Oligomerization of Rac1 GTPase Mediated by the Carboxyl-terminal Polybasic Domain*

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The Rho family GTPase Rac1 mediates a variety of signal transduction processes leading to activation of NADPH oxidase, actin cytoskeleton reorganization, transcription activation, and stimulation of DNA synthesis. In this study, Rac1 was found to form a reversible monomer and oligomer in both the GDP- and GTP-bound states in vitro and in cells. Mutational analysis and peptide competition experiments showed that the unique C-terminal domain of Rac1 consisting of six consecutive basic residues (amino acids 183–188) is required for the homophilic interaction. Oligomerization of Rac1-GTP led to a self-stimulatory GTPase-activating protein (GAP) activity, resulting in a significantly enhanced intrinsic GTP hydrolysis rate of Rac1-GTP. Deletion or mutation of the polybasic residues drastically decreased its intrinsic GTPase activity and resulted in a loss of the self-stimulatory GAP activity. In the oligomeric state, Rac1 became insensitive to the RhoGAP stimulation, albeit maintaining the responsiveness to the guanine nucleotide exchange factor. The ability of the Rac1 C-terminal mutants to activate the effector p21<sup>cdc42</sup>/rac-activated kinase-1 correlated with their oligomerization states, suggesting that oligomer formation potentiates effector activation. Furthermore, the oligomer-to-monomer transition of Rac1-GDP could be driven effectively by interaction with the Rho guanine nucleotide dissociation inhibitor. Building on previous characterizations of Rac1 interaction with regulatory proteins and effectors, these results suggest that Rac1 may employ yet another means of regulation by cycling between the monomeric and oligomeric states to effectively generate a transient and augmented signal.

Rho family GTPases Rac1, Cdc42, and RhōA belong to the Ras superfamily of monomeric small GTP-binding proteins. They regulate a wide spectrum of cellular functions, ranging from cell growth and cytoskeletal organization to secretion (1–4). The physiological significance of Rho family GTPases is highlighted by increasing evidence for their involvement in human diseases such as cancer, hypertension, and inflammation (5–7).

The multiple biological functions of Rho family proteins are mediated through a tightly regulated GTP-binding/GTP-hydrolyzing cycle (8, 9). Three classes of regulatory proteins are known to be involved in the regulatory process. The guanine nucleotide exchange factors (GEFs)<sup>3</sup> catalyze the exchange of bound GDP for GTP, resulting in activation of the GTPases, whereas the GTPase-activating proteins (GAPs) stimulate the intrinsic GTP hydrolysis by Rho GTPases, leading to the rapid conversion to the GDP-bound inactive state. The Rho guanine nucleotide dissociation inhibitors (RhoGDIs) preferentially bind to the GDP-bound form of Rho proteins and prevent both spontaneous and GEF-catalyzed release of the nucleotide. In addition, RhoGDI recognizes the isoprenoid moiety of the GTPases and is capable of solubilizing the membrane-associated proteins. In the GDP-bound form, the Rho proteins interact with their specific effector or target molecules to trigger diverse cellular responses. Among a growing panel of the small G-protein targets, the serine/threonine protein kinases p21<sup>cdc42</sup>/rac-activated kinases (PAKs) were found to be activated upon binding to the GDP-bound form of Rac1 and Cdc42 (10, 36), and they have been implicated in a number of signaling pathways downstream of Rac1 and Cdc42, including c-Jun N-terminal kinase activation and cytoskeletal reorganization (36).

Among the Rac subfamily, the Rac1 GTPase shares >90% amino acid identity with its closest relatives, Rac2 and Rac3, and much functional redundancy has been expected among the three Rho GTPase members (11). For example, both Rac1 and Rac2 can be substituted in vitro effectively as a component of the NADPH oxidase complex. In addition to differences in tissue distributions (Rac1 and Rac3 are ubiquitous, whereas Rac2 expression is restricted to hematopoietic cells) (11, 12), however, two lines of evidence suggest that Rac1 may play a distinct role in cells. Neutrophils from Rac2 knockout mice are defective in many actin-based functions as well as in cell proliferation and survival even though Rac1 expression remains high (13). In vitro, Rac1 is able to bind to and stimulates the kinase activity of PAK1 ~4–5-fold better than Rac2 (14). These functional differences are reflected in their structures, which differ significantly only at the C termini. Most notably, Rac1 contains a unique C-terminal domain consisting of six consecutive basic amino acids, whereas this domain is disrupted by three neutral amino acids in the Rac2 and Rac3 sequences. This region of the molecule is not structured in the tertiary model of available Rho GTPases (28–30) and has been suggested to have a role in the membrane localization of Rac1 (15) and in interaction with effector proteins (14). How the unique feature of this region in Rac1 might contribute to the distinct function of Rac1 remains unclear.

Besides the interaction with regulatory proteins and effector molecules, a few Rho GTPases have been found to interact with...
homologically among themselves. We have previously shown that Cdc42 and Rac2 may form homodimers and that the dimerization event elicits a self-stimulatory GTPase-activating activity of the GTPases similar to the RhoGAP effect (16). An arginine residue in the C-terminal domain of these GTPases was shown to confer the catalytic GAP activity (17). These observations suggest that certain small GTPases may involve self-association as an additional mode of regulation. Similar suggestions have been made for other small and large G-proteins. For example, ADP-ribosylation factor-1 has been shown to exist in a functional dimeric or tetrameric form (18). A recent study with Ras suggests that dimerization at the plasma membrane is essential for Raf-1 activation (19). In analogy, homodimerization or oligomerization in large GTPases of the Mx family, which are interferon-induced GTPases (76 kDa) responsive to viral infections, has been known to regulate their GTP-binding and GTP-hydrolyzing cycle, yielding a highly efficient self-regulatory large GTPase complex (20, 21). Whether a similar homophilic interaction occurs between Rac1 GTPases and how such an interaction would affect the regulatory cycle of Rac1 are of particular interest to us.

In this study, we present evidence that Rac1 forms a reversible monomer and oligomer under physiologically relevant conditions and that its C-terminal polybasic domain is an essential determinant for oligomer formation. The biochemical results reported here, combined with our previous knowledge of Rac1 regulation, lead to a model in which the Rac1 oligomeric state may contribute to the generation of a transient and augmented signal.

**EXPERIMENTAL PROCEDURES**

**Materials**—GDP, GTP, GTP-S, and bacterial purine-nucleotide phosphorylase were purchased from Sigma. All radiolabeled nucleo-
tides were obtained from PerkinElmer Life Sciences. 2-Amino-6-mer-
captopo-7-methyluridine ribonucleoside (MESG) was synthesized as de-
scribed previously (22). The polypeptides corresponding to the C-terminal 12 amino acids of Rac1 and Cdc42 (PVKRRKRRKLL and PEKKRSRRCVLL, respectively) were custom-synthesized by Bio-Syn-
thesis, Inc. (Lewisville, TX). The anti-Myc and anti-HA monoclonal antibodies were obtained from Sigma and Roche Molecular Biochemi-
cals, IN), respectively.

**DNA Constructs and Recombinant Protein Preparations**—The Rac1 C-terminal mutants were generated by polymerase chain reaction-directed mutagenesis using internally derived oligonucleotides encoding the desired sequences. Rac1–8 represents a truncated form of Rac1 with deletion of the last eight residues of the C terminus. The Rac1–5Q mutant contains five Gln residues substituted for the corresponding residues (amino acids 183–187) of Rac1. The Rac1-Cdc42 mutant contains amino acids 183–188 of Cdc42 substituted for the corresponding residues of Rac1 in the Rac1 backbone. The mutant cDNAs were sequence-confirmed and subcloned into the pGEX-KG vector, pET28a vector, pVL1392-His6, pCMV-6-Myc vector, or pKH3 vector to be expressed as the GST, His6, or (HA)3 fusions in mammalian cells. The pKH3-Cdc42 and pKH3-RhoA constructs were as previously described (46). The pCMV6-Myc-PK1 construct was a kind gift from Dr. J. Chernoff (Fox Chase Cancer Center).

The preparation of recombinant GTPases was performed as de-
scribed (23). The E. coli-expressed proteins were in GST- or His6-tagged form and were purified by affinity chromatography on glutathione-agarose or Ni2+-agarose beads. The insect cell expression of His6-Rac1 was carried out by previously described procedures (24). The N-termi-
al GST- or His6-tagged sequences were cleaved by thrombin digestion when necessary. The post-translationally modified form of Rac1 was purified from the membrane fraction of the Sf9 cells infected with the recombinant baculovirus encoding His6-Rac1. RhoGDI and the GAP domain of p50GAP were containing amino acids 205–439 were expressed as GST fusions in E. coli as described. The quality of the proteins used in all assays was judged by SDS-polyacrylamide gel electrophoresis. Protein concentrations were estimated from Coomassie Blue-stained gels and/or by using BCA protein assay reagents (Pierce).

For transient expression in COS-7 cells, the pCMV6-Myc and pHK3 constructs were transfected into COS-7 cells grown to ~80% confluence in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum using LipofectAMINE regent (Life Technologies, Inc.). 48 h post-transfection, the cells were harvested for the complex formation assays.

**Gel Filtration**—A Superdex 200HR 10/30 column (10 x 30 cm; Am-
ersham Pharmacia Biotech) was used to analyze the homophilic interaction of the small GTPases in combination with a Bicinchoninic acid (BioRad) kit for protein quantification. The column was equilibrated with 50 mM HEPES (pH 7.6), 1 mM DTT, 100 mM NaCl, and 2 mM MgCl2 and was calibrated with molecular mass standards containing thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), chymotrypsigen A (25 kDa), and ribonuclease A (13.7 kDa) (Amersham Pharmacia Biotech). The samples were loaded in a volume of 0.1 ml, and elutions were performed at the indicated buffer conditions.

**GTPase Activity Assays**—The radioactive filter binding assays measuring the retention of γ32P-GTP-bound Rac1 were carried out as described (25). Briefly, recombinant Rac1 was preloaded with γ32P-GTP in 100 µl of buffer containing 50 mM HEPES (pH 7.6) and 0.1 mM EDTA for 10 min at ambient temperature before the addition of MgCl2 to a final concentration of 1 mM. An aliquot of γ32P-GTP-loaded Rac1 was mixed with reaction buffer containing 50 mM HEPES (pH 7.6), 0.2 mg/ml bovine serum albumin, 1 mM DTT, and 1 mM MgCl2. At different time points, the reaction was terminated by filtering the reaction mixture through nitrocellulose filters, followed by washing with 10 ml of ice-cold buffer containing 50 mM HEPES (pH 7.6) and 10 mM MgCl2. The radioactivity retained on the filters was then subjected to radioactive counting.

The MESG/phosphorylase system monitoring the free γ-P, released from the G-protein-bound GTP was based on the method described by Webb and Hunter (26) and has been applied to the measurement of Rho GTPases (17, 23, 27). Briefly, a 0.8-mL solution containing 50 mM HEPES (pH 7.6), 1 mM MgCl2, 0.2 mM MESG, 10 units of purine-nucleotide phosphorylase, 200 µM GTP, and the indicated amount of GFP-preloaded recombinant GTPase was mixed in a 4-mm width, 10-mm path length cuvette, and the time courses of the absorbance change at 360 nm were recorded. The concentrations of P, in the solution released from the G-protein-bound GTP is proportional to the net absorbance change by a factor of the extinction coefficient εmax εmax = 11,000 M–1 cm–1 at pH 7.6 (26). The kinetic data analysis was carried out as previously described (23).

**Immunoprecipitation and Kinase Activity Assay**—COS-7 cells transfected with the Myc-Rac1, HA-Rac1, HA-Cdc42, HA-RhoA, or Myc-PAK1 construct alone or in combination were harvested 48 h post-transfection. Cells were washed with ice-cold phosphate-buffered saline; lysed in 20 mM Tris-HCl (pH 7.4), 0.5% Triton-X100, 100 mM NaCl, 1 mM DTT, 1 mM MgCl2, 10 µg/ml leupeptin, and 10 µg/ml aprotinin; and centrifuged at 14,000 x g for 10 min at 4 °C. Protein concentration was confirmed by Western blotting of the cell lysates. For the immunoprecipitation assay, HA-tagged Rac1 was precipitated with anti-HA monoclonal antibody immobilized on protein A-Sepharose (Roche Molecular Biochemicals) from the lysates after a 2-h incubation under constant agitation. The immunoprecipitates were washed three times with the lysis buffer and subjected to anti-Myc or anti-HA Western blotting. To carry out the PAK1 kinase assay (14), the anti-Myc-PAK1 immunoprecipitates were incubated in a 60-µl reaction mixture containing 50 mM HEPES (pH 7.5), 2 mM MgCl2, 2 mM MnCl2, 0.2 mM ATP, and 20 µM γ32P-ATP in the absence or presence of various Rac1 mutants preloaded with GTP-S for 20 min at 30 °C. The reaction was terminated by the addition of an equal volume of 2X Laemmli buffer and subjected to gel electrophoresis and autoradiography/phosphorimaging analysis.

**RhoGDI Competition Assay**—To examine the effect of RhoGDI on the homophilic interaction of Rac1, the isoprenylated forms of His6-Rac1 and HA-Rac1 were generated from the membrane fraction of Sf9 insect cells and COS-7 cells, respectively, by extraction with 0.5% CHAPS. Immobilized His6-Rac1 was incubated with HA-Rac1 in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 50 mM NaCl, 0.02% CHAPS, and various doses of Rho-GDI. After three washes with the incubation buffer, the His6-Rac1 coprecipitates were then subjected to anti-HA Western blot analysis.

**RESULTS**

**Rac1 Exists in Reversible Monomeric and Oligomeric Forms**—We have previously described the dimerization properties of a few Rho family GTPase members, including RhoA, Rac2, and Cdc42 (16, 17). These Rho proteins contain two or more positively charged basic residues in the C-terminal region that were found to be essential for dimer formation (17). As a
A distinct member of the Rho family, Rac1 contains six consecutive basic residues (183KKRKRK188) in this region. Initially, we were interested to test whether Rac1 would conform to the similar dimer-forming property of Rac2 and Cdc42. The gel filtration profile of purified Rac1 shows that Rac1 was eluted in two peak fractions that correspond to estimated molecular masses of 25 and 600 kDa, respectively (Fig. 1A), suggesting that Rac1 could exist in a mixture consisting of a monomer and higher order oligomer under the physiologically relevant buffer conditions. Both the GDP- and GTPγS-bound Rac1 proteins yielded similar gel filtration profiles, indicating that the nucleotide-binding state of Rac1 does not affect the monomer-oligomer distribution. When the gel filtration pattern of Rac1 was compared with that of other small GTPases such as Ras and Cdc42, it became clear that the oligomerization property is unique to Rac1 since both Ras and Cdc42 (Fig. 1A), as well as the Rho GTPases including RhoA, RhoB, Rac2, and TC10 (data not shown), were detected exclusively in the monomeric form or in a mixture of monomer and dimer. To rule out the trivial explanation that oligomer formation was due to irreversible aggregation of Rac1, we performed further gel filtration analysis at varying salt and/or Mg²⁺ concentrations. Increasing the NaCl concentration from 50 to 300 mM resulted in a gradual shift of the overall population of Rac1 from oligomer to mono-
mer, whereas the divalent Mg$^{2+}$ ion also appeared to have a dramatic effect on the monomer-oligomer distribution such that $>70\%$ Rac1 oligomer was disassembled into monomer when the Mg$^{2+}$ concentration was raised from 0.2 to 30 mM as exemplified in two such conditions in Fig. 1A. Moreover, the monomer-to-oligomer transition was dependent on the initial Rac1 concentrations (submicromolar) (data not shown). These results indicate that the oligomer and monomer of Rac1 exist in a reversible equilibrium.

To further establish that Rac1 is capable of oligomerization, a complex formation assay was performed using immobilized GST-Rac1-GTPγS in an incubation with purified His$_6$-tagged Rac1-GTPγS, Cdc42-GTPγS, or RhoA-GTPγS. SDS-polyacrylamide gel electrophoresis analysis of GST-Rac1 coprecipitates revealed that whereas GST-Rac1 bound to Cdc42 or RhoA at a barely detectable level, it was capable of a tight interaction with His$_6$-Rac1 (Fig. 1B). The binding affinity between GST-Rac1 and His$_6$-Rac1 was sensitive to the buffer ionic strength since the amount of His$_6$-Rac1 coprecipitates was markedly reduced at increasing concentrations of NaCl or MgCl$_2$. These results confirm that Rac1 is capable of forming an oligomer and further suggest that oligomerization is mostly homophilic in nature.

To determine whether Rac1 oligomerization could occur in a cellular background, we coexpressed two N-terminal tagged forms of Rac1, HA-Rac1 and Myc-Rac1, in COS-7 cells. As a comparison, HA-Cdc42 or HA-RhoA was also coexpressed with Myc-Rac1 in these cells. Cellular HA-Rac1, HA-Cdc42, or HA-RhoA was immunoprecipitated from the cell lysates using an anti-HA monoclonal antibody immobilized on protein A-Sepharose beads, and the presence of associated Myc-Rac1 was detected by anti-Myc Western immunoblotting. As shown in Fig. 1C, the HA-Rac1 immunoprecipitates readily associated with Myc-Rac1 under the condition that no nonspecific binding of Myc-Rac1 to the anti-HA antibody complex was visible. In contrast, HA-RhoA did not appear to associate with Myc-Rac1 under similar conditions, whereas HA-Cdc42 displayed weaker binding to Myc-Rac1 (Fig. 1C). Thus, cellular Rac1 may form a stable homo-oligomer, raising the possibility that this feature of Rac1 may be involved in its regulation.

The C-terminal Polybasic Domain of Rac1 Is Involved in Oligomerization—The polybasic motif located immediately upstream of the isoprenylation CAAX box at the C terminus appears to be conserved among the Rho family GTPases (Fig. 2A). The recently available three-dimensional structures of RhoA, Rac1, and Cdc42, however, do not provide clues as to how this region is folded with regard to the overall structural backbone because truncation of this region was necessary for the crystallization conditions or NMR data collection process (28–30). Our previous sequence analysis revealed that the presence of two or more Lys or Arg residues in this region correlates with the homophilic interaction of the Rho family members RhoA and Cdc42 (16). To examine whether this region is involved in Rac1 oligomerization, two different experimental approaches were taken. First, a pair of polypeptides corresponding to the C-terminal region of Rac1 and Cdc42 (amino acids 183–191), respectively, were synthesized and used in a competition assay. Incubation of the Rac1-derived polypeptide (PVKKKKRRKCLLKL) with Rac1 caused a concentration-dependent disassembly of the Rac1 oligomer, and at 300 μM, the oligomer was completely shifted to monomer (Fig. 2B). In contrast, a peptide corresponding to the C-terminal residues of Cdc42 (PEPKKKSSRCVLL) displayed only a marginal effect on the oligomer- monomer distribution of Rac1 at the similar concentrations (data not shown), suggesting that the Rac1 polypeptide effect is specific for the Rac1 homophilic interaction. Second, a set of C-terminal mutants of Rac1 were constructed and tested for their effects on oligomerization (Fig. 2A). The Rac1-Cdc42 chimera (Rac1-Cdc42 mutant), which contains the Rac1 backbone with the C-terminal eight residues of Cdc42 substituted for the corresponding residues of Rac1. The Rac1–8 mutant represents a truncated form of Rac1 with deletion of the last eight residues (positions 181–191) from the C terminus. The Rac1-5Q mutant contains five neutral Gln residues substituted for basic amino acids 184–188 of Rac1. The basic residues lysine and arginine are underlined. B, a C-terminal polypeptide derived from Rac1 corresponding to residues 181–191 induces disassembly of the Rac1 oligomer in a dose-dependent manner. The gel filtration conditions were similar to those described in the legend to Fig. 1A. C, the Rac1 mutants (Rac1-Cdc42, Rac1-5Q, and Rac1–8) show gel filtration profiles distinct from that of wild-type Rac1. Standard elution conditions as described in the legend to Fig. 1A were applied.
The Rac1 oligomer displays significantly higher intrinsic GTP hydrolysis activity compared with the monomer. A, time courses of $\gamma^32P$GTP hydrolysis are compared between the isolated Rac1 oligomer and monomer at $-2 \mu M$ at 20 °C. The Rac1 monomer and oligomer species were collected from the corresponding gel filtration fractions as shown in Fig. 1A. The GTPase reactions were performed in buffer containing 50 mM HEPES (pH 7.6), 1 mM DTT, 50 mM NaCl, and 1 mM MgCl$_2$. The kinetic data fit best into a single exponential equation to yield intrinsic rate constants. B, the C-terminal mutants of Rac1 (20 μM) display distinct GTPase reaction rates as determined by the MESG/phosphorylase assay. The concentration of released γ-P$_i$ in the reaction solution was calculated by a factor of the extinction coefficient ε$_{360}$ nm = 11,000 M$^{-1}$ cm$^{-1}$ from the absorbance change of the phosphorylase coupling reactions (26). C, dose-dependent GTP hydrolysis time courses of Rac1 measured by the MESG/phosphorylase assay. D, concentration dependence of the intrinsic GTPase rate of Rac1 compared with that of the Rac1-8 mutant. The apparent GTP hydrolysis rates of Rac1 at various concentrations were derived by the best fit of the kinetic data from C.

As demonstrated above, the oligomerization state of Rac1 is sensitive to the ionic strength of the buffer conditions. The distribution of Rac1 isoforms at different concentrations of NaCl was revealed by the gel filtration profiles, with the percentage of oligomer steadily decreasing at increasing NaCl concentrations (Fig. 1A). In parallel, the GTP hydrolysis rate of Rac1 was found to decrease according to increasing NaCl concentrations (Fig. 1B). These results further suggest that the oligomerization state of Rac1 could account for the enhanced ability to hydrolyze GTP.

To test directly the intrinsic GTPase-activating potential of Rac1, the GDP-bound or GTPγS-bound Rac1 and Rac1 mutants were titrated into Rac1-GTP, and the GTPase reactions were monitored by measuring γ-P$_i$ release from Rac1-GTP. The addition of Rac1-GDP did not cause any change in γ-P$_i$ release, whereas the addition of Rac1-GTPγS resulted in a significant enhancement of γ-P$_i$ release, similar to that seen upon the addition of p50 RhoGAP under these conditions (Fig. 1C). The GTPγS-bound Rac1-Cdc42 mutant produced a partial enhancement compared with the effect brought about by Rac1-GTPγS, and the Rac1-8 and Rac1-5Q mutants had no detectable effect (Fig. 1C). These results indicate that the activated form of Rac1 presents a GAP activity for Rac1-GTP, and this self-stimulatory GAP activity is dependent upon the homophilic binding ability of Rac1. The apparent GTPase-activating effect of Rac1-GTPγS was not affected by isoprenylation since the post-translationally modified form of Rac1 purified from S9 insect cells (Fig. 1D, Rac1$^{\text{Ih}}$) behaved similarly to E. coli-produced Rac1. The observed oligomerization-elicted self-stimulatory GAP activity of Rac1 provides a rational for the dose-dependent fast rate of intrinsic GTPase activity, and the requirement of the C-terminal domain for this activity can be attributed to the presence of Arg$_{166}$ in the region, which have previously shown to constitute a "built-in arginine figure" in forming a transition state of the GTPase-activating reaction of other Rho family GTPases such as Cdc42 and Rac2 (17).

The Rac1 Oligomer Is Insensitive to RhoGAP Stimulation—p50 RhoGAP has been shown to potently catalyze GTP hydrolysis by Rho family GTPases including Rac1 (23, 27). To characterize the interaction of the Rac1 oligomer and monomer with RhoGAP, we measured the rate of γ-P$_i$ release from Rac1-GTP
under low salt conditions, which favor the formation of the
Rac1 oligomer, and from Rac1−8, which maintains in the
monomeric state under these conditions, in the absence or
presence of p50RhoGAP. Although p50 RhoGAP effectively
stimulated the rate of GTP hydrolysis of Rac1
by 10-fold, it was
unable to significantly increase the GTPase activity of Rac1,
which displayed a higher intrinsic GTPase rate due to the
self-stimulatory GAP activity (Fig. 6A), indicating that the
oligomeric form of Rac1 is insensitive to p50RhoGAP. The appar-
tent preference of RhoGAP for monomeric Rac1 was further
examined quantitatively by measuring the initial rate of
\( g\)-Pi release from the GTP-bound GTPases as a function of the
GTPase concentrations in the presence of a catalytic amount of
RhoGAP (Fig. 6B). Fitting of the data by a modified Michaelis-
Menten equation (23) yielded the kinetic parameters
\( K_m \) and
\( k_{\text{cat}} \) (Table I). The catalytic efficiencies
\( \frac{k_{\text{cat}}}{K_m} \) of p50RhoGAP
for Rac1−8 were determined to be ~8-fold higher than for Rac1
at 21.4 ± 2.7 min\(^{-1}\) \( \mu M\)\(^{-1}\) compared with 2.6 ± 0.4 min\(^{-1}\) \( \mu M\)\(^{-1}\),
respectively, and the difference in
\( K_m \) seems to be a major factor for the varied ability of RhoGAP to elicit the GAP activity
for Rac1. We suspect that the detected residue RhoGAP activity
for Rac1 under the assay conditions was due to a partial dis-
sociation of oligomer to monomer. Interestingly, although
poorly responsive to the RhoGAP stimulation, Rac1, under
similar low salt conditions, or the isolated Rac1 oligomer from
the gel filtration column remained fully reactive with a Rac-
specific GEF, TrioN (data not shown). These results suggest
that the Rac1 oligomer may rely primarily on the self-stimula-
tory GAP activity rather than the interaction with a RhoGAP to
return to the GDP-bound basal state and indicate that oligo-
meric Rac1-GDP can be subject to reactivation upon GEF
catalysis.

**Fig. 4.** The intrinsic GTPase activity of Rac1 correlates with
its oligomerization state in a salt concentration-dependent
manner. A, the distribution of the Rac1 oligomer and monomer at
various NaCl concentrations. Aside from the specified NaCl concentra-
tions, the gel filtration conditions were similar to those described in the
legend to Fig. 1A. The ratios of oligomer versus monomer were deter-
mined by measuring the areas of the corresponding elution peaks of the
respective gel filtration profiles. B, salt concentration dependence of the
intrinsic rates of GTP hydrolysis by Rac1. The GTPase reactions were
measured by the MESG/phosphorylase coupling reactions and by the
[\( g\)-\( ^32P\)]GTP filter binding assays. The apparent rate constants were
derived by the best fit of the time courses of Rac1-GTP hydrolysis into
a single exponential equation.

**Fig. 5.** Rac1 possesses a self-stimulatory GAP activity medi-
dated by the C-terminal domain. \( \gamma\)-P release by 5 \( \mu M\) Rac1-GTP were
determined at the 5-min time point in the absence or presence of GDP-
or GTP\( ^{\gamma\text{S}}\)-loaded Rac1 (5 \( \mu M\)), various Rac1 mutants (5 \( \mu M\), or
p50RhoGAP (50 nM) in buffer containing 50 mM HEPES (pH 7.6), 1 mM
DTT, 100 mM NaCl, and 2 mM MgCl\(_2\). Rac1* indicates the isoprenylated
form of Rac1.
observations of PAK1 activation using various Rac1 C-terminal mutants and Rac1/Rac2 chimeras were reported before (14), in light of our data on the oligomerization states of Rac1 and its mutants under the low salt conditions that were employed in the PAK1 kinase assays, we interpret these results as more likely an indication of a synergistic effect of Rac1 oligomerization on the effector activation rather than that the C-terminal region of Rac1 constitutes an additional PAK1 effector-binding site as proposed previously (14).

RhoGDI Induces Disassembly of the Rac1-GDP Oligomer—Aside from RhoGAP and GEF, RhoGDI represents another major class of regulators for Rac1 function. One of the major roles of RhoGDI is thought to be the regulation of the distribution of Rho GTPases between the membrane compartments and cytosol, where at the latter location each Rho family member is in complex with RhoGDI at a 1:1 stoichiometry (8, 9). It was therefore of interest to determine whether RhoGDI contributes to the regulation of the oligomerization state of Rac1. To this end, we took an Ni\textsuperscript{2+}-agarose affinity-based approach to test directly the effect of RhoGDI on the complex formation between immobilized His\textsubscript{6}-Rac1 purified from the Sf9 insect cell membranes and HA-tagged Rac1 isolated from the membrane fraction of COS-7 cells transiently transfected with the HA-Rac1 construct. Both of these Rac1 species were therefore in isoprenylated form and were capable of interacting with RhoGDI. In agreement with the above co-immunoprecipitation results (Fig. 1C), His\textsubscript{6}-Rac1 formed a stable complex with HA-Rac1 (Fig. 8, lane 2). The addition of increasing amounts of GST-RhoGDI to the incubation mixtures resulted in a dose-dependent inhibition of the complex formation between His\textsubscript{6}-Rac1 and HA-Rac1 (Fig. 8, lanes 3–5) such that at 10 \mu M GST-RhoGDI, the homophilic interaction between the two populations of Rac1-GDP became undetectable. In parallel, the sample containing 10 \mu M GST instead of GST-RhoGDI did not affect the binding interaction between the Rac1 species (Fig. 8, lane 2).

**Table 1**

Kinetic parameters of p50\textsuperscript{RhoGAP} that regulate Rac1 and Rac1–8

The p50\textsuperscript{RhoGAP}-catalyzed GTPase reactions of Rac1 and Rac1–8 as analyzed in Fig. 6 by nonlinear regression yielded the \( K_m \) and \( k_{cat} \) values listed. GAP reactions were carried out at 20 °C in 50 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM MgCl\textsubscript{2}, 0.2 mM MESG, 200 \mu M GTP, and 10 units of purine-nucleotide phosphorylase. The results are representative of three independent measurements.

|        | \( K_m \) (\mu M) | \( k_{cat} \) (min\textsuperscript{-1}) | \( k_{cat}/K_m \) (min\textsuperscript{-1} \mu M\textsuperscript{-1}) |
|--------|-------------------|----------------|--------------------------|
| Rac1   | 30.5 ± 3.8        | 52.3 ± 10.6    | 1.7 ± 0.4                |
| Rac1–8 | 4.48 ± 0.8        | 95.9 ± 12.0    | 21.4 ± 2.7               |

**Fig. 7.** The C-terminal domain of Rac1 is required for full activation of PAK1. Myc-PAK1 was expressed in COS-7 cells and immunoprecipitated from the cell lysates using immobilized anti-Myc antibody. The immunoprecipitates were incubated with similar amounts of wild-type or mutant Rac1 proteins (5 \mu M) preloaded with GTP\textsubscript{S} in kinase reaction buffer containing 50 mM HEPES (pH 5.0), 2 mM MgCl\textsubscript{2}, 2 mM MnCl\textsubscript{2}, 0.2 mM DTT, and 20 \mu M [\gamma\textsuperscript{32P}]ATP for 20 min. **Upper panel,** PAK1 autophosphorylation was visualized by autoradiography after SDS-polyacrylamide gel electrophoresis. **Lower panel,** the bar graph indicates the relative kinase activity of PAK1 stimulated by wild-type Rac1 and its mutants. The results shown are representative of three separate experiments.
Immobilized His6-Rac1-GDP (were subjected to anti-HA Western blot analysis after extensive m of increasing amounts of GST-RhoGDI (2, 4, and 10 µM (lanes 3–5, respectively)) or 10 µM GST (lane 6). The His6-Rac1-GDP coprecipitates were subjected to anti-HA Western blot analysis after extensive washes.

Thus, RhoGDI may be involved in the disassembly process of the Rac1 oligomer and actively participate in the oligomer-to-monomer transition as well as the membrane-to-cytosol translocation of Rac1.

**DISCUSSION**

In this study, we have characterized a unique biochemical property of the Rac1 GTPase, self-assembly into large oligomers. We found that Rac1 exists reversibly in a monomeric state and an oligomeric state, with a dissociation constant in the submicromolar range. Although the Rac1 responsiveness to RhoGAP is diminished in the oligomeric state, the Rac1 oligomer remains fully functional in the interaction with its GEF, TrioN. The oligomerization process brings on the self-stimulatory GAP activity, which is mostly responsible for Rac1 down-regulation, and appears to optimize the effector (PAK1)-activating potential. Interaction with RhoGDI results in the disassembly of the Rac1 oligomer and cycles Rac1 to the cytosol in the RhoGDI-Rac1 complex. These results suggest a novel mode of regulation for Rac1 function.

**Oligomerization of Rac1 and the Structural Determinants Involved**—Our gel filtration and complex formation results clearly show that Rac1 may exist in an oligomeric form under physiologically relevant buffer conditions. The immunoprecipitation experiments in cells coexpressing two distinct populations of Rac1 demonstrate that the oligomerization occurs in vivo (Fig. 1). The observation that the Rac1 monomer and oligomer are in a reversible equilibrium further suggests that the oligomeric form of Rac1 may bear physiological significance.

There is a growing body of evidence suggesting that certain GTP-binding proteins may undergo an oligomeric state in carrying out their cellular functions. For example, it has been recognized that many members of the large GTPase family including Mx, dynamin, and yeast Vps1p exist natively in large oligomers and aggregates (20). Among the members of the Ras small GTPase superfamily, ADP-ribosylation factor-1 has been found in functional dimeric or tetrameric forms (18, 20). Ras itself has recently been suggested to form a dimer at the plasma membrane that appears to be essential for Raf-1 activation (19). Our previous studies have demonstrated that the Rho GTPases Cdc42, RhoA, and Rac2 are capable of forming homodimers, and the dimerization may play a role in the negative regulation of the GTPases (16, 17). Interestingly, Rac2 homodimer formation was recently detected in the neutrophils of a patient with Kostmann syndrome, suggesting that the homophilic interaction of the GTPase may be involved in the functional regulation (7).

Most members of the Rho protein family contain a stretch of two to six polylysine and/or arginine residues immediately N-terminal of the isoprenylation motif, the CAAX box. This polybasic domain has been implicated in the dimer formation of Cdc42 and RhoA (16, 17). Here we present evidence that the corresponding region of Rac1 consisting of six consecutive basic residues is essential for its oligomerization. This was first supported by a peptide competition assay in which the polypeptide corresponding to the C-terminal polybasic domain of Rac1 was able to effective disassemble the Rac1 oligomer to the monomer (Fig. 2B). Mutational analysis of the polybasic residues revealed that removal of or substitutions in the polybasic domain led to the exclusive monomeric form of Rac1 or a gradual shift from the oligomer to monomer, providing the secondary support for the involvement of this region in the oligomerization. Combined with the previous observations, it appears that the Rho proteins containing multiple basic residues (Lys or Arg) in the C-terminal domain, including Cdc42, RhoA, Rac2, RhoC, and Rac1, may form either a dimer or higher oligomer and that other Rho proteins lacking the consecutive basic residues in this region, including TC10 and RhoB, are exclusively monomeric. The homophilic binding interaction among these proteins seems to correlate with the number of charged residues in the C-terminal domain. In the absence of available information on the tertiary structure of this domain, we speculate that the positively charged nature of the polybasic domain could provide the contact sites that stabilize the dimer or oligomer conformation by interacting with a negatively charged region of the neighboring molecules. Given that the homophilic interactions are guanine nucleotide state-specific, it is possible that the binding sites of the polybasic motif of one GTPase would include the switch regions of the adjacent molecules, joining together two or more molecules in a linear, asymmetric configuration. Such a model is consistent with the self-stimulatory GAP activity associated with the oligomerization process of Rac1 and is in line with our previously proposed model of Cdc42 dimer configuration in which Arg<sup>106</sup> of the C-terminal domain functions effectively as an “arginine finger” to stabilize the transition state of the GTP hydrolytic core of the immediately adjacent molecule (17). Such an oligomer configuration would be distinct from the proposed oligomer structure of the high molecular mass G-protein Mx, which depends on the homophilic interaction through several regions of the molecule that include a conserved self-assembly motif in the amino-terminal moiety and two amphipathic helices at the C-terminal end (20, 21, 31) and would be different from the dimer configuration of Ras, in which case a lipid moiety was expected to be involved in the dimerization (19). Further studies by mutagenesis and by the structural biology approach are needed to resolve the self-associated state of Rac1 and other Rho GTPases.

**Self-stimulatory GAP Activity of Rac1-GTP Elicited by Oligomerization**—Although sharing a high degree of sequence homology in the GTP-binding core and switch regions, Rho family proteins behave differently in their ability to hydrolyze GTP. Rac1 appears to display the highest intrinsic GTPase activity among the Rho GTPases examined, including RhoA, Rac2, and Cdc42; and its GTP-hydrolyzing potential is dependent upon its membrane localization. For example, it has been proposed that Rac1 has to interact with GEF molecules in order to be hydrolyzed. However, in the monomeric state, Rac1 behaves like Ras and RhoA, with a slow intrinsic GTPase reaction rate at 0.02 min<sup>−1</sup>, whereas the isolated Rac1 oligomer shows an 8-fold higher basal activity than the monomer. The C-terminal truncation or substitution mutants of Rac1, known to form the monomer only (Fig. 2C), displayed a turnover number for GTP hydrolysis that is indistinguishable from that of the Rac1 monomer. These results indicate that the oligomeric state of Rac1 correlates with the GTPase activity and suggest that the enhanced GTP-hydrolyzing ability may be attributed to the
self-association ability of Rac1. Indeed, direct demonstration of GTPase-activating activity was provided by the Rac1-GTPγS-elicited GTPase-activating potential, which resulted in a significant stimulation of γ-Pi, release from Rac1-GTP, similar to the effect of RhoGAP, indicating that the active form of Rac1 presents a specific GAP activity for its own form. The self-stimulatory GAP activity is apparently dependent on the oligomerization ability of Rac1 since the mutant forms of Rac1 that are no longer capable of oligomerization or that have a weaker tendency to oligomerize suffered complete or partial loss of the self-stimulatory GAP activity. Furthermore, the isoprenylation modification of the CAAX sequences does not interfere with either the oligomer formation or the GTPase-activating activity of Rac1 because a similar extent of stimulation of Rac1 GTPase activity was observed when the post-translationally modified form of Rac1 was examined (Fig. 5). Thus, similar to Cdc42 and Rac2, Rac1 may utilize the oligomerization-associated self-stimulatory GAP activity for its negative regulation.

A conserved, surface-exposed arginine residue in RhoGAPs, termed the arginine finger, has been implicated in the GAP-catalyzed GTPase reaction of Rho GTPases (32, 33). The arginine residue from GAP protein appears to contribute to the stabilization of the transition state of the GTPase reaction and therefore is directly involved in catalyzing the cleavage of γ-Pi from bound GTP. We have previously observed that an arginine residue in the polybasic domain of Cdc42, Arg₁₈₆, functions as a built-in arginine finger to elicit the self-stimulatory GAP activity. This arginine residue is also present at the corresponding position of Rac1 protein. We expect that an arginine residue of the polybasic domain, either Arg₁₈₄ or Arg₁₈₆, may undertake the task, similar to Arg₁₈₆ in Cdc42, to participate in the GTPase reaction of an adjacent Rac1 molecule and to stimulate its GTPase activity. The fact that the Rac1-GTP oligomer is insensitive to RhoGAP stimulation is also consistent with the possibility that the critical arginine residue of the attacking Rac1-GTP would compete with the arginine finger provided by RhoGAP for access to the Rac1-GTP substrate and further supports that the Rac1-GTP oligomer employs mostly the self-stimulatory GAP activity, rather than depending on a RhoGAP, to arrive at the basal, GDP-bound state efficiently. Interestingly, such a self-stimulatory GTPase-activating activity has been a common feature found in the large GTPase (67 kDa) family including Mx, dynamin, and yeast Vps1p, the endogenous GTPase activities of which were significantly enhanced upon oligomerization (34, 35).

Oligomerization of Rac1 Contributes to the Effector PAK1 Activation—The PAK1 serine/threonine kinase represents one of the best characterized Rac1 effectors (10, 36). Rac1 binding to the p21-binding domain of Pak1 results in the release of the auto-inhibition of the kinase domain, leading to enhanced autophosphorylation and subsequent activation of Pak1. Previous structure-function studies of Rac1 have mapped the regions containing residues 26–45 and 143–175 as two primary Pak1-interactive sites (37). Peptide competition studies have also implicated the C-terminal domain of Rac1 as a potential site for NADPH oxidase activation (38, 39). A recent structure complex between Cdc42 and the polybasic domain of Pak1 depicts multiple contact sites involving the switch I, switch II, α₁, and α₅ regions of Cdc42, but a direct involvement of the C-terminal domain could not be observed since this region of Cdc42 was deleted in the Cdc42-Pak1 complex (40). Using a Rac1 mutation and the Rac1/Rac2 chimera approach, Knaus et al. (14) found that the C-terminal polybasic domain of Rac1 is also important for Pak1 activation. The difference at the C termini of Rac1 and Rac2, in particular, could account for the up to 5-fold variation in Pak1-activating ability (14). Here we have made a similar observation that the polybasic domain is required for the full potential of Rac1 to activate Pak1. Optimal activation of Pak1 was observed with the full-length Rac1 stimulation, whereas the mutations or truncation of the C-terminal polybasic residues led to a decreased activity of Rac1 in Pak1 activation (Fig. 7). Under the Pak1 kinase assay conditions (low salt), we expect that Rac1 mostly exists in the oligomeric form. Therefore, we interpret the effect of various Rac1 C-terminal mutations on Pak1 activity to be in good correlation with their ability to form an oligomer, and the oligomeric state of Rac1 is optimal for Pak1 activation. The recently available Pak1 tertiary structure reveals that a homodimeric conformation of the Rac1/Cdc42-interactive binding domain is involved in maintaining Pak1 in the auto-inhibited state (41). Our results suggest that the oligomeric form of Rac1 may have an advantage in breaking open the Pak1 dimer to relieve the auto-inhibition and raise the possibility that the self-assembled Rac1-GTP oligomer may be an addition to the Rac1-GTP monomer in activation of a selective subset of Rac1 downstream effectors.

Recent studies by del Pozo et al. (42) showed that expression of a constitutively active mutant of Rac1 that lacks a membrane-targeting sequence fails to activate Pak1 in adherent cells, suggesting that membrane co-localization of Rac1 and Pak1 is essential for Pak1 activation. Upon serum stimulation, Rac1 was found to be enriched in membrane microdomains made of rafts and caveolae (43). This process could lead to an increase in the local concentration of Rac1 (44). Accumulation of active Rac1 on the membrane would then initiate a chain reaction among Rac1 molecules that results in the formation of oligomers, which in turn results in an enhanced binding of Rac1 to the plasma membrane. Our biochemical results described here support the possibility that self-assembly of Rac1 into an oligomer is an important event in the plasma membrane for the increased coupling of activated Rac1 to Pak1. Alternatively, the oligomerization of Rac1 may provide multiple effector interaction sites that could function cooperatively in effector activation. It remains a challenge to demonstrate that Rac1 oligomerization occurs at a specific plasma membrane site and that the Rac1 oligomer plays a role in the effector recruitment and/or activation in vivo. The recently described in vivo fluorescence energy transfer method (45) that allows monitoring of small G-protein interactions in live cells may prove useful to address such issues.

RhoGDI Drives the Oligomer-to-Monomer Transition of Rac1—The function of RhoGDI is 2-fold: countering the GEF activity to inhibit GDP dissociation from the GTPases and solubilizing the GDP-bound Rho proteins from the membrane compartment to cycle to the cytosol (8). The cytoplasmic pool of Rac1 is found in complex with RhoGDI, indicating that RhoGDI is a critical regulator of the subcellular localization of Rac1. The Rac1 oligomer remained reactive with RhoGDI, but the presence of excess RhoGDI led to the inhibition of oligomerization and the disassembly of the Rac1 oligomer (Fig. 8). Such a mode of RhoGDI interaction with the Rac1 oligomer can be rationalized by the recently available structure of RhoGDI in complex with Cdc42, in which the hydrophobic pocket of RhoGDI engulfs the isoprenoid moiety of Cdc42, and the lid of the lipid-binding pocket provides extended contacts with the C-terminal polybasic residues of Cdc42 (9). Applying the RhoGDI-Cdc42 interaction to the Rac1 situation, one would predict that the high affinity interaction of RhoGDI with Rac1 initiated through hydrophobic lipid binding could efficiently compete with the hydrophilic binding of a neighboring Rac1 polybasic domain, which is essential for maintaining the ol-
gomer configuration. The resulting product of the RhoGDI intervention would be the solubilized Rac1 from a membrane environment, and meanwhile, the oligomer configuration would be broken open by the formation of a RhoGDI-Rac1 complex at a 1:1 molar ratio. An additional function of RhoGDI therefore might be to allow Rac1 to cycle between the oligomeric and monomeric states.

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