interaction between Cdc42Hs and RhoGDI is mediated through the Rho insert region

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Members of the Rho subfamily of GTP-binding proteins contain a region of amino acid sequence (residues 122–134) that is absent from other Ras-like proteins and is termed the Rho insert region. To address the functional role of this domain, we have constructed a Cdc42Hs/Ras chimera in which loop 8 from Ha-Ras was substituted for the region in Cdc42Hs that contains the 13-amino acid insert region. Our data indicate that the insert region of Cdc42Hs is not essential for its interactions with various target effector molecules or for interactions with the guanine nucleotide exchange factor, Db, or the Cdc42 GTPase-activating protein (GAP). However, the regulation of GDP dissociation and GTP hydrolysis on Cdc42Hs by the Rho GDP-dissociation inhibitor (GDI) is extremely sensitive to changes in the insert region, such that a Cdc42Hs/Na-Ras chimera that lacks this insert is no longer susceptible to a GDI-induced inhibition of GDP dissociation and GTP hydrolysis. The insensitivity to GDI activity is not due to the inability of the GDI molecule to bind to the Cdc42Hs/Na-Ras chimera, and in fact, the GDI is fully capable of stimulating the release of this chimera from membranes.

A variety of biological signaling events are mediated through a number of GTP-binding proteins, which serve as molecular switches that transduce incoming signals to downstream targets. In particular, the Rho subfamily of GTP-binding proteins (which includes Cdc42Hs, Rac, and Rho) has received a great deal of attention in the past few years. These proteins were shown to be implicated in the signal transduction pathways of various external stimuli (e.g. platelet-derived growth factor, phorbol esters, bradykinin (1, 2)) and to be responsible for triggering a variety of cellular responses that influence cytoskeletal architecture, cell cycle progression, cell polarization, and cellular motility (e.g. see Refs. 3–5). At present, two distinct types of signaling cascades, which are initiated by the activation of Rho subfamily GTP-binding proteins, have been delineated. One involves the stimulation of soluble serine/threonine kinases, designated the p21-activated kinases (PAKs) (6, 7) and culminates in the activation of the nuclear mitogen-activated protein kinases, the c-Jun kinase (JNK1) and p38 (7, 8), in a manner reminiscent of the Ras-mediated Raf/Mek/Erk protein kinase cascade (9). A second and seemingly independent class of signaling events translates the activation of Rho subfamily GTP-binding proteins into cytoskeletal rearrangements (10). These events may involve both direct interactions between the GTP-binding proteins and cytoskeletal elements (e.g. the interaction between Rac1 and tubulin (11)) and interactions between the GTP-binding proteins and target molecules that serve to mediate effects on actin and myosin (e.g. WASP (12), IQGAP (13, 14), and Rho kinase (15, 16)).

Activation and deactivation processes of signaling through Rho-like GTP-binding proteins are similar to those observed for other small GTP-binding proteins as well as for heterotrimeric G proteins. Thus, activation is achieved via GTP → GDP exchange (mediated by external factors, termed GEFs), and deactivation is mediated through GTP hydrolysis and further accelerated by interactions with regulators termed GAPs (17). Unique to the Rho (and Rab) family members is the existence of a third type of regulator, called a guanine nucleotide dissociation inhibitor (GDI). These proteins inhibit GDP dissociation as well as GTP hydrolysis (18, 19). Additionally, they were shown to facilitate the release of the GTP-binding proteins from membranes (18), such that it is felt that virtually all of the cytosolic Rho subfamily GTP-binding proteins are complexed to GDIs (20).

While considerable advances have recently been made in the identification of the different biological activities mediated by Rho-like GTP-binding proteins, the molecular mechanisms underlying these responses are still largely unknown. Rho family members share significant homology with other GTP-binding proteins both in amino acid sequence and in structural motifs (21, 22). Thus, all GTP-binding proteins seem to share a common structural unit (exemplified by the structure of Ras), which is responsible for nucleotide binding and hydrolysis, accompanied by regions of specific effector/regulator interactions. For example, the switch 1 and 2 regions (residues 32–45 and 60–70, respectively), which show dramatic changes upon the transitions between the GTP- and GDP-bound states, are highly conserved among all of the small GTP-binding proteins, thus pointing to common mechanisms for target/effector and regulatory protein interactions that are supplemented by residues that ensure specificity. However, one clear deviation from the common folding motifs of small GTP-binding proteins is a 13-amino acid insertion, unique to the Rho subfamily proteins. In this report, we study the functional role of this insert region in Cdc42Hs and show that it is essential for the regulation of guanine nucleotide exchange by the RhoGDI.

EXPERIMENTAL PROCEDURES

Chimeras—The ΔL8-Cdc42Hs mutant contains a short sequence of loop 8 of Ha-Ras (123AARTVES127) in place of the insert region of Cdc42Hs (residues 120–139); its construction was described earlier (14). The activated (GTPase-defective) version of this mutant was produced by an otherwise identical procedure, except for the template DNA, which was Cdc42Hs (Q61L) in pET15b.
Cdc42Hs-GDI Interactions

CAAX-Ras is a replacement mutation of c-Ha-Ras in which the COOH-terminal 19 residues (173-LNPDPESPGCMCKCVLS202) of Ras are replaced by the eight COOH-terminal residues of Cdc42Hs (180-KSRRCVLL184). The Cdc42Hs-Ras construct was prepared by the polymerase chain reaction using a pET15b upstream primer (sense, 5'-CTA TAG GGA AAT TGT GAG CGG-3'); c-Ha-Ras in pET15b as a template, and a downstream primer encoding the eight COOH-terminal amino acids of Cdc42Hs (antisense, 5'-GTC AAG CTT GCC AAG AAC AAA CAG AAG CCT ATC ACT CCA-3').

FC-Ras is a replacement mutation in c-Ha-Ras in which the hexahistidine tag is replaced with that of Cdc42Hs (identical to CAA-Ras described above) and loop 8 of Ras (residues 121–127) is replaced with the Rho insert region (residues 120–139 from Cdc42Hs). This construct was generated using an overlapping polymerase chain reaction, with CAA-Ras (see above) as a template, external pET15b vector primers (sense 5'-CTA TAG GGA AAT TGT GAG CGG-3'; antisense, 5'-GCC ACC TGA CTT CTA AGA AAC CTC-3'), and two internal primers (sense, 5'-GAG AAG CCT GCC AAG AAC AAA CAG AAG CCT ATC ACT CCA CGG CAG GCC CAG GAC CTT GCT-3'; antisense 5'-GTT GCT GGG CAG AAG CTT CCT ATC ACT ATG GCC ACA CCT AAT AGT AGA GGG GTC ATC TCT CAG GTC ACA-3').

Expression and Purification of Recombinant Proteins—Histidine-tagged recombinant proteins and their mutants were produced in Escherichia coli and purified using Ni2+ affinity chromatography followed by thrombin proteolysis of the tag and further purification on Q Sepharose (27). Cloning of wild type and ΔL8 Cdc42Hs into the insect cell baculovirus vector was achieved using the restriction sites XhoI and BamHI. Cloning of pET15b, purification of the ~800-kilobase pair fragment (which includes the hexahistidine tag), and ligation into the insect cell transfer vector pVL1392 digested with the same enzymes. Cloning of CaaX-Ras and FC-Ras into pVL1392 was achieved similarly using the enzymes BglII and EcoRI. Recombinant virus was produced and used for infection of Sf21 insect cells according to the manufacturer's instructions (PharMingen, San Diego, CA). Frozen insect cells pellets were lysed by Dounce homogenization in 20 mM Tris–Cl, pH 8.0, 6 mM EDTA, 1 mM dithiothreitol, 10 μM GTPγS, 1 mM NaN3, 0.5 mM phenylmethanesulfon fluoride, and 25 μg/ml (each) of leupeptin and aprotinin, followed by low speed centrifugation (10 min, 1,500 rpm, 4 °C, IEC CRU-5000 centrifuge) to remove cell debris. The membrane fraction was then pelleted (12,000 rpm, Beckman JA20 rotor, 20 min), washed twice in Tris (pH 8.0), 0.1% CHAPS, 40 mM imidazole, 0.5 M NaCl, and 1 μg/ml each, of leupeptin and aprotinin, and solubilized in the same buffer supplemented with 0.4% (w/v) CHAPS. The solubilized membranes were then cleared by centrifugation and loaded onto a Ni2+-diaminocaproic acid-agarose column (1 ml) equilibrated with 20 mM Tris, pH 8.0, 0.5 mM NaCl, 1 mM EDTA, 1 μg/ml aprotinin and leupeptin, 0.1% CHAPS, and 5 mM imidazole. After extensive washing with 20 mM Tris (pH 8.0), 0.1% CHAPS, 40 mM imidazole, 0.5 μM NaCl, and 1 μg/ml aprotinin and leupeptin, the protein was eluted using the same buffer supplemented with 0.3 M NaCl and 200 mM imidazole.

GST-RhoGDI was expressed in E. coli and purified using glutathione-agarose as described earlier (18). Recombinant Cdc42-GAP and Dbl proteins were prepared as outlined by Leonard et al. (23).

Additional Biochemical Studies—Mammalian cell culture, transient transfections, immunoprecipitations, and PKA activity assays were described earlier (7). Dissociation and hydrolysis assays with radioactive nucleotides were done as described earlier (18). Antibodies directed against IQGAP and also the recombinant (insect cell-produced) GRD domain of IQGAP were generous gifts of Dr. M. Hart (Onyx Pharmaceuticals, Richmond, CA).

RESULTS AND DISCUSSION

Amino acid sequence alignment of the various Rho subtype proteins with Ha-Ras and other members of the Ras subfamily is illustrated in the diagram (18). The unique COOH-terminal 19 residues from Cdc42Hs are unique to Cdc42Hs, Rac, and other members of the subfamily (24, 25). In the recently described x-ray crystallographic structure of Rac1 bound to GMP-PNP, this insert region was shown to form a separate (mostly helical) structural element attached to a Ras-like GTPase core (26). Since the biological activities and the molecular interactions of members of the Rho subfamily are significantly different from those of Ras and since the insert is unique to all Rho proteins, we have investigated the role of this region in Cdc42Hs by constructing a chimeric mutant (designated here as ΔL8) in which the “Rho insert” region of Cdc42Hs (residues 120–139) is replaced by a short sequence from loop 8 in Ha-Ras (residues 121–127). The amino acid sequence alignment of Ha-Ras and Cdc42Hs in this region was derived using the program DNAStar. A complete sequence alignment of the peptides can be found in Setucliff et al. (25). The amino acid sequence of the ΔL8 protein in the insert region. Normal type denotes amino acids that originate from the Cdc42Hs sequence, while outlined residues originate from the Ha-Ras protein.

Interactions of ΔL8 with Targets of Cdc42Hs—It has become increasingly clear in the past few years that the activation of the subfamily GTP-binding proteins leads to the stimulation of multiple target/effector molecules, resulting in divergent phenotypic responses. One established signaling cascade involves the direct interaction of GTP-bound Cdc42Hs with Rac1 with members of the PAK family of soluble serine/threonine kinases (6, 7). To test the role of the Rho insert in PAK activation, we have directly assayed the ability of the recombinant ΔL8 mutant of Cdc42Hs to activate the auto- and exophosphorylating activity of mPAK-3 in vitro. In these studies, mPAK-3 was first transiently expressed in COS-7 cells and then immunoprecipitated using a hemagglutinin tag (7) and incubated with either the wild-type Cdc42Hs or the ΔL8 protein (complexed with either GTPγS or GDP) in the presence of MgCl2, [γ-32P]ATP, and the phosphorylation substrate myelin basic protein. Following SDS-polyacrylamide gel electrophoresis and visualization of the phosphorylated products by autoradiography, it was evident that the activation of mPAK-3 by either wild-type Cdc42Hs or ΔL8 was essentially identical, thus ruling out the participation of the insert region of Cdc42Hs in the binding or activation of this target (Fig. 2).

It is now apparent that mPAK-3 is only one of a large number of proteins that represent putative targets for Cdc42Hs and Rac and possess a homologous domain that mediates their interaction with the GTP-binding proteins, designated the PBD (p21-domain binding; Ref. 6) or CRIB motif (Cdc42- and Rac-interacting binding motif; Ref. 28). Indeed, we have directly compared the binding of the wild-type GST-Cdc42Hs fusion protein and the GST-ΔL8 fusion protein with the CRIB domain-containing targets ACK (for activated-Cdc42 kinase; Ref. 29) and WASP (for Wiscott-Aldrich syndrome protein; Ref. 30) as assessed by their co-precipitation following the addition of glutathione-agarose. The results of these studies verified that the absence of the insert region in the ΔL8 mutant does not affect the association of Cdc42Hs with either of these putative target molecules (data not shown). Two recently identified targets for Cdc42Hs represent the two isoforms of IQGAP, a ubiquitous 190-kDa protein that is encoded by a single gene that contains highly conserved domains referred to as G1 and G2 (21). IQGAP1, the larger isoform of IQGAP, contains a G1 domain in the C-terminus, a novel C-terminal domain, and two copies of the Dbl homology (DH) domain. IQGAP2 contains a G2 domain in the N-terminal region of the protein and a single DH domain. These two proteins were selected for this study because IQGAP1 and IQGAP2 have been shown to interact with Cdc42Hs and Rac, respectively, and are thought to be involved in the assembly of the actin cytoskeleton (21). IQGAP1 and IQGAP2 also have been reported to be involved in cell motility, lamellipodium formation, and mitogenesis (21).

A. Cdc42: 115TQIDLDDPSTIEKLANKQKDITPETAEKL

Ras: 116MKCDAA-----------RVVERSQAQDLA

ΔL8: 115TQIDLDDPSTIEKLAN

Fig. 1. The Rho insert region of Cdc42Hs, A, amino acid sequences of Cdc42Hs and Ha-Ras were aligned using a Clustal algorithm in the program DNAStar. A complete sequence alignment of the proteins can be found in Setucliff et al. (25). The amino acid sequence of the ΔL8 mutant from the insert region. Normal type denotes amino acids that originate from the Cdc42Hs sequence, while outlined residues originate from the Ha-Ras protein.
Cdc42Hs-GDI Interactions

Hydrolysis rates were measured by monitoring the time-dependent increase in tryptophan fluorescence following EDTA loading with GTP as described by Leonard et al. (27). Reactions were initiated by the addition of 5 mM MgCl₂ followed by the addition of catalytic amounts of the Cdc42GAP. Nucleotide exchange was measured by loading the protein with [3H]GDP and measuring protein-bound radioactivity using the nitrocellulose filtration assay.

### Table I

|                      | GTP hydrolysis (min⁻¹) | GDP exchange (s⁻¹) |
|----------------------|------------------------|--------------------|
| Wild type            | 1.45                   | 3.6 × 10⁻⁶          |
| ΔL8                  | 1.38                   | 1.07 × 10⁻⁶         |

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RhoGDI does not exhibit any of these effects on E. coli-expressed GTP-binding proteins, it was assumed that the geranyl-geranylation of Cdc42Hs (or Rac or RhoA) at its carboxy-terminal CAAX motif was required for interactions with the GDI. However, at present little is known regarding the specific mode of binding that occurs between the GDI and Cdc42 (or related GTP-binding proteins) and whether other regions, aside from the carboxyl-terminal domains of the GTP-binding proteins, are necessary for these interactions. In these studies, we have used insect cell baculovirus expression systems to obtain modified, membrane-bound wild-type Cdc42Hs and the ΔL8 protein and compared their abilities to functionally couple to the RhoGDI.

We first tested the ability of the RhoGDI to release the ΔL8 and wild-type Cdc42Hs proteins from insect cell membrane preparations. In this assay, isolated insect cell membranes, containing post-translationally modified Cdc42Hs, were incubated with the GST-RhoGDI fusion protein. Membrane-bound (pellet) and soluble (supernatant) fractions were then separated using high speed centrifugation, and the respective fractions were resolved on SDS-polyacrylamide gel electrophoresis and Western blotting. As shown in Fig. 3, in the absence of RhoGDI, all of the Cdc42Hs protein remains associated with the membrane pellet, whereas in the presence of GDI, a significant portion of Cdc42Hs is extracted to the soluble supernant. It is clear from Fig. 3 that the ΔL8 mutant behaves in an identical manner to wild-type Cdc42Hs in this assay. Thus, removal of the surface loop formed by residues 120–134 did not impair the ability of the GDI to mediate the release of Cdc42Hs from membrane fractions. These results then indicate that the GDI regulatory protein can both bind to the ΔL8 protein and induce the necessary conformational changes within the GTP-binding protein to cause its dissociation from membranes.

We then tested whether the RhoGDI was able to influence guanine nucleotide binding to the ΔL8 protein. For this purpose, purified insect cell-expressed wild-type Cdc42Hs and the ΔL8 protein were first complexed with [3H]labeled GDP and then diluted into a buffer containing 7 mM EDTA and excess unlabeled GDP (18, 19). Aliquots were then taken at different time points, and the amount of protein-bound radioactive nucleotide was measured by filtration and scintillation counting. As shown in Fig. 4A, in the absence of GDI, the bound (radioactive) nucleotide was rapidly replaced by unlabeled GDP (t1/2 = 1.6 min), whereas preincubation of Cdc42Hs with RhoGDI greatly inhibited GDP dissociation from the wild-type protein (t1/2 = 12.2 min). However, surprisingly, the GDI was not able to inhibit GDP dissociation from the ΔL8 protein. The [3H]GDP bound to ΔL8 was displaced rapidly (t1/2 < 1 min), even when the GTP-binding protein was incubated with a significant (20-fold) excess of RhoGDI. We have consistently found that EDTA-induced nucleotide dissociation is more rapid from the ΔL8 mutant protein than from wild-type Cdc42Hs (by ~1.5-fold; see Fig. 4). Presumably, in the absence of Mg2+ ions, the insert...
region contributes to the binding of GDP, and therefore, nucleotide dissociation is more rapid upon deletion of this domain.

During the initial characterization of RhoGDI, it was shown that the membrane-releasing activity was not influenced by the nature of the nucleotide bound to Cdc42Hs. On the other hand, inhibition of nucleotide dissociation was preferentially observed with GDP-bound Cdc42Hs. However, complex formation between Cdc42Hs-GTP and RhoGDI markedly inhibited GTP hydrolysis by Cdc42Hs (both intrinsic and GAP-stimulated activity). We have tested whether the presence of RhoGDI affects GTP hydrolysis by the ΔL8 protein. Purified, insect cell-expressed wild-type and ΔL8 proteins were complexed to [γ-32P]GTP, and the GTPase reaction was initiated by the addition of MgCl₂ in the presence or absence of RhoGDI. As seen in Fig. 5A, the GTPase activity of the wild-type Cdc42Hs protein was markedly inhibited by RhoGDI. With the ΔL8 protein (Fig. 5B), however, no inhibition of the GTP hydrolytic reaction occurred, even when a large excess of RhoGDI was added.

Given the marked effect of the ΔL8 mutation on the functional coupling of Cdc42Hs to RhoGDI, we were interested in assessing the contribution of the insert region to the overall binding interaction between the two proteins. Toward this end, we have estimated the relative efficiency of the wild-type and ΔL8 Cdc42Hs proteins in competing the functional assay shown in Fig. 4. The results of this experiment are shown in Fig. 6. Both wild-type Cdc42Hs and the ΔL8 protein show the same dose-dependent competition for RhoGDI (i.e. the concentration for 50% competition was ~2 μM). Thus, we conclude that the Cdc42Hs(ΔL8) exhibits an essentially identical affinity toward the RhoGDI as the wild-type protein.

Conclusions—Taken together, our results clearly show that the Rho insert region does not serve as an essential motif for binding a variety of Cdc42Hs targets, nor for the functional interactions of either the GEF (Dbl) or the Cdc42-GAP. However, it does play a critical role in the ability of the RhoGDI to influence nucleotide exchange and inhibit GDP dissociation. Thus, to our knowledge, this represents the first demonstration of a regulatory role for the Rho insert region. In the related Ras protein, substitution and deletion mutations within the insert region (residues 124–135) were shown to result in the loss of productive interactions between the GTP-binding protein and the Rac-specific target, the NADPH oxidase (24). It was not reported whether these mutants of Rac were also defective in their interactions with GDI, but given the high degree of sequence homology between the two proteins in the insert region (70% identity), it is likely that GDI-induced inhibition of nucleotide exchange will occur in Rac (and RhoA) as well.

Apparently, the Rho insert region is not involved in binding the GDI, since we can clearly show that the GDI will catalyze the release of the ΔL8 protein from membranes (e.g. Fig. 3). Furthermore, the ΔL8 protein appears equivalent to wild-type Cdc42Hs in its ability to compete for RhoGDI (Fig. 6). The requirement for post-translational modification for the interactions of Cdc42Hs (or Rac, RhoA) with the RhoGDI strongly suggests that the carboxyl-terminal region of these GTP-binding proteins serves as a GDI-binding site. The question arises whether the insert region and carboxyl-terminal are sufficient structural requirements for interaction with RhoGDI. Toward this end, we have constructed protein chimeras between Cdc42Hs and Ras (which does not interact with RhoGDI). We found that the addition of the insert region (residues 122–134 from Cdc42Hs) to the analogous location in loop 8 of Ras (i.e. replacing residues 120–126), together with the substitution of the carboxyl terminus of Ras with that of Cdc42Hs (8 amino acids).
acids, including the CVLL modification site), did not render GDI sensitivity to Ras (data not shown). Thus, additional residues on Cdc42Hs (or Rac, RhoA) play a role in the interface between the GTP-binding protein and the GDI.

Based on existing data and the results we report here, it is possible to construct a working model for the interactions between Cdc42Hs and RhoGDI as follows. RhoGDI binds to the COOH-terminal region of Cdc42Hs, and this interaction is sufficient for stimulating the release of Cdc42Hs from membranes. As an outcome of GDI-binding, a conformational change occurs at the Rho insert region, which is required for inhibiting GDP dissociation and GTP hydrolysis, possibly by inducing a strain on that loop, such that it will cover the nucleotide-binding pocket. The absence of the insert region in Ras may explain why this GTP-binding protein, while sensitive to GEFs and GAPs, has not been shown to be sensitive to a GDI-like regulator. It will be interesting to see how this mode of GDI-mediated regulation for Rho-like GTP-binding proteins will compare with the mechanisms underlying the regulation of Rab subfamily proteins by their GDI regulators.

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