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A single residue can modify target-binding affinity and activity of the functional domain of the Rho-subfamily GDP dissociation inhibitors

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ABSTRACT **The GDP dissociation inhibitors (GDIs) represent an important class of regulatory proteins for the Rho- and Rab-subtype GTP-binding proteins. As a first step toward identifying the key functional domain(s) on the Rho-subtype GDI, truncations of the amino and carboxyl termini were performed. Deletion of the final four amino acids from the carboxyl terminus of Rho GDI or the removal of 25 amino acids from the amino terminus had no significant effect on the ability of the GDI to inhibit GDP dissociation from the Rho-like protein Cdc42Hs or on its ability to release Cdc42Hs from membrane bilayers. However, the deletion of 8 amino acids from the carboxyl terminus of Rho GDI eliminated both activities. To further test the importance of the carboxyl-terminal domain of the Rho GDI molecule, chimeras were constructed between this GDI and a related protein designated LD4, which is 67% identical to Rho GDI but is less potent by a factor of 10–20 than Rho GDI in functional assays with the Cdc42Hs protein. Two sets of chimeras were constructed that together indicated that as few as 6 amino acids near the carboxyl terminus of Rho GDI could impart full GDP dissociation inhibition and membrane dissociation activities on the LD4 molecule. Further analysis of this region by site-directed mutagenesis showed that a single change at residue 174 of LD4 to the corresponding residue of Rho GDI (i.e., Asp-174 → Ile) could impart nearly full (70%) Rho GDI activity on the LD4 molecule.**

The regulation of the GTP-binding/GTPase cycles of Ras and related low molecular mass GTP-binding proteins represents a key event in a number of biological activities. GDP-releasing factors (GRFs) like the SOS protein (1), CDC25 (2), Ras GRF (3), and the *dbl* oncogene product (4) increase the rate of dissociation of GDP from Ras-like proteins and stimulate the formation of the GTP-bound state, while GTPase-activating-proteins (GAPs), like the Ras GAP (5), the neurofibromin protein (NF1) (6), and various Rho GAPs (e.g., bcr, chimerin) (6, 7), accelerate the hydrolysis of bound GTP to GDP. A third class of regulatory proteins are the GDP dissociation inhibitors (GDIs), which were named for their ability to prevent the release of GDP from low molecular mass GTP-binding proteins. Thus far, GDIs have been identified for members of the Rho- and Rab-subtype GTP-binding proteins (8, 9). The Rho-type GDI, $M_r \approx 28$ kDa, has been shown to inhibit GDP dissociation from the RhoA, Rac1, Rac2, and Cdc42Hs proteins (8, 10). This GDI also inhibits the GTPase activities of these Rho-type GTP-binding proteins (11–13) and stimulates their release from biological membranes (10). Recently, a protein that is 67% identical to Rho GDI and highly expressed in hematopoietic cells was identified (14). The gene encoding this protein was originally cloned by subtractive hybridization from the pluripotent leukemic cell line K562, and therefore has been named *LD4*, for leukemic D4. The LD4 protein is less potent by a factor of 10–20 than Rho GDI in its ability to inhibit GDP dissociation or to release Cdc42Hs from membranes (15), and its true physiological target remains to be identified.

In contrast with the extensive structure–function information that has been obtained for the GRFs and GAPs, virtually nothing is known about the regions of the GDI molecules that are involved in their interactions with GTP-binding proteins. As a first step toward mapping key functional domains on Rho GDI, we generated recombinant GDI molecules that were truncated at the amino and carboxyl termini and assayed their abilities to inhibit GDP dissociation from Cdc42Hs and to stimulate the release of Cdc42Hs from membranes. To complement the results obtained with the truncated GDI molecules, we then prepared Rho GDI/LD4 chimeras and have identified a region on Rho GDI that confers upon the LD4 protein higher capability for functional coupling to Cdc42Hs. This information was used for the rational design of several point mutants, which demonstrate that a single residue is responsible for much of the difference in the efficacies of these two molecules.

MATERIALS AND METHODS

Construction of the Rho GDI Deletion Mutants. The carboxyl-terminal deletions were constructed as follows. PCR was performed according to the conditions recommended by Perkin–Elmer/Cetus except for the addition of 15% (vol/vol) glycerol into the reaction mixture. The template was plasmid pGST-GDI, which contains an in-frame fusion between the genes encoding glutathione *S*-transferase (GST) and Rho GDI (10). The 5′ oligonucleotide in these reactions hybridizes upstream of the start of the GDI coding sequence in the pGST-GDI plasmid. The 3′ oligonucleotides used in the reactions were designed to introduce a stop codon after amino acid residue 196 or 200 in the Rho GDI coding sequence. This primer also contains a *Hin*dIII restriction enzyme site directly after the stop codon. The PCR products were digested with *Eco*RI (a site found between the 5′ primer and the start of the Rho GDI coding sequence) and *Hin*dIII and ligated into pGEX-KG (GST fusion vector) (16). The ligated DNA was electroporated into *Escherichia coli* strain JM101. Plasmid minipreps of single constructs were sequenced to verify the constructs. The amino-terminal deletion was constructed in a similar manner except for the oligonucleotides used in the PCR

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Abbreviations: GDI, GDP dissociation inhibitors; GRF, GDP-releasing factor; GST, glutathione *S*-transferase.

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reaction. The 5′ oligonucleotide contains an *Eco*RI site and hybridizes to Rho GDI beginning at amino acid 26. The 3′ oligonucleotide hybridizes downstream of the *Hin*dIII site (which is downstream of Rho GDI in pGST-GDI).

Construction of pGST-LD4, pGST-mD4, and the Chimeric Plasmids. The plasmid encoding the GST-LD4 fusion was constructed as follows. PCR was performed with plasmid pCA1040, which contains the cDNA for LD4 (14). The 5′ oligonucleotide contains an *Xba* I site and hybridizes to the first three codons of LD4. The 3′ oligonucleotide hybridizes to the last five codons of LD4. The 3′ PCR product was digested with *Xba* I and *Sal* I and ligated to pGEX-KG digested with *Xba* I and *Xho* I. Using this strategy results in a fusion join-point that is identical to the join-point of pGST-GDI. The same strategy was used to construct pGST-mD4, except the template was the murine D4 cDNA described previously (15) and the oligonucleotides hybridized to mD4 specifically. The general strategy for the construction of the chimeras is as follows. The pGST-GDI and pGST-LD4 plasmids described above were used as templates to amplify the desired fragment of the gene. The 5′ oligonucleotides contained an *Eco*RI site, and the 3′ oligonucleotides contained a *Hin*dIII site. The resulting products could then be cloned into the pGEX-KG vector. The fragments were then spliced together in the correct order by using specific restriction enzyme sites engineered into the fragments through the PCR primers. The first chimeric site (found in all chimeras) is an *Nhe* I site. The second site (found in chimeras C and D) is an *Nru* I site. All of the above constructions used 15% glycerol in the PCR reaction, were electroporated into *E. coli* strain JM101, and were sequenced.

Point mutants were constructed in the chimera A background by using the Amersham Sculptor kit and oligonucleotides that were specific for the desired changes. To obtain single-stranded DNA, the 3′ LD4 fragment was cloned into M13-mp19. After mutagenesis, the fragment was cloned behind the Rho GDI fragment of chimera A.

Expression and Purification of Wild-type and Mutant Fusion Proteins. GST-GDI, GST-LD4, GST-mD4, and all chimeric and truncated proteins were expressed and purified as described (10). Fractions of pure fusion protein were either used immediately (in the case of the deletion analysis) or after storing at −20°C (in the case of the analysis of the chimeric proteins, point mutants, and GST-mD4). Proteins that contained the amino terminus of LD4 (i.e., GST-LD4 and chimera B) were sensitive to *E. coli* proteolytic enzymes, so that as much as 50% of the total protein was present as proteolyzed fragments (with the remaining 50% representing the parent molecule). Because the amino-terminal region of LD4 contains several protease-sensitive sites, chimeras C and D and all single and double mutants (see Fig. 5*B*) were made with an amino terminus from Rho GDI. The result of the GDP-dissociation assay for chimera A shows that this region alone is not sufficient for activity (see Fig. 3). All other fusion proteins showed very little proteolysis, so that >95% of the total protein existed as the parent molecule as judged by SDS/PAGE. Protein concentrations were determined by Bradford assay with rabbit IgG (Sigma) as the standard. In those cases where some proteolytic breakdown occurred, the protein concentration was judged by comparison with wild-type GST-GDI on SDS/PAGE.

GDP Dissociation Inhibition Assays and Membrane Dissociation Assays. The effects of the fusion proteins on the dissociation of radiolabeled GDP from Cdc42Hs were assessed by a filter binding assay as described (10), except that the amount of platelet-purified Cdc42Hs used in each assay was ≈7 ng. The fusion proteins were tested for their ability to release membrane-bound Cdc42Hs into the soluble phase as described (10), except that membranes were prepared from *Spodoptera frugiperda* insect cells infected with a recombinant baculovirus encoding the Cdc42Hs protein. After incubation of the membranes (containing ≈9 ng of Cdc42Hs) with the indicated amount of the fusion protein for 25–30 min (this time was constant for any set of comparison assays), the membranes were centrifuged for 10 min in an Eppendorf microcentrifuge tube at 16,000 × *g*. The Cdc42Hs released from the membranes was determined by quantitative Western blotting using ^{125}I-labeled protein A.

RESULTS AND DISCUSSION

The primary aim of these studies was to identify the regions of the Rho-subtype GDI that are responsible for the interaction of this molecule with a low molecular weight GTP-binding protein such as Cdc42Hs. As a first step toward identifying these contact sites, short amino- and carboxyl-terminal deletions of Rho-GDI were made to determine the importance of these terminal regions for the inhibition of GDP dissociation from Cdc42Hs and for releasing Cdc42Hs from membranes. Fig. 1 shows three deletion mutants of the Rho GDI cDNA that were constructed by PCR amplification of the appropriate region. Primers were synthesized to allow amplification of the DNA encoding residues 26–204 (mutant NΔ25), 1–196 (CΔ8), and 1–200 (CΔ4). The primers also contained restriction sites to allow the PCR products to be ligated into the pGEX-KG plasmid vector and then expressed as GST fusion proteins in *E. coli*.

The recombinant wild-type and truncated GST-GDI proteins were first tested for their ability to inhibit GDP dissociation from Cdc42Hs. Wild-type Rho GDI yielded a dose-dependent inhibition of GDP dissociation with an IC_{50} value

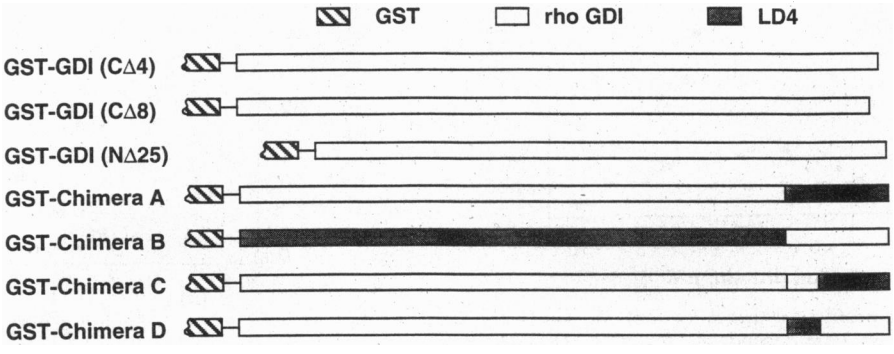

FIG. 1. Predicted fusion protein products of the Rho GDI deletion constructs and the Rho GDI/LD4 chimeras. The two carboxyl-terminal deletion mutants (CΔ4 and CΔ8) contain the first 196 and 200 amino acids of Rho GDI fused to GST. The amino-terminal mutant, NΔ25, contains residues 26–204 of Rho GDI fused to GST. Chimera A contains 171 amino-terminal residues of Rho GDI linked to the carboxyl-terminal 33 residues of LD4, while chimera B contains 168 amino-terminal residues of LD4 and 33 carboxyl-terminal residues of Rho GDI. Chimera C contains the first 183 residues of Rho GDI linked to the final 24 residues of LD4, and chimera D has residues 169–176 of LD4 replacing the analogous sequence in Rho GDI (172–179). See Fig. 4 for the exact carboxyl-terminal residues in these constructs.

of ≈0.3 μM (Fig. 2*A*), similar to results obtained previously with Rho GDI purified from bovine brain (10). Both the NΔ25 and the CΔ4 mutants also were effective inhibitors of GDP dissociation from Cdc42Hs, with IC_{50} values of ≈0.7 μM (Fig. 2*A*). However, when eight amino acids were removed from the carboxyl terminus of Rho GDI, the ability of this protein to inhibit GDP dissociation was completely eliminated.

The recombinant GDI proteins were then assayed for their ability to stimulate the release of Cdc42Hs from membrane bilayers (Fig. 2*B*). The recombinant (wild type) Rho GDI catalyzed the release of Cdc42Hs from membranes with a dose range similar to that required for the inhibition of GDP dissociation; in these experiments, the apparent K_d for the Rho GDI–Cdc42Hs interaction was ≈0.7 μM. The NΔ25 and CΔ4 mutants yielded results that were essentially identical to those obtained with the wild-type Rho GDI. However, the CΔ8 mutant was again incapable of activity. These findings indicated that the carboxyl-terminal region of Rho GDI contains residues that influence (either through direct interaction or via the maintenance of an appropriate tertiary structure) the functional coupling of the GDI to Cdc42Hs. Given that both the inhibition of GDP dissociation and the membrane dissociation activity were influenced by the carboxyl-terminal (8 amino acid) truncation to the same degree, it seems likely that these different activities are mediated through a common domain on the Rho GDI molecule.

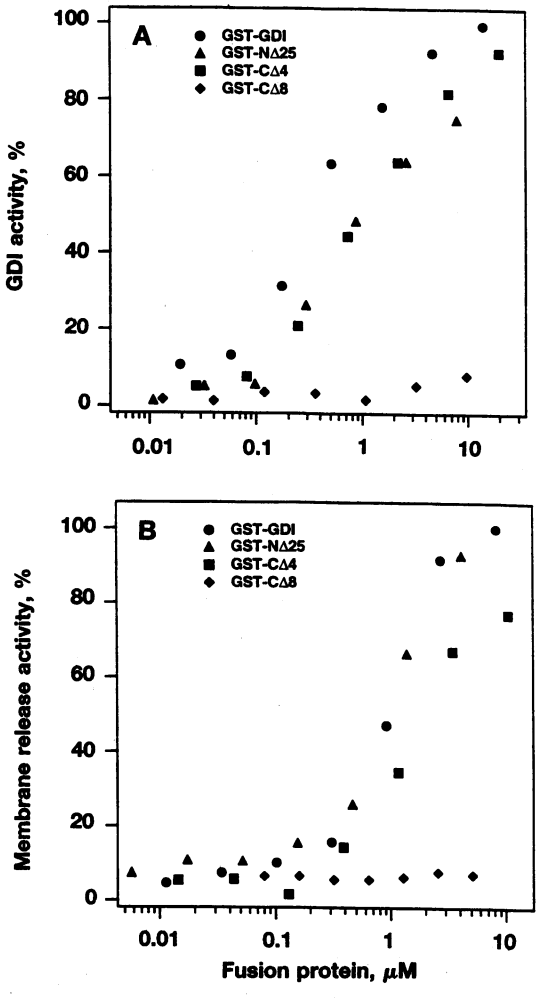

FIG. 2. Dose–response profiles for truncation mutants. The wild-type GST-Rho GDI shows dose-dependency for both the GDP dissociation inhibition assay (*A*) and the membrane release assays (*B*). In both cases, GST-GDI (CΔ8) shows no activity, while the other truncation mutants have nearly wild-type activity levels. Assays were carried out as described in *Materials and Methods*, and activities are expressed as a percentage of the response seen with a saturating dose of GST-GDI.

To examine further the importance of the carboxyl-terminal domain in the functional activities of Rho GDI, we constructed chimeras of Rho GDI and the related LD4 protein. Previously, we had shown that despite the high degree of sequence identity between Rho GDI and the LD4 protein, the latter was significantly less effective than Rho GDI in inhibiting GDP dissociation and in triggering the release of Cdc42Hs from membranes (15). Thus, these proteins were ideal candidates for a chimera study aimed at determining whether a specific domain from Rho GDI could impart a full GDI function on to the LD4 molecule. Since the deletion studies outlined above suggested the importance of the carboxyl-terminal domain in Rho GDI function, the PCR was used to generate two GST-fusion proteins: one that contained 84% of the full-length sequence of Rho-GDI and a carboxyl terminus composed of just 16% of the LD4 protein (designated GST-chimera A in Fig. 1) and a second that contained the amino-terminal 84% of the LD4 protein and the carboxyl-terminal 16% of Rho GDI (designated GST-chimera B).

The dose–response profiles for the inhibition of GDP dissociation from Cdc42Hs, obtained by using the wild-type Rho GDI and LD4 proteins and the two chimeric proteins (A and B), are shown in Fig. 3. The results highlight the differences in the effectiveness of the wild-type Rho GDI and LD4 proteins when assaying their abilities to inhibit GDP dissociation; specifically, the IC_{50} values from the dose–response curves for Rho GDI and LD4 differ by at least an order of magnitude. However, when 33 carboxyl-terminal amino acids of Rho GDI were substituted for the corresponding residues for LD4 (chimera B), essentially full GDI activity was obtained (IC_{50} ≈ 0.7 mM). The reciprocal chimera that contained the amino-terminal 171 amino acids of Rho GDI and the carboxyl-terminal 33 amino acids of LD4 (chimera A) was less effective by a factor of 15 than either the wild-type Rho GDI or chimera B when assaying for the inhibition of GDP dissociation (Fig. 3). Essentially identical differences were observed when comparing the abilities of chimeras A and B to stimulate the dissociation of Cdc42Hs from membranes (data not shown). It should be noted that chimera A appears less potent than wild-type GST-LD4 in these assays (Fig. 3). One possibility for this difference is that the carboxyl terminus within the chimeric protein is less accessible for binding Cdc42Hs compared with

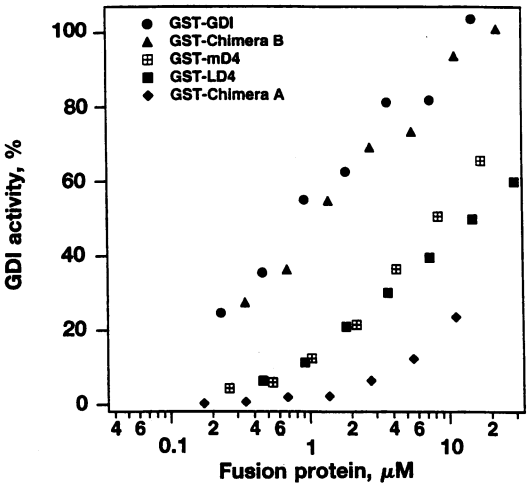

FIG. 3. Dose–response profiles for chimeric proteins. The wild-type and chimeric GDI proteins were tested for their ability to inhibit the dissociation of [α-^{32}P]GDP from Cdc42Hs as described in text. The efficacy of the GDIs in this assay is dependent on the carboxyl-terminal 33 amino acids. Activities are expressed as a percentage of the response seen with a saturating dose of GST-GDI.

the carboxyl terminus of the GST-LD4 molecule. Nevertheless, taken together, the results presented in Fig. 3 provide verification that a limited region from the carboxyl-terminal domain of Rho GDI (comprising 33 amino acids) is capable of imparting full GDI activity within the LD4 molecule.

It is interesting that the final 33 residues of Rho GDI and LD4 are very well conserved between these two proteins (see Fig. 4). Residues 197–200 in Rho GDI, which when deleted result in a complete loss of activity toward Cdc42Hs, differ from the corresponding residues for LD4 in only a single conservative change of a threonine (position 197 in Rho GDI) for a serine residue (position 194 in LD4). Thus, it seemed unlikely that this region could account for the significant differences in functional activity exhibited by Rho GDI versus LD4 toward Cdc42Hs. Rather, we suspected that this region may be necessary to stabilize the tertiary structure and/or to influence the accessibility of another interaction domain located upstream but within the carboxyl-terminal 33 amino acids of the GDI molecule.

Inspection of the Rho GDI and LD4 sequences points to an obvious difference between Rho GDI at positions 172 and 173 and LD4 at the corresponding positions 169 and 170. In LD4 the two residues at these positions are Gln-Asp, whereas in all other family members, including the murine homolog of LD4, mD4, and the recently cloned LD4 counterpart from normal human cells (C.N.A. and B.L., unpublished data), the residues are Arg-Gly (see Fig. 4). However, when mD4 was expressed in *E. coli* as a GST fusion protein and assayed for its ability to inhibit GDP dissociation from Cdc42Hs, it yielded a dose–response profile essentially identical to that for GST-LD4, with an apparent IC_{50} value of 7–8 μM (Fig. 3). Thus, the Gln-Asp → Arg-Gly changes, alone, do not account for the differences in functional activity exhibited by the Rho GDI and LD4 molecules.

We next examined other obvious differences in the carboxyl-terminal domains of Rho GDI and LD4—specifically, the substitution of an arginine present in Rho GDI (Arg-180) for phenylalanine at the corresponding position (Phe-177) in LD4, and the substitution of a threonine present in Rho GDI (Thr-187) for the glutamine residue (Gln-184) in LD4. The background for these mutations and all subsequent constructs is chimera A because typically it is expressed more stably in *E. coli* relative to constructs that contain the N-terminal 168 amino acids from LD4 (see *Materials and Methods*). However, neither of these single substitutions showed any GDI activity above that of chimera A (data not shown). Thus, we constructed two additional chimeras in attempting to delineate further the region on the GDI molecule responsible for its functional specificity. One chimera contained the first 171 amino acids from Rho GDI, residues 172–180 from the carboxyl-terminal 33 amino acid residues of Rho GDI, and the final 24 amino acids from LD4 (designated chimera C in Fig. 4). The other chimera contained the first 171 amino acids from Rho GDI, then 8 amino acid residues from LD4, followed by the final 25 amino acids from Rho GDI (chimera D). Chimera C was a fully functional GDI for Cdc42Hs, whereas chimera D, which contained just 8 amino acids unique to the carboxyl terminus of LD4, was much less effective and essentially identical to the LD4-containing chimera A (Fig. 5*A*).

Taken together, the results presented in Figs. 3 and 5*A* indicate that residues 172–180 of Rho GDI are responsible for its higher efficacy (relative to LD4) in inhibiting GDP dissociation from Cdc42Hs (or in triggering the release of Cdc42Hs from membranes). Within this region of nine amino acids, there are six differences between Rho GDI and LD4 (see boldface residues in Fig. 4). As outlined above, neither changing the Gln-Asp pair (positions 169–170 in LD4) to an Arg-Gly pair (positions 172 and 173 in Rho GDI) nor changing the phenylalanine (position 177 in LD4) to arginine (position 180 in Rho GDI) could account for the differences in functional activity between Rho GDI and LD4. It also seemed unlikely that the conserved threonine (position 171 in LD4)-to-serine (position 174 in Rho GDI) change could explain these differences. Thus, we examined if the remaining differences—namely, asparagine at position 176 in Rho GDI versus histidine at position 173 in LD4 or isoleucine at position 177 of Rho GDI versus asparagine at position 174 of LD4, might be responsible for the differences in the abilities of these proteins to act as GDIs for Cdc42Hs. The results in Fig. 5*B* show that

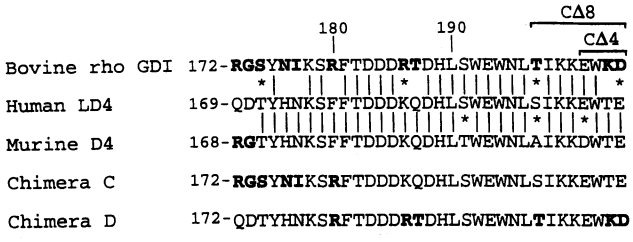

FIG. 4. The carboxyl-terminal 33 residues of wild-type and chimeric GDI proteins. The sequences shown represent the 33 amino acids switched between Rho GDI and human LD4 in chimeras A and B. Also shown are the 4 and 8 amino acids deleted in the two carboxyl-terminal truncation mutants. Boldface letters represent residues unique to bovine Rho GDI.

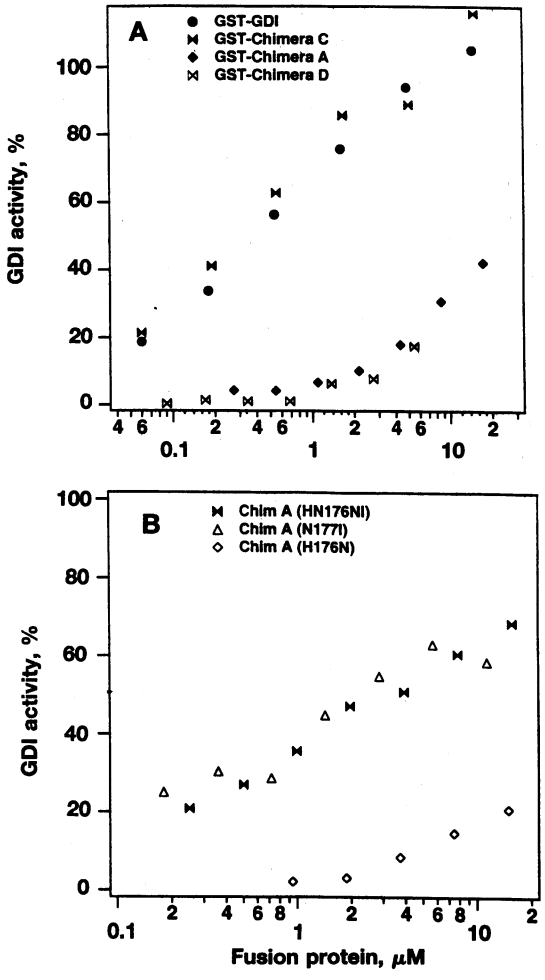

FIG. 5. Further delineation of the key residues of the carboxyl-terminal domain of the GDI. (*A*) Effects of chimeras C, A, and D and the wild-type GST-Rho GDI on the dissociation of GDP from Cdc42Hs. Assays were carried out as described in Fig. 2*A*. (*B*) The effects of single and double point mutations made in a background of chimera (Chim) A. Mutant numbers refer to Rho GDI residue number.

changing the histidine in chimera A to an asparagine (normally present in Rho GDI) did not convert this chimera to a fully functional GDI. However, changing both the histidine and asparagine (positions 173 and 174) in chimera A to asparagine and isoleucine restored nearly complete GDI activity to chimera A. Moreover, this was accomplished by just the single substitution of an isoleucine residue for an asparagine residue, suggesting that Ile-177 in Rho-GDI is largely responsible for the enhanced activity exhibited by this regulatory protein for Cdc42Hs (relative to LD4). An examination of the dose–response profiles for LD4 (Figs. 3 and 5*A*; also see ref. 15) indicates that it is a less effective GDI for Cdc42Hs, compared with Rho GDI because of a weakened binding affinity. At increasing concentrations of LD4, its GDI activity for Cdc42Hs approaches the maximal activity measured with Rho GDI. This then suggests that residues 172–180 of the Rho GDI, and particularly Ile-177, represent the determinants that influence high-affinity binding to Cdc42Hs and to related proteins (Rho, Rac) that show an enhanced functional interaction with Rho GDI versus LD4.

There is increasing evidence that the class of GDI regulatory proteins are involved in a diverse range of biological activities. The Rab-subfamily GDIs play a role in vesicular transport, neuronal secretion, and share homology with a subunit of geranylgeranyltransferase and the choroideraemia gene product (17–20). Rho GDI mediates the movement of Rho and related proteins between membrane and cytosolic compartments (18, 21) during actin stress fiber formation and membrane ruffling and cell migration and morphological changes. Rho GDI also forms a complex with Rac that is essential for the stimulation of the NADPH oxidase system in neutrophils (22–24). In all cases, these activities require the binding of the GDI to a specific GTP-binding protein. Our results provide an insight into the structural basis for the binding and function of two GDIs, the Rho GDI and the related LD4 molecule. Although there is no recognizable homology between the Rab-subtype GDIs and Rho-subtype GDIs, it is possible that the Rab GDIs have analogous domains dictating specific binding to the Rab proteins.

Rho GDI is ubiquitously expressed, whereas LD4 is preferentially expressed at very high levels in hematopoietic cells of all lineages. It remains to be established whether these GDIs act on a different repertoire of GTP-binding proteins or if they bind to the same GTP-binding proteins but with different affinities. The high level of expression of the LD4 molecule in hematopoietic cells could facilitate its binding to Cdc42Hs (or to Rac or Rho). However, since Rho GDI also is expressed in hematopoietic cells, it seems likely that these two proteins interact with distinct targets (GTP-binding proteins), as specified by the carboxyl-terminal domains of the GDI molecules. Along these lines, it is tempting to anticipate the identification of additional proteins, related to Rho GDI that contain similar differences in the carboxyl-terminal domain and thereby demonstrate distinct functional specificities.

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