Uncoupling of Inhibitory and Shuttling Functions of Rho GDP Dissociation Inhibitors*

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Rho GDP dissociation inhibitors (rhoGDIs) are postulated to regulate the activity of small G proteins of the Rho family by a shuttling process involving the extraction of Rho from donor membranes, the formation of the inhibitory cytosolic Rho/rhoGDI complexes, and delivery of Rho to target membranes. However, the role of rhoGDIs in site-specific membrane targeting or extraction of Rho is still poorly understood. Here we investigated the molecular functions of two rhoGDIs, the specific rhoGDI-3 and the less specific but well studied rhoGDI-1, in HeLa cells using structure-based mutagenesis of the rhoGDI protein. We identified two sites in rhoGDI, which form conserved interactions with their Rho target, whose mutation results in the uncoupling of inhibitory and shuttling functions of rhoGDIs: D66GDI-3 (equivalent to D45GDI-1), a conserved residue in the helix-loop-helix/GDI/switch 1 Rho interface, and D206GDI-3 (equivalent to D185GDI-1) in the β-sandwich GDI/switch 2 Rho interface.

Mutations of both sites result in the loss of rhoGDI-3 or rhoGDI-1 inhibitory activity but not of their ability to form cytosolic complexes with RhoG or Cdc42 in vivo. Remarkably, the mutants were detected at Rho-induced membrane ruffles or protrusions where they co-localized with RhoG or Cdc42, likely identifying for the first time the site of extraction of a Rho protein by a rhoGDI in vivo.

We propose that these mutations act by modifying the steady-state kinetics of the shuttling process regulated by rhoGDIs, such that transient steps at the cell membranes now become detectable. They should provide valuable tools for future investigations of the dynamics of membrane extraction or delivery of Rho proteins and their regulation by cellular partners.

Small G proteins of the Rho family regulate many aspects in the normal cell such as polarity, motility, vesicular traffic, cell cycle progression, and Ras-mediated cell transformation (1–3). They are also involved in human diseases such as infections (4) and cancer development and progression through their up-regulation rather than activating point mutations in contrast to Ras proteins (5, 6), thus showing the importance of Rho-regulatory proteins (7). Rho proteins function as molecular switches, combining a GDP/GTP and a cytosol/membrane alternation. The GDP/GTP switch is controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins and determines the conformational differences between inactive and active Rho proteins, while the cytosol/membrane alternation is regulated by Rho GDP dissociation inhibitors (rhoGDIs).

RhoGDIs are postulated to function in this cycle by shuttling Rho proteins between membranes, where these proteins are attached by an isoprenoid moiety located in their C terminus, and the cytosol (for a review, see Ref. 8). This cyclic activity involves the extraction of Rho proteins from a donor membrane, the formation of a cytosolic Rho/rhoGDI complex, and the delivery to a target membrane. Whether rhoGDIs act in this process as subsidiary transporters of inactivated Rho proteins or as essential actors for Rho activity is currently being debated (9–11). An essential role of rhoGDI is supported by the observation that the Rac1/rhoGDI-1 complex is crucial for Rac1-mediated regulation of the NADPH oxidase system in neutrophils (12) and that rhoGDI-1 appears to be critical for Cdc42-mediated cellular transformation (10). A subsidiary role was in contrast suggested by the fact that nascent prenylated Cdc42 mutant, unable to bind to rhoGDI-1, could target to Golgi membranes and be activated to induce filopodia (9) and that an activated mutant of Rac1 stimulates membrane ruffling in rhoGDI-1(−/−) cells (11).

Yet the most studied rhoGDIs (rhoGDI-1 and D4/LyGDI) have never been observed in vivo at their sites of extraction or delivery, making their mechanism and its coordination with the action of other Rho regulators difficult to elucidate. Most studies of membrane extraction and delivery have so far involved in vitro experiments. Delivery of Rac1 to membranes was analyzed using reconstituted isoprenylated Rac1/rhoGDI-1 complexes in the presence of liposomes, showing that Rac1 must dissociate from rhoGDI-1 prior to its activation by its GEF Tiam and that this delivery does not involve the GEF (13). This role of rhoGDI as an inhibitor of GEF-stimulated GDP/GTP exchange is also supported by the crystal structures of Rho/rhoGDI complexes where the binding site for the GEF overlaps the interface for rhoGDI (14–17). Structural studies also show that rhoGDI has a modular organization with an N-terminal helix-loop-helix domain that binds to the switch 1, which appears to fold only upon binding to the Rho protein (18, 19), and a C-terminal β-sandwich domain that binds to the switch 2 and carries the hydrophobic binding site for the geranylgeranyl group attached to Rho. This, together with fluorescence kinetics following the extraction of Cdc42 from liposomes (20), suggested that the molecular activity of rhoGDIs could be separated into the recognition of the Rho protein through its

* This work was supported by the CNRS and by grants from the “Association pour la Recherche sur le Cancer.” The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: GEF, guanine nucleotide exchange factor; rhoGDI, Rho guanine nucleotide dissociation inhibitor; wt, wild type; HA, hemagglutinin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DO, dropout medium; BD, binding domain; ERM, Ezrin, Radixin, Moesin, and Merlin.
nucleotide-sensitive switch regions and a step of extraction of the isoprenyl group from the membrane.

Various studies support that extraction from and delivery to membranes of Rho proteins by rhoGDIs in vivo may be assisted by additional regulations. This includes the phosphorylation of Cdc42 or RhoA proteins by protein kinase A, which facilitates their extraction by rhoGDI-1 (21, 22), or that of rhoGDI-1 by protein kinase C (23, 24) or by p21-activated kinase (Pak1) (25), which facilitates the dissociation of Rac from rhoGDI-1 and its delivery to membranes. Likewise, a set of investigations suggests that several cellular components could act as displacement factors to release Rho proteins from rhoGDI, permitting membrane targeting and activation of Rho. Recent experiments show that direct interaction of rhoGDI-1 with the neurotrophin receptor (p75NTR) initiates the activation of RhoA by facilitating the release of prenylated RhoA from rhoGDI-1 (26). In addition, the tyrosine kinase Etk disrupts the interaction between RhoA and rhoGDI-1 through the interaction between the pleckstrin homology domain of Etk and RhoA (27). Moreover phosphoinositides (28) and proteins from the ERM (Ezrin, Radixin, Moesin, and Merlin) family (29-31) have also been suggested to act as dissociation factors facilitating Rho release from the complex.

While structural data have provided a robust background for investigating the Rho-rhoGDI interactions by mutagenesis, in vivo studies of rhoGDIs mutants are made difficult because the rhoGDI family has only three members as compared with at least 20 Rho family members. Notably rhoGDI-1 (referred to as GDI-1 hereafter) and D4/LyGDI (also named rhoGDI-2) have a broad range of activity toward several Rho proteins in vivo (8, 32, 33). Accordingly the cellular effects of these rhoGDIs and their mutants could be difficult to interpret as several Rho targets are likely to be affected. However, we identified and characterized a third mammalian rhoGDI (rhoGDI-3 or rhoGDI-γ) (34, 35) that displays a unique specificity for RhoG in vivo (36). Expression of RhoGDI-3wt (referred to as GDI-3 hereafter) was shown to inhibit the phenotypes associated with activated RhoG (36), which include the translocation of RhoG to the cell periphery and the formation of ruffles and lamellipodia (37, 38). Moreover, although it forms soluble complexes with RhoG, GDI-3 is also able to sequester RhoG at the Golgi where both proteins co-localize (36). This is likely to be the site of RhoG delivery by GDI-3 as RhoG was found to be activated by its GEF at the perinuclear region (38). GDI-3 has a 25-amino acid extension at its N terminus compared with rhoGDI-1 and D4/LyGDI that is responsible for its unconventional ability to target itself and RhoG to the Golgi apparatus (34, 36).

Here we report a structure-based mutagenesis study of GDI-3 in HeLa cells, taking advantage of the fact that GDI-3 is the most specific rhoGDI regulator known to date and that the RhoG/GDI-3 system presents a unique readout of its activity in vivo. Mutations were introduced to interfere with GDI-3 interactions with the switch 1 and switch 2 regions of RhoG, and GDI-3 mutants were analyzed for their ability to inhibit RhoG-induced phenotypes, that is formation of membrane ruffles and detection of RhoG at the cell periphery. They were also analyzed for their ability to form cytosolic complexes, and in a displacement of GDI-1 mutants to Cdc42-induced membrane protrusions where they co-localize with RhoG, likely identifying for the first time the site of extraction of a Rho protein by a rhoGDI in vivo.

Equivalent mutations introduced in GDI-1 and assayed in co-expression with wild type Cdc42 resulted in a similar loss of GDI-1 inhibitory activity, despite their ability to form cytosolic complexes, and in a displacement of GDI-1 mutants to Cdc42-induced membrane protrusions. We propose that these novel mutations act by modifying the steady-state kinetics of the shuffling process regulated by rhoGDIs such that the transient extraction step now becomes detectable. They should provide valuable tools for future investigations of the dynamics of membrane extraction or delivery of Rho proteins and their regulation by cellular partners.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies against Myc and HA epitopes were from Sigma (clone 9E10 and HA-7, respectively). The secondary antibodies against mouse IgG or rabbit IgG linked to horseradish peroxidase were from American Biosciences. The secondary immunoreagents Alexa Fluor 647 and 488 were from Molecular Probes (Leiden, The Netherlands). The Dynabeads (goat anti-mouse IgG) were purchased from Dynal (Compiègne, France). Restriction endonucleases for molecular biology were New England Biolabs. Mutations were performed using the Pfu Turbo polymerase, nucleotide mixtures, and buffers from Stratagene.

Cell Constructs—For expression in eukaryotic cells, the GDI-3 sequence containing a Myc tag at the C terminus was cloned in the pCDNA3.1/neo (+) plasmid (Invitrogen) as described previously (36). GDI-1 was cloned in pcDNA3/myc vector to obtain a protein with a Myc tag at the N terminus. GDI-3 and GDI-1 point mutations were generated by the QuikChange method using the iCyClerTM from Bio-Rad with primers designed to insert mutations at the desired position. The pcDNA3.1- RhoG(3wt) was a generous gift of P. Port (Centre de Recherches de Biochimie Macromoléculaire, CNRS, Montpellier, France). pFBL23-RalB and pGAD1318-RalBD yeast expression clones were kind gifts of J. Camonis (Institut Curie, Paris, France). The yeast expression vectors containing Cdc42 and GDI-1 were kind gifts of M.-C. Dagher (Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, Commissariat à l’Énergie Atomique, Grenoble, France). For expression in E. coli, Cdc42 cDNA was subcloned into the pET28a(+) plasmid.

Cell Culture and Transfection—HeLa cells were grown on 100-mm tissue dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotic/antimycotic mixture (Invitrogen) as described in Ref. 36. Cells were transfected by electroporation as explained previously (39). Briefly cells from subconfluent cultures were harvested using trypsin/EDTA (Invitrogen) and washed once using PBS, and then resuspended in tetracycline solution (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄, 0.75 mM MgCl₂, 25 mM HEPES, pH 7.6, 5 mM MgCl₂, 2 mM ATP, 5 mM glutathione). For each electroporation pulse, cells were resuspended in 100 μl of cytosol solution containing 20 μg of each expression vector, then gently spread into 100-mm culture dishes, and incubated for 24 h at 37°C in 5% CO₂. In the case of GDI-1 and Cdc42 expression vectors, 20 μg of HA-Cdc42 vector and only 10 μg of the Myc-GDI-1wt vector was used as an amount of 20 μg turned out to be toxic for the cells. Consequently the same amount of GDI-1 mutants was used. For immunofluorescence studies, transfected cells were plated in individual wells on Labtek multichamber glass slides (VWR International).

Subcellular Fractionation and Immunoprecipitation—Cells were harvested 24 h after transfection using trypsin/EDTA, washed with PBS, and homogenized at 4°C in 500 μl of TMD buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol, and a mixture of proteases inhibitors) complemented with 0.25 mM sucrose. Nuclei and cellular debris were pelleted by centrifugation at 1000 × g for 10 min at 4°C, and the resulting postnuclear supernatant was fractionated by centrifugation at 100,000 × g for 1 h at 4°C to obtain the cytosolic fraction (S100) and the membrane-containing pellet (P100). For subcellular fractionation studies, the S100 fraction was concentrated, and proteins were denatured by boiling in Laemmli sample buffer. The P100 fraction was directly resuspended and denatured by boiling in Laemmli sample buffer. Immunooisolation experiments were performed on the cytosolic fraction S100 using Dynal magnetic beads, Dynabeads (M450) coated with goat anti-mouse IgG, as described previously (36). Bound and unbound components from each fraction were denatured by boiling in Laemmli sample buffer.

Electrophoresis and Western Blot—Proteins contained in S100, P100 fractions, and immunocomplexes were subjected to 13% SDS-PAGE and electrotransferred onto Hybond ECL nitrocellulose membrane (Amer sham Biosciences). Immobilized proteins were analyzed by immunode tection using specific primary antibody (diluted from 1:2500 to 1:5000).
GDI-3 is in DC mutated sites. B), Arg-159/Lys-162 views of the Asp-66 (RhoG/GDI-3 complex (11) and close-up A filters to be processed for minus tryptophan and leucine agar and then patched on Whatmann mated with AMR70 transfected cells. Diploid cells were selected on DO minus leucine. L40 transfected cells were and grown in DO minus tryptophan. The AMR70 yeast strain was pFBL23 plasmids containing either GDI-3wt or GDI-3 mutant cDNAs dropout medium (DO). The L40 yeast strain was transfected with lithium acetate protocol and grown at 30 °C in appropriate complete expression (36). Since the structure of the RhoG/GDI-3 complex is not known, we took advantage of the high sequence identity between GDI-3 and GDI-1 (65% taking into account the helix-loop-helix module and the β-sandwich domains) and RhoG and Cdc42 (61%) to generate a model of the complex based on the high resolution crystal structure of the Cdc42/GDI-1 complex (15). The model was generated with the 3DPSSM server (www.sbg.bio.ic.ac.uk/~3dpssm/) and restricted to conserved structured domains, excluding the specific N-terminal extension of GDI-3. Remarkably, despite the specificity of GDI-3 for RhoG, amino acids that form its interface with the core of RhoG are essentially identical to those in the Cdc42/GDI-1 complex except for the substitution of Pro-66Cdc42 for Thr-69RhoG in the switch 2 domain. In contrast, residues in GDI-3 that differ from GDI-1 are mostly exposed to solvent, which is particularly striking in the helix-loop-helix module whose Rho-interacting side is invariant with regard to GDI-1 and has almost no residue in common in its exposed face. Whether these differences reflect interactions with distinct partners remains to be established. The high conservation of residues within the RhoG/GDI-3 interface ensures on the other hand that their interactions are reliably predicted in the homology model. Three locations were therefore targeted for mutagenesis on the basis of the model (Fig. 1). First, mutations were introduced at Asp-66GDI-3 (equivalent to Asp-45 in GDI-1) in the helix-loop-helix module to disrupt its interactions with the switch 1 of

![Figure 1. Structure-based mutagenesis of GDI-3.](image-url)
RhoG. Asp-66GDI-3 forms a hydrogen bond with Thr-35RhoG, a residue that is conserved in most small GTP-binding proteins where its interactions with GTP and its associated Mg\(^{2+}\) ion act as springs to organize the active conformation (40). This ThrRhoG/AspGDI hydrogen bond, observed in all three Rho/RhoGDI crystal structures, was therefore regarded as a determinant of the preference of RhoGDIs for the GDP-bound form of Rho proteins (15). Asp-66GDI-3 was mutated to alanine either alone or with an additional S68IGDI-3 mutation (Ser-47GDI-1), a residue that establishes a set of hydrogen bonds with the main chain of Ile-36RhoG.

A second mutation was designed to disrupt the interaction of the switch 2 region of RhoG with the C-terminal \(\beta\)-sandwich of GDI-3. Asp-206GDI-3 was changed to either alanine to remove a salt bridge with Arg-66RhoG from the switch 2 or isoleucine to create an additional steric hindrance. This mutation is the counterpart on GDI of the mutation of Arg-66Cdc42 to glutamate or alanine, which prevented Cdc42 from forming stable cytosolic complexes with GDI-1 and from being removed from membranes (9, 10). However, Arg-66Cdc42 is involved in multiple interactions with GEFs, GTPase-activating proteins, and effectors (41) such that the interpretation of the mutated phenotype as the result of the sole GDI effect is difficult.

Finally we explored the contribution of positively charged residues in the C-terminal \(\beta\)-sandwich domain that may guide rhoGDI or its complex with geranylgeranylated Rho relative to negatively charged membranes through electrostatic interactions (42). Two such residues, Arg-159GDI-3 and Lys-162GDI-3, were selected on strand \(\beta\)6 in close proximity to the geranylgeranyl binding pocket and were simultaneously changed to alanine.

**Fig. 2.** Asp-66\(^{\text{GDI-3}}\) and Asp-206\(^{\text{GDI-3}}\) mutations impair the inhibitory activity of GDI-3 and displace GDI-3 to the cell periphery. A–F, HeLa cells co-expressing Myc-tagged GDI-3 proteins with HA-RhoGwt are shown. Co-transfections were made with 20 \(\mu\)g of each plasmid. Fixed cells were permeabilized (0.25% saponin) 24 h post-transfection before antibody treatment and confocal microscope analysis. GDI-3 proteins were visualized by monoclonal anti-Myc antibody followed by Alexa Fluor 647 secondary antibody. RhoG was stained by polyclonal anti-HA antibody followed by secondary Alexa Fluor 488 antibody. For single RhoG expression (in G) cells were transfected with 20 \(\mu\)g of RhoG construct. All images represent one single optical section (depth of the field, 0.4 \(\mu\)m). Arrows indicate the peripheral co-localization of RhoGwt and GDI-3 mutants in membrane ruffles (B–E). Cells shown are representative of more than 100 observed cells. Bars show 10 \(\mu\)m as indicated.
In contrast, the Asp-66 or Asp-206 mutants of GDI-3 did not inhibit RhoG-induced cell morphology. In all cells expressing these mutants, HA-RhoG was not only detected around the nucleus and the Golgi apparatus but also found, with a punctate distribution, throughout the cytoplasm and in RhoG-induced membrane ruffles (Fig. 2, B–E, lower panels). The morphology of these cells was similar to those expressing HA-RhoG alone (Fig. 2G), and the peripheral cell localization of RhoG is a hallmark of its activation at the perinuclear region followed by its transport to the cell periphery (37, 38). The finding that RhoG was able to be activated and to translocate to the cell periphery in the presence of these mutants indicates that they were defective in their inhibitory activity.

The Asp-66 and Asp-206 Mutations Displace GDI-3 to the Cell Periphery—The most striking phenomenon was that these GDI-3 mutants co-localized with RhoG at peripheral membrane ruffles (Fig. 2, B–E, arrows). To investigate whether the localization of GDI-3 mutants at the cell periphery is dependent on RhoG co-expression, we analyzed their intracellular distribution in HeLa cells when expressed alone (Fig. 3A). As already described, GDI-3wt localized mainly to the Golgi apparatus, and the R159A/K162A GDI-3 double mutation did not affect this localization. The GDI-3D66A, GDI-3D206A, and GDI-3D206I mutants displayed a marked cytoplasmic localization with a reticular distribution in more than 75% of the examined cells. A concomitant localization at Golgi was also clearly identified for these mutants, but no localization could be seen at the cell periphery. Thus, the peripheral localization of GDI-3 mutants seen in cells co-transfected with HA-RhoGwt seems to be contingent on the presence of RhoG in these cells (see Fig. 2). Intriguingly, the double mutant D66A/S68I, when expressed alone (Fig. 3A), appeared to be depleted from the Golgi apparatus in all of the analyzed cells. The integrity of the Golgi apparatus in cells expressing this mutant was, however, not affected as shown by a Golgi marker, FITC-labeled lectin from *L. culinaris* (Fig. 3B, lower panel, arrows). Moreover GDI-3D66A/S68I acted as GDI-3D66A when co-expressed with RhoG and co-localized with RhoG at the Golgi apparatus and at RhoG-induced membrane ruffles (see Fig. 2C). To summarize, use of the Asp-66 and Asp-206 mutants showed for the first time a rhoGDI at sites of Rho localization at the cell periphery. Dependence of this localization on RhoG co-expression suggests an interaction of the GDI-3 mutants with RhoG.

To further characterize the membrane/cytosol partition of the GDI-3 mutants, cells expressing only Myc-tagged GDI-3 proteins were lysed in the absence of detergent, and post-nuclear supernatants were submitted to classical cell fractionation and analyzed by Western blotting. As shown in Fig. 4, wild type GDI-3 and the D206I mutant distributed into both the cytosolic and the pellet fractions, confirming their observation in the cytosol and on Golgi by confocal microscopy. Despite its absence from the Golgi apparatus, the D66A/S68I mutant was also found, as were GDI-3wt and D206I mutant, in the pellet fraction (Fig. 4). This could possibly be explained by an interaction of GDI-3 proteins not only with the Golgi apparatus but also with other intracellular compartments such as vesicles or with a cytoskeletal component.

The D66A/S68I double mutant, which failed to be observed at the Golgi apparatus when expressed alone, displayed a slightly lower apparent molecular weight on SDS-PAGE following cell fractionation (Fig. 4). This phenomenon could be due to a cleavage at the N terminus of the molecule as the protein is detected by its C-terminal Myc epitope, which excludes a C-terminal proteolysis. This may explain why this mutant appeared depleted from Golgi as the N-terminal extension in GDI-3 is necessary for its Golgi targeting (36). Interestingly co-expression with RhoG prevented this proteolysis (see co-immunoprecipitation below), thus explaining why this mutant behaved as the D66A mutant under these conditions.

The GDI-3 Mutants with Impaired Inhibitory Activity Form Complexes with RhoG in HeLa Cells—The conditional co-localization of GDI-3 mutants with RhoG at the cell periphery suggests that their interactions with RhoG are not abolished by the mutations. To investigate this possibility, we performed co-immunoprecipitation experiments of cytosolic subcellular fractions. HeLa cells were co-transfected with equal amounts of HA-RhoG and either GDI-3wt or one of the GDI-3 mutants tagged with a Myc epitope, and cytosolic fractions were incu-
bated with magnetic beads coated with an anti-Myc antibody. Proteins contained in bound and unbound fractions were next revealed by Western blot using anti-HA and anti-Myc antibodies for detection of RhoG and GDI-3, respectively (Fig. 5A). Remarkably all GDI-3 mutants were able to form cytosolic complexes with RhoG (Fig. 5A, upper panel). Quantitative analysis by optical densitometry of co-immunoprecipitated RhoG from three independent experiments did not reveal significant differences between GDI-3wt and either of the GDI-3 mutants (Fig. 5B). In particular, the mutants with impaired inhibitory activity (D66A, D66A/S68I, D206A, and D206I) retained their ability to form cytosolic complexes, thus uncoupling these two components of GDI-3 activity in vivo.

As mutations of Asp-66, Ser-68, and Asp-206 residues are expected to impair Rho/GDI protein-protein interfaces, we analyzed the extent of destabilization of the complex formed between these mutants and RhoG by the yeast two-hybrid mating assay as an alternative method to study protein-protein interactions (Fig. 5C). Interactions were detected by activation of the HIS3 and of LacZ reporter genes, and diploids co-expressing Ral-LexA DBD and RalBD-GAL4-AD were used as a positive control (43) (Fig. 5C, lower panel). Co-expression of RhoG and either GDI-3wt (Fig. 5C, upper panel) or GDI-3R159A/K162A the double mutant (Fig. 5C, lower panel) also induced the activation of both reporter genes, in agreement with the interactions found between these proteins in HeLa cell cytosol. In contrast, none of the other four GDI-3 mutants (D66A, D66A/S68I in the upper panel, and D206A or D206I in the lower panel of Fig. 5C) was able to interact with RhoG in the two-hybrid system. We deduce that although RhoG/GDI-3 mutant complexes form in the cell cytoplasm and persist in co-immunoprecipitation assays, they are probably less stable than the wild type complex. Thus, analysis of interactions between two proteins under less physiological conditions, i.e. the two-hybrid assay, revealed differences between wild type and mutant RhoG/GDI-3 complexes that were otherwise not detected in living cells.

The Effect of the Asp-66GDI-3 and Asp-206GDI-3 Mutations Can Be Generalized to the Regulation of Cdc42 by GDI-1—In view of the above results with GDI-3, the mutations equivalent to Asp-66GDI-3 (Asp-45GDI-1) and Asp-206GDI-3 (Asp-185GDI-1) were introduced in the less specific GDI-1 regulator and analyzed for their effect on wild type Cdc42. We controlled that transient expression of N-terminally HA-labeled Cdc42 induces the formation of microspikes in HeLa cells (Fig. 6A, arrowhead).
as already described (44, 45). Cdc42 was found to localize not only at a perinuclear region, shown to be essentially the Golgi apparatus (9, 46), but also at the cell periphery (Fig. 6A, arrows). This latter localization has already been observed for activated or fast cycling Cdc42 mutants (10). Co-expression with GDI-1wt tagged with an N-terminal Myc epitope redistributed Cdc42 into the cytoplasm (Fig. 6B), and Cdc42-induced microspikes were no longer detected as already described (9, 47). Remarkably, as found for GDI-3 and RhoG, both GDI-1D45A (Fig. 6C) and GDI-1D185A (Fig. 6D) were displaced to the cell periphery (Fig. 6A, C, D, arrows) when co-expressed with wild type Cdc42 and were at the same time unable to inhibit the formation of Cdc42-induced microspikes (Fig. 6D, arrows). Thus, when co-expressed with the GDI-1 mutants, Cdc42 was detected at the Golgi and throughout the cytoplasm and concentrates at the cell periphery.

We next analyzed the ability of GDI-1D45A and GDI-1D185A to form co-immunoprecipitable complexes with Cdc42. HeLa cells were co-transfected with the HA-Cdc42-coding vector and MycGDI-1wt or MycGDI-1D45A or MycGDI-1D185A vectors. Following classical cell fractionation, cytosolic fractions were incubated with magnetic beads coated with the monoclonal anti-HA antibody, and HA-Cdc42/MycGDI-1 complexes were isolated. Proteins contained in bound and unbound fractions were analyzed by Western blot (Fig. 7A).

**Fig. 6.** GDI-1D45A and GDI-1D185A do not inhibit Cdc42 activation and relocation. A, transient expression of HA-Cdc42wt 24 h after transfection with 20 μg of plasmid. B–D, HeLa cells co-expressing Myc-tagged GDI-1 proteins and HA-Cdc42 are shown. Co-transfections were carried out with 20 μg of HA-Cdc42-coding plasmid and 10 μg of GDI-Myc-coding plasmids (see “Experimental Procedures”). GDI-1 proteins were visualized by monoclonal anti-Myc antibody followed by Alexa Fluor 647 secondary antibody. Cdc42 was stained by polyclonal anti-HA antibody followed by secondary Alexa Fluor 488 antibody. All images represent one single optical section (depth of the field, 0.4 μm). Arrows indicate peripheral co-localizations of Cdc42 and GDI-1 mutants. Arrowheads indicate microspikes. Bars are 10 μm as indicated.

three independent experiments, adjusted for the total amount of expressed HA-Cdc42 in each case, revealed that only about 30% of the total MycGDI-1wt proteins were found in complex with HA-Cdc42 (Fig. 7B). This low percentage, compared with that found for the RhoG/GDI-3wt complex (∼60%), could be explained by the high expression level of endogenous GDI-1 in HeLa cells as found using the anti-GDI-1 antibody (data not shown), thus competing with MycGDI-1 for binding to HA-Cdc42. As for GDI-3, the two GDI-1 mutants also co-localized with Cdc42p at the tips of small buds during the G1/S phase of the cell cycle and at the mother bud neck region (38). We have shown that GDI-3wt targets RhoG to the Golgi apparatus (delivery site) where it inhibits its activation with a concomitant disappearance of RhoG from the cell periphery (extraction site) (36). Taking advantage of this phenotypic readout that does not require mutations in RhoG, we investigated the molecular coupling between the inhibitory and shutting functions of GDI-3 by the in vivo study of its mutants.

The crystal structures of Rho/GDI complexes show that rhoGDIs form bimodal interactions with Rho proteins, suggesting a possible hierarchy between the N-terminal helix-loop-helix/GDI/switch 1 Rho interface and the GDI C-terminal β-sandwich interacting with the switch 2 and the isoprenyl group of Rho (15–17). Since the switch 1 and switch 2 are involved in the interactions of Rho proteins with almost all their regulators and effectors (41), mutations were introduced in GDI-3, thus affecting only the function of this regulator. Based on homology modeling of the RhoG/GDI-3 structure, mutations were therefore designed to impair polar or charged interactions between invariant residues in each interface separately, and GDI-3 mutants were analyzed in vivo for their ability to inhibit RhoG activity and to transfer RhoG into cytosolic complexes (summarized in Table I). We found that the GDI-3D66A or GDI-3D66A/S68I mutant in the helix-loop-helix/GDI/switch 1 Rho interface and GDI-3D206A or GDI-3D206I mutant in the β-sandwich/GDI/switch 2 Rho interface lost their ability to inhibit the formation of RhoG-induced ruffles as identified by confocal immunofluorescence, but they retained their ability to form cytosolic complexes as detected by co-immunoprecipitation. Remarkably these GDI-3 mutants could now be observed at the cell periphery where they co-localized with RhoG to membrane ruffles. This localization was dependent on the co-expression of RhoG, suggesting that a direct interaction of these mutants with RhoG is likely to be responsible for their displacement to the cell periphery. To generalize the functional importance of Asp-66 and Asp-206 amino acids of GDI-3 to another known GDI, mutations equivalent to D66GDI-3 (D45GDI-1) and

### DISCUSSION

We have investigated here how rhoGDIs shuttle Rho family members between extraction and delivery sites. Except the Golgi targeting of rhoGDI-3, the mammalian GDI-1 or D4/LyGDI has never been observed at either an extraction or a delivery site in vivo, making it difficult to understand how to establish their role in specific targeting of Rho proteins and their spatiotemporal coordination with other regulators like GEFs, ERM proteins, or protein kinases. Interestingly rhoGDI in Saccharomyces cerevisiae, Rd1p, has recently been found to be co-localized with Cdc42p at the tips of small buds during the G1/S phase of the cell cycle and at the mother bud neck region during cytokinesis (48).

In the case of RhoG and GDI-3, the extraction and delivery sites appear to be distinct. When activated at the perinuclear region by the GEF1 domain of the exchange factor Trio, RhoG translocates to the cell periphery in a microtubule-dependent manner and induces membrane ruffles and lamellipodia (37, 38). We have shown that GDI-3wt targets RhoG to the Golgi apparatus (delivery site) where it inhibits its activation with a concomitant disappearance of RhoG from the cell periphery (extraction site) (36). Taking advantage of this phenotypic readout that does not require mutations in RhoG, we investigated the molecular coupling between the inhibitory and shutting functions of GDI-3 by the in vivo study of its mutants.

The crystal structures of Rho/GDI complexes show that rhoGDIs form bimodal interactions with Rho proteins, suggesting a possible hierarchy between the N-terminal helix-loop-helix/GDI/switch 1 Rho interface and the GDI C-terminal β-sandwich interacting with the switch 2 and the isoprenyl group of Rho (15–17). Since the switch 1 and switch 2 are involved in the interactions of Rho proteins with almost all their regulators and effectors (41), mutations were introduced in GDI-3, thus affecting only the function of this regulator. Based on homology modeling of the RhoG/GDI-3 structure, mutations were therefore designed to impair polar or charged interactions between invariant residues in each interface separately, and GDI-3 mutants were analyzed in vivo for their ability to inhibit RhoG activity and to transfer RhoG into cytosolic complexes (summarized in Table I). We found that the GDI-3D66A or GDI-3D66A/S68I mutant in the helix-loop-helix/GDI/switch 1 Rho interface and GDI-3D206A or GDI-3D206I mutant in the β-sandwich/GDI/switch 2 Rho interface lost their ability to inhibit the formation of RhoG-induced ruffles as identified by confocal immunofluorescence, but they retained their ability to form cytosolic complexes as detected by co-immunoprecipitation. Remarkably these GDI-3 mutants could now be observed at the cell periphery where they co-localized with RhoG to membrane ruffles. This localization was dependent on the co-expression of RhoG, suggesting that a direct interaction of these mutants with RhoG is likely to be responsible for their displacement to the cell periphery. To generalize the functional importance of Asp-66 and Asp-206 amino acids of GDI-3 to another known GDI, mutations equivalent to D66GDI-3 (D45GDI-1) and

### Table I

Summary of GDI-3 and GDI-1 mutant activity toward RhoG and Cdc42

| GDI/Rho interface targeted by mutagenesis | Inhibitory activity | Cell phenotype | Rho GTPase localization | rhoGDI localization | rhoGDRho interaction |
|------------------------------------------|-------------------|---------------|------------------------|-------------------|---------------------|
| GDI-3wt                                  | +                 | Cell death, round and smooth cells | Golgi/ER | Golgi | Cytoplasm | + + + |
| GDI-3D66A                                | -                 | RhoG-induced ruffles and lamellipodia | Ruffles | Golgi/ER | Cytoplasm | + + - |
| GDI-3D66A/S68I                           | -                 | RhoG-induced ruffles and lamellipodia | Ruffles | Golgi/ER | Cytoplasm | + + - |
| GDI-3D206A                               | -                 | Cell death, round and smooth cells | Golgi/ER | Cytoplasm | + + + |
| GDI-1wt                                  | +                 | Cell death, round and smooth cells | Cytoplasm | + + + |
| GDI-1D45A                                | -                 | Cdc42-induced microspikes | Cell periphery/Microspikes | Golgi | Cytoplasm | + - |
| GDI-1D185A                               | -                 | Cdc42-induced microspikes | Cell periphery/Microspikes | Golgi | Cytoplasm | + - |

*The Rho GTPase is RhoG in the case of CDI-3 and Cdc42 in the case of GDI-1.

b Cytosolic complexes are marked (+ +) if interaction is comparable to that of RhoG/CDI-3wt or Cdc42/GDI-1wt, and (+) if lower.

c Text in bold refers to the most striking phenotype. ER, endoplasmic reticulum. Nt, N terminus; Ct, C terminus.
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D206\textsubscript{GDI-3} (D185A\textsubscript{GDI-1}) were introduced in GDI-1, and the less specific but well studied Cdc42/GDI-1 system was analyzed. We found that, as in the RhoG/GDI-3 system, GDI-1 mutants were displaced to Cdc42-induced microspikes and were able to form cytosolic complexes. Thus, we identified novel mutations that uncouple the inhibitory and shuttling functions of rhoGDIs and that displace detectable amounts of both GDI-1 and GDI-3 to the site of action of their Rho targets in vivo.

Mutations in rhoGDIs were introduced at conserved positions where they are expected to weaken either the helix-loop-helix\textsubscript{GDI}/switch 1\textsubscript{Rho} or the \textit{β}-sandwich\textsubscript{GDI}/switch 2\textsubscript{Rho} interfaces. Accordingly neither the GDI-3 mutants nor their corresponding GDI-1 mutants could form complexes with Rho proteins in the yeast two-hybrid assay. However, the mutants retained their ability to form cytosolic complexes in vivo although to a lesser extent in the case of Cdc42/GDI-1 as compared with wild type GDI-1, and probably membrane-localized complexes could form at the cell periphery as well. This is in partial disagreement with mutations in Cdc42 and Rac1 targeting the same site in the \textit{β}-sandwich\textsubscript{GDI}/switch 2\textsubscript{Rho} interface as D185A\textsubscript{GDI-1}, which resulted in the complete loss of the cytosolic complexes (9–11). The unexpected robustness of the Rho/GDI protein-protein interface to our novel GDI mutants suggests that interfering with one of the interfaces does not affect the formation of the other one and that these interfaces could form separately. However, we could not at this stage establish a hierarchy between these two interfaces as both yielded essentially similar effects. Separable roles for both interfaces are consistent with the partial opening of the RhoAGDI-1 complex by phosphoryositides as reported (28) and the observation, by fluorescence resonance energy transfer in vitro, that extraction of Cdc42 by GDI-1 is a biphasic reaction (20) proposed to be accounted for by the formation of the Rho/GDI and the lipid/GDI interfaces (15). Our data suggest that the formation of the GDI/Rho interface itself may take place in two steps, respectively corresponding to the formation of the helix-loop-helix\textsubscript{GDI}/switch 1\textsubscript{Rho} interface, possibly detecting the nucleotide status, and the formation of the \textit{β}-sandwich\textsubscript{GDI}/switch 2\textsubscript{Rho} interface, possibly coordinated to the positioning of rhoGDI relative to the membrane and to the extraction of the Rho isoprenyl tail. The function of this separation of the GDI action in two steps is not known at the moment, but it could in principle allow the simultaneous binding of a third partner before its full binding to or dissociation from Rho.

GDI-3, which is unique to mammals, differs from GDI-1 and D4/LyGDI by its N-terminal extension that is essential for its unconventional localization to the Golgi and its ability to locate RhoG to this compartment (36). Surprisingly our GDI-3 mutants where the conserved interface with the switch 1 and switch 2 is impaired resulted in less drastic phenotypes as compared with the deletion of the unique N terminus or the reduction of its hydrophobic surface by a double mutation (36). Notably these N-terminal mutants, which are unable to target RhoG to Golgi, were not displaced to the cell periphery in the presence of RhoG, and they displayed a marked decrease in the stability of their cytosolic complexes as compared with the novel mutants, suggesting that their interactions with RhoG may be more severely impaired. This critical role of the N terminus in stabilizing the RhoGGDI-3 complex may explain why co-immunoprecipitation did not detect a difference between wild type and the novel GDI-3 mutants, while a slight but significant (30%) decrease was measured in the case of Cdc42/GDI-1. Yet we do not have an explanation for the molecular role of the extension at this stage, and current data yield conflicting results. On the one hand, the N terminus of GDI-3 did not interact with RhoG in the two-hybrid assay (data not shown). On the other hand, the behavior of the double mutation D66AS68I in the present study could be in agreement with an interaction between the N-terminal extension of GDI-3 and RhoG. Indeed the establishment of RhoG/GDI-3 complexes protected GDI-3\textsubscript{D66AS68I} from the N-terminal proteolysis found in HeLa cells when this mutant was expressed alone. In addition, we cannot exclude interactions of this N-terminal putative helix with other partners that could stabilize these mutant complexes extracted from HeLa cells.

Our analysis of the effect of the mutations suggests that the loss of inhibition by the GDI-3 mutants and their displacement to the cell periphery may result from modified steady-state kinetics of the shuttling cycle. A partial opening of the RhoG/GDI-3 complexes, facilitated by our mutations, could assist the disengagement of RhoG from GDI-3 at the Golgi where it is not trapped, unlike when expressed with GDI-3wt (36), thus permitting activation of RhoG. Symmetrically it could also impair the extraction of RhoG from membranes at the cell periphery, resulting in non-productive interactions of GDI-3 with RhoG at the membrane, yielding longer residence at the cell periphery on average and allowing detection by confocal immunofluorescence. Besides we do not exclude that the destabilization of the bimodal interface may be more permissive to unusual interactions, such as for instance the formation of RhoG-GTP-GDI-3 complexes.

Alltogether we propose that differences in the kinetics of individual steps of the rhoGDI functional cycle, introduced by our novel mutations, do not impair the overall shuttling process but reveal for the first time transient steps as they become rate-limiting. The specificity and the phenotypes associated with the RhoG/GDI-3 system allowed these intermediates to be identified using wild type RhoG with either wild type or mutant GDI-3, which contributed respectively to the identification of the probable site of RhoG delivery at the Golgi apparatus (36) and of RhoG extraction site from ruffles at the cell periphery (this work). This was confirmed by mutants of the more promiscuous GDI-1, co-expressed with wild type Cdc42, resulting in the detection of this regulator at one of the sites of action of Cdc42, namely the cell periphery near the plasma membrane.

These mutations should now provide valuable tools for future investigations of still unresolved issues of the dynamics of the shuttling events regulated by rhoGDIs. In the case of RhoG, this includes the molecular mechanism of targeting to Golgi mediated by the N terminus of GDI-3 but also the possible implication of GDI-3 in shutting RhoG to distinct compartments to act on different effectors like kinectin, initially detected at the endoplasmic reticulum (49), and Dock180/ELMO, which is found at the cell periphery (50). More generally, interferers that slow the kinetics of the shuttling mechanism should allow novel insight into the traffic of rhoGDIs to the sites of Rho extraction and delivery and their coordination with other regulatory partners such as guanine nucleotide exchange factors.

Acknowledgments—We are indebted to S. Brown and C. Talbot (Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France) for confocal microscopy facilities. We thank P. Fort and A. Blangy (Centre de Recherches de Biochimie Macromoléculaire, Montpellier, France), M.-C. Dagher (Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, Commissariat à l’Energie Atomique, Grenoble, France), and J. Camonis (Institut Curie, Paris, France) for their kind gifts of plasmids.

REFERENCES

1. Bar-Sagi, D., and Hall, A. (2000) Cell 103, 227–238
2. Ridley, A. J. (2003) Traffic 2, 303–310
3. Qualmann, B., and Mellor, H. (2003) Biochem. J. 371, 233–241
4. Boquet, P., and Lemichez, E. (2003) Trends Cell Biol. 13, 238–246
5. Sahai, E., and Marshall, C. (2002) Nat. Rev. Cancer 2, 133–142
6. Malliri, A., and Collard, J. (2003) Curr. Opin. Cell Biol. 15, 583–589
7. Jiang, W. G., Watkins, G., Lane, J., Cunnick, G. H., Douglas-Jones, A., Mokbel, K., and Mansel, R. E. (2003) Clin. Cancer Res. 9, 6423–6440
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8. Olofsson, B. (1999) *Cell. Signal.* 11, 545–554
9. Gibson, R., and Wilson-Delfosse, A. (2001) *Biochem. J.* 359, 285–294
10. Lin, Q., Fuji, R., Yang, W., and Cerione, R. (2003) *Curr. Biol.* 13, 1469–1479
11. Gandhi, P., Gibson, R., Tong, X., Miyoshi, J., Takai, Y., Konieczkowski, M., Sedor, J., and Wilson-Delfosse, A. (2004) *Biochem. J.* 378, 409–419
12. Di-Poi, N., Faure, J., Gribou, S., Molnar, G., Pick, E., and Dagher, M. (2001) *Biochemistry* 40, 10014–10022
13. Robbe, K., Otto-Bruc, A., Chardin, P., and Antiony, B. (2003) *J. Biol. Chem.* 278, 4756–4762
14. Snyder, J., Worthylake, D., Rossman, K., Betts, L., Pruitt, W., Siderovski, D., and Wilson-Delfosse, A. (2002) *Traffic* 3, 342–358
15. Hoffman, G. R., Nassar, N., and Cerione, R. A. (2000) *Cell 100*, 345–356
16. Schaffrath, K., Stephan, I., Jensen, O. N., Illenberger, D., and Gierschik, P. (2000) *Nat. Struct. Biol.* 7, 122–126
17. Gossner, Y., Faure, J., Fieschi, F., Vignal, E., and Pehay-Peyroula, E. (2001) *Biochemistry* 40, 10007–10013
18. Grizot, S., Faure, J., Fieschi, F., Vignal, E., Dagher, M., and Pebay-Peyroula, E. (2001) *Biochemistry* 40, 623–633
19. Mangeat, P., Roy, C., and Martin, M. (1999) *EMBO J.* 18, 1744–1750
20. Lang, P. F., Geisbert, M., Delespine-Carmagnat, R., Stancou, R., Pouchelet, M., and Bertoglio, J. (1996) *EMBO J.* 15, 510–519
21. Forget, M., Desroisiers, R., Gingras, D., and Beliveau, R. (2002) *Biochem. J.* 361, 243–254
22. Mehta, D., Rahman, A., and Malik, A. (2001) *J. Biol. Chem.* 276, 22614–22620
23. Price, L. S., Langeslag, M., ten Klooster, J. P., Hordijk, P. L., Jalink, K., and Collard, J. G. (2003) *J. Biol. Chem.* 278, 39413–39412
24. DerMardirossian, C., Schnelzer, A., and Bokoch, G. (2004) *Mol. Cell* 15, 117–127
25. Yamashita, T., and Toyohara, M. (2003) *Nat. Neurosci.* 6, 461–467
26. Faure, J., Vignal, E., and Dagher, M. (1999) *Eur. J. Biochem.* 262, 879–889
27. Takahashi, K., Sasaki, T., Mammoto, A., Takai, K., Kameyama, T., Tsukita, S., and Takai, Y. (1997) *J. Biol. Chem.* 272, 23371–23375
28. Maeda, M., Matsui, T., Imamura, M., Tsukita, S., and Tsukita, S. (1999) *Oncogene* 18, 4788–4797
29. Mangeat, P., Roy, C., and Martin, M. (1999) *Trends Cell Biol.* 9, 187–192
30. Fukumoto, Y., Kaibuchi, K., Hori, Y., Fujikura, H., Araki, S., Ueda, T., Kikuchi, A., and Takai, Y. (1999) *Oncogene* 5, 1321–1328
31. Scherle, P., Behrens, T., and Staudt, L. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 7568–7572
32. Zaleman, G., Closson, V., Camonis, J., Honore, N., Rousseau-Merck, M. F., Tatavion, A., and Olofsson, B. (1996) *J. Biol. Chem.* 271, 30366–30374
33. Acer, C. N., Manor, D., Ko, J. L., Zhu, S. C., Horiuchi, T., VanAelst, L., Cerione, R. A., and Lim, B. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 4279–4284
34. Brunet, N., Morin, A., and Olofsson, B. (2002) *Traffic* 3, 342–358
35. Gauthier-Rouviere, C., Vignal, E., Meriane, M., Roux, P., Montcourier, P., and Fort, P. (1998) *Mol. Biol. Cell* 9, 1379–1394
36. Blangy, A., Vignal, E., Schmidt, S., Debant, A., Gauthier-Rouviere, C., and Fort, P. (2000) *J. Cell Sci.* 113, 729–739
37. Morin, A., Picari, R., and Tixier-Vald, A. (1996) *Mol. Cell. Endocrinol.* 117, 59–73
38. Vetter, I. R., and Withehofer, A. (2001) *Science* 294, 1299–1304
39. Badii, R., Bokoch, G., Lian, L., and Roberts, G. (2001) *J. Biol. Chem.* 276, 26214–26220
40. Golovanov, A., Chuang, T., DerMardirossian, C., Barsukov, I., Hawkins, D., Badii, R., Bokoch, G., Lian, L., and Roberts, G. (2001) *J. Mol. Biol.* 305, 121–135
41. Bauer, B., Mirey, G., Vetter, I. R., Garcia-Ranea, J. A., Valencia, A., Withehofer, A., Camonis, J. H., and Codd, R. H. (1999) *J. Biol. Chem.* 274, 17763–17770
42. Hall, A. (1998) *Science* 279, 509–514
43. Ikeda, N., Nakashima, H., Tanaka, Y., Tachibana, K., and Takai, Y. (2001) *Oncogene* 20, 3457–3463
44. Erickson, J. W., Zhang, C. J., Kahn, R. A., Evans, T., and Cerione, R. A. (1996) *J. Biol. Chem.* 271, 26850–26854
45. Michaelson, D., Silitui, J., Murphy, G., D'Eustachio, P., Rush, M., and Philips, M. R. (2001) *J. Cell Biol.* 152, 111–126
46. Richman, T., Toenjes, K., Morales, S., Cole, K., Wasserman, B., Taylor, C., Koster, J., Whelihan, M., and Johnson, D. (2002) *Curr. Genet.* 45, 339–349
47. Vignal, E., Blangy, A., Martin, M., Gauthier-Rouviere, C., and Fort, P. (2001) *Mol. Cell. Biol.* 21, 8022–8034
48. Katoh, H., and Negishi, M. (2003) *Biochemistry* 42, 461–464
Uncoupling of Inhibitory and Shuttling Functions of Rho GDP Dissociation Inhibitors
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J. Biol. Chem. 2005, 280:4674-4683.
doi: 10.1074/jbc.M409741200 originally published online October 28, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409741200

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