cdc42T35A or RhoA T37A’s responsiveness to Dbl (14). This behavior differs from Cdc42T35A sensitivity to Cdc42 or RhoA T37A sensitivity to Vav3 and Lbc (14, 36), which is inactive to the respective GEFs. Given that Thr35 serves to maintain a network of interaction in the Mg\(^{2+}\)-nucleotide complex (37–39), these results imply that the essential Mg\(^{2+}\) cofactor plays different roles in the catalysis of various GEF reactions. Of the other switch I mutants tested, Rac1D38A and Rac1E39A lost binding activity to the GEFs, suggesting that both residues may participate in GEF docking. It appears therefore that the switch I region contributes to both the GEF binding and the GEF catalysis of GDP release.

We have also come to a similar conclusion for the role of the switch II region of Rac1 in the GEF reactions. In the complex of Rac1 and Tiam1, Tyr47 anchors switch II by burying it in a hydrophobic pocket formed by Tiam1 residues Leu1204, Ala1228, Ser1229, and Asn1232 and Rac1 residues Gln61, Asp63, and Leu67 (15). The loss of binding of Y64A, Q61L, and L67A mutants to the GEFs supports the importance of this anchoring interaction and agrees with the RasY64A mutational effect on the Sos interaction (27). It is possible that the highly conserved Tyr47 residue among Ras superfamily members shares such a role in their respective GEF reactions. On the other hand, the Rac1A59G mutant remained fully reactive toward the GEF stimulations, which again is consistent with the RasA59G responsiveness to Sos (27), suggesting that if displacement of Mg\(^{2+}\) by Ala59 is involved in the GEF-mediated GDP dissociation, it would be a minor rather than major factor in catalysis. Unexpectedly, mutants D65A and L70A/S71A of Rac1 were found to retain at least partial GEF binding activity yet displayed undetectable catalytic response toward the GEFs. Either these mutants have suffered more global distortions of conformation that may have led to the abolishment of GEF responsiveness, or these switch II residues are partly involved in the GDP/GTP exchange process. Given that similar mutants made in Cdc42 and RhoA yield distinct reactive profiles toward various GEFs (14, 36), the later interpretation is favored at this time.

The structure of Rac1-Tiam1 suggests that the GEF first interacts with a conformationally rigid portion of Rac1 (strand β2–3 and residues 65–74 of switch 2) to provide sufficient binding energy, which is then followed by alteration of the conformations of switch I and the remainder of switch II to destabilize nucleotide binding (15). Our Cdc42/Rac1 chimera studies firmly point to the region between residues 53 and 70 of Rac1 as a harbinger for the structural determinant(s) dictating selective coupling of the G-protein with the GEFs. Biochemical and cell biological characterization of the point mutants made in this region and beyond by swapping corresponding residues of Rac1 with those of Cdc42 provided convincing evidence that Trp56 of Rac1 serves as the specificity cue for the GEF recognition. Significantly, Trp56 appears to be the only residue in Rac1 allowing the subset of GEFs to discriminate it from Cdc42, since the Cdc42F56W mutant acts like wild type Rac1 in reconstituted GEF reactions in vitro and in cells. Utilizing one single amino acid of Rho GTPase to specify GEF interactions, Trp56 of Rac1 in particular, as we have shown here for TrioN, GEF-H1, and Tiam1 regulations, may not be a generalized phenomenon for other Rho GTPase-GEF reactions. Oncogenic Dbl protein, for example, functions equally well on Cdc42 and Cdc42F56W, indicating that residue 56 of Cdc42 is not involved in specifying Dbl recognition. Vav proteins (Vav1–Vav3), which specifically interact with RhoA (and Rac1) but not Cdc42, appear to involve two amino acids in the β2β3 region of RhoA (Asp49 and Glu54) to ensure specific substrate recognition (36), while another RhoA-specific GEF Lbc oncoprotein requires the unique Lys57 and Asp76 for RhoA for functional coupling (14). In addition, certain exchange factors including Vav3 and Cdc42 may utilize other structural cues in their substrates (e.g. Lys114 of RhoA and Glu116 of Cdc42, respectively) to specify GDP/GTP exchange catalysis (14, 36). It is likely that activation of each Rho family G-protein by a specific GEF engages in distinct features. It should also be noted that although Trp56 of Rac1 alone is sufficient for the GEF discrimination against Cdc42, it apparently does not serve a similar role for GEF discrimination against Rho. Other

2 Y. Gao and Y. Zheng, unpublished observation.
residues that differ between Rac and Rho are required to define Rac/Rho specificity toward GEFs.

In contrast with the highly related sequences of Rac1 and Cdc42, the DH domain sequences of the Rac1-specific GEFs are quite diverse. For example, the sequences of the DH domain of Tiam1 are no more related to those of TrioN and GEF-H1 than to the DH domain of other GEFs such as Lbc or Dbl. Analysis of the sequences of helix $\alpha_2$ and surrounding amino acids that interact with $\beta_2/\beta_3$ of Rac1 and may dictate substrate specificity from the GEF point of view reveals no obvious clue on how the Rac1 selectivity is conferred through this region of the DH domain. Actually, this area is among the most variable region in DH domains. Detailed mutagenesis studies are required to identify the sites on the GEFs that enable specific recognition of small G-protein substrates.

Many Dbl family GEFs as well as their Rho GTPase substrates have been implicated in a variety of biological processes including cell proliferation, motility, apoptosis, and neuronal morphogenesis (1, 2, 5). In addition, there is increasing appreciation of a close relationship between the activation status of Rho GTPases and human pathological conditions such as cancer (40). Therefore, the GEF-Rho GTPase interface may represent an attractive target site to design specific inhibitors that might bear therapeutic value (41). To this end, we have initiated a search for specific inhibitors that block the GEF-Rac1 interaction. Based on the GEF-discriminative nature of Trp56 of Rac1, we have tested the potential inhibitory effect of a polypeptide derived from Rac1 containing this critical residue in the GEF binding assay. The results show that at a concentration higher than 100 $\mu$M, it can specifically interfere with the GEF binding activity, with rac(G12V) showing an inhibitory effect on TrioN, GEF-H1, and Tiam1, but not the effector PAK1. In contrast, similar peptide containing a Trp56 to Phe56 mutation displayed no effect on the GEF-Rac1 binding, giving a promising sign suggesting that a chemical compound mimicking the Trp56 function of the peptide may be explored for anti-Rac1 signaling function. Thus, our structure-function characterization of Rac1 not only provides insight on how the small GTPase is regulated by the GEFs; the knowledge gained may also be combined with future chemical biology and biological studies to design and identify more effective inhibitors of Rac1 function.

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Rac1 Trp56 Specifies Interaction with a Subset of GEFs
Trp$^{56}$ of Rac1 Specifies Interaction with a Subset of Guanine Nucleotide Exchange Factors
Yuan Gao, Jingchuan Xing, Michel Streuli, Thomas L. Leto and Yi Zheng

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Fibroblast growth factor-2 represses platelet-derived growth factor receptor-α (PDGFR-α) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical cis-acting element in the proximal PDGFR-α promoter.

Michelle R. Bonello and Levon M. Khachigian

Page 2378, Fig. 1B: Panel B was incorrect as printed although the legend is correct. The correct Fig. 1B is shown below.

Trp₅₆ of Rac1 specifies interaction with a subset of guanine nucleotide exchange factors.

Yuan Gao, Jinchuan Xing, Michel Streuli, Thomas L. Leto, and Yi Zheng

Jinchuan Xing’s first name was misspelled. The correct spelling is shown above.