Rab Proteins Form in Vivo Complexes with Two Isoforms of the GDP-dissociation Inhibitor Protein (GDI)*

(Received for publication, September 2, 1994, and in revised form, October 7, 1994)

Chunzhi Yang‡, Vladimir I. Slepnev, and Bruno Goud§

From the Unité de Génétique Somatique (URA CNRS 361), Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

GTPases of the Rab family play a key role in the regulation of vesicular transport in eukaryotic cells. Several accessory proteins that regulate their GDP/GTP cycle as well as their subcellular localization have been identified within the past few years. The best known is Rab3A GDP dissociation inhibitor protein (GDI), originally identified as an inhibitor of GDP dissociation from Rab3A, a Rab protein specifically expressed in neuronal and neuroendocrine cells. Recent studies have pointed out a role of Rab3A GDI as a chaperone of several Rab proteins during their cycling between cytosol and membranes and Rab3A GDI has been considered so far as a general regulator of Rab function. However, cDNAs encoding potential isoforms of this protein, called GDI β and GDI-2, have been recently isolated. In this study, we have characterized cytosolic Rab protein complexes in various cell types and tissues using Mono Q chromatography. We show that in rat brain and in insulin-secreting RINm5F cells, the majority of Rab proteins are complexed with Rab3A GDI. In contrast, in Chinese hamster ovary cells, they are mainly complexed to a protein that we have identified as GDI β. In rat liver cytosol, Rab proteins form complexes with both isoforms. We also show that the proportion of Rab proteins complexed with either isoform depends on the relative abundance of Rab3A GDI and GDI β in the cytosol. These findings suggest that GDI isoforms are either redundant or could be involved in the fine control of Rab function.

Over 30 members of the ras-related Rab GTPase family have been identified within the past few years in a wide variety of eukaryotic cells, including yeast Saccharomyces cerevisiae (Sec4 and Ypt proteins) (for review, see Refs. 1 and 2). Being associated with distinct compartments of both the biosynthetic/secretory and the endocytic pathways, Rab proteins are thought to play an important role in the regulation of vesicular traffic. They cycle between a cytosolic "inactive" (GDP-bound) form and a membrane-associated "active" (GTP-bound) form. This cycle could specify accurate docking and/or fusion of transport vesicles with their acceptor membranes (for review, see Refs. 3–5). An unresolved question is to know how Rab proteins fulfill their function in the context of proteins such as the v- and t-SNARES recently shown to be involved in the docking/fusion machinery (6, 7).

Several accessory proteins which regulate the nucleotide state of Rab proteins and their subcellular localization have been identified. They include the mammalian protein Mss4 and the yeast protein Dse4 which exhibit guanine-nucleotide exchange factor-like activities (8–9). A putative smg p25A/Rab3A guanine-nucleotide exchange factor has also been identified in brain cytosol (10). Less is known about the proteins that might stimulate the very low intrinsic GTPase activity of Rab proteins. Although Rab GTPase-activating protein-like activities have been detected in various cell or tissue extracts (10, 11), only Gyp 6 which encodes for a GTPase-activating protein specific for Ypt6, the yeast homolog of Rab6, has been cloned (12). Another protein, rabphilin, which shares homology with the synaptic vesicle integral membrane protein synaptotagmin, can inhibit GTPase activating protein-stimulated GTPase of Rab3A, but its precise function is still unclear (13).

So far, the best characterized Rab accessory protein is Rab3A GDI.1 This protein was originally discovered as an inhibitor of the release of GDP from the isoprenylated form of smg p25A/Rab3A, a Rab protein preferentially expressed in neuronal and neuroendocrine cells (14). Rab3A GDI forms cytosolic complexes with Rab3A and in vitro inhibits the binding to and promotes the dissociation of the GDP-bound form of Rab3A from membranes (15, 16). In contrast to the cellular specificity of Rab3A, Rab3A GDI mRNA is expressed in all tissues, although at lower levels than in the brain (17). In addition, Rab3A GDI is not specific for this protein as it has also been shown to inhibit GDP release from Sec4 and Rab11 and to extract a broad range of Rab proteins from membranes (18–20). Furthermore, cytosolic complexes formed between Rab proteins and Rab3A GDI have been detected in various cell types, including insulin-secreting RINm5F and Chinese hamster ovary (CHO) cells (16, 21).

Based on the above results, it has been suggested that Rab3A GDI acts as a general regulator of Rab function (20). Its role would be to both inhibit GDP release and to act as a chaperone of Rab proteins during their cycle between cytosol and membranes. However, two new cDNAs coding for GDI-like proteins have been very recently cloned from a rat brain library and a mouse skeletal muscle library, referred to as GDI β and GDI-2, respectively (22, 23). GDI β has been shown to inhibit GDP release from Rab3A and Rab11 and GDI-2 can extract Rab4 and Rab5 from membranes (22, 23). These findings then raise the question of whether differences in function between Rab3A

1 The abbreviations used are: GDI, GDP dissociation inhibitor protein; QFF, Sepharose Q Fast Flow; EGS, ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester); CHAPS, N-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; CHO, Chinese hamster ovary.

* This study was supported in part by grants from the Human Frontier Science Program (RG-386/92), the Université Pierre et Marie Curie, and an Economic European Community Concerted Action Grant BIO2-CT92-0205. C. Yang and V. I. Slepnev contributed equally to this work. 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.

‡ Fellow from the Association Française contre les Myopathies.

§ To whom correspondence should be addressed: Unité de Génétique Somatique (URA CNRS 361), Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France. Tel: 33-1-45-68-55-68; Fax: 33-1-43-61-31-71.
GDI and these GDI-like proteins exist. To address this point, we have used Mono Q chromatography to characterize cytosolic Rab protein complexes in various cell types and tissues. We show that, while the majority of Rab proteins are complexed with Rab3A GDI in brain and RINm5F cells, they are complexed with GDI β in CHO cells. In rat liver cytosol, about half of the Rab proteins were found to be complexed with Rab3A GDI, the other half being associated with GDI β. Interestingly, the proportion of cytosolic Rab proteins complexed with either Rab3A GDI or GDI β appears to correlate with the respective amounts of both proteins in the different cytosols analyzed in this study.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mono Q HR 5/5 and PC 1.6/5, Superdex HR 75 columns, and Q Fast Flow (QFF) resin were obtained from Pharmacia-France. QFF chromatography was performed with a fast protein liquid chromatography system (Pharmacia Biotech Inc.) and other columns were adapted to the Smart System (Pharmacia). (±)-[3H]GTP (specific activity, 3000 Ci/mmol), [3H]-Protein A, and the ECL kit were purchased from Amersham.

**Antibodies**—Rabbit antibodies raised against Rab1A, Rab2, Rab4A, and Rab6 proteins were described elsewhere (25, 26). Rabbit anti-Rab3A GDI antibody (raised against a synthetic COOH-terminal peptide of Rab3A GDI) was kindly provided by Dr. Dr. Jean-Pierre Abastado, Institut Pasteur (France) and with the pSV2-histidinol plasmid (a gift of Dr. Bernard Amersham, Amersham, UK) and the rabbit antibody against Rab3A was a gift of Dr. Marino Zerial (EMBL, Germany) and the rabbit antibody against Rab3A was a gift of Dr. François Darchen (IBPC, Paris). We generated for this study a rabbit polyclonal monoclonal antibody 9E10 specific against the c-myc proto-oncogene product (27), was used to detect myc epitope-tagged Rab6. Mouse monoclonal antibody 9E10, reactive with the epitope EKQKLISEEDLN derived from the c-myc proto-oncogene product (27), was used to detect myc epitope-tagged Rab6.

**Preparation of Cytosols**—CHO and RINm5F cells were trypsinized when grown on Petri dishes or collected by centrifugation when grown in suspension and washed twice in phosphate-buffered saline. All subsequent steps were carried out at 4 °C. Cells were resuspended in Hepes buffer (30 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors containing 1 μg/ml final concentration of leupeptin, aprotinin, chymostatin, antipain, and pepstatin) and homogenized using a Branson-type homogenizer as described previously (25). Mouse brains and rat livers were obtained immediately after slaughter, washed several times with ice-cold Hepes buffer, and then homogenized with a Dounce homogenizer. Cell or tissue homogenates were centrifuged for 10 min at 600 g to remove cell debris, nuclei, and aggregates and then for 1 h at 4 °C at 150,000 × g. High speed supernatants were considered as cytosols and the corresponding pellets as membrane fractions.

**Mono Q Chromatography**—Cytosols were filtered through a 0.22-μm filter (Milipore) and then applied to a 1-ml Mono Q HR 5/5 column. After washing with 2 ml of Hepes buffer, elution was performed with a linear gradient of NaCl (0-400 mM) in the same buffer at a flow rate of 0.4 ml/min. 0.5-ml fractions were collected and analyzed for their content in small GTP-binding proteins and Rab proteins.

**Cross-linking Experiments**—About 1 mg of cytosolic proteins from CHO, 0.1 mg of cytosolic proteins from mouse brain, and aliquots of the different fractions obtained after Mono Q chromatography were preincubated with 0.1 mM GDP for 15 min at 30 °C. 5 mM EGS (ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid) was then added for 45 min at 30 °C. The reaction was stopped by the addition of 10 mM monoothanolamine.

**Purification of GDI β from CHO Cells**—About 250 mg of cytosolic proteins were prepared from 3 × 10⁷ CHO cells grown in roller flasks. The extract was loaded onto a 15-ml QFF column equilibrated in buffer A (30 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 0.5% CHAPS, 0.1 mM phenylmethylsulfonyl fluoride). After washing with 30 ml of buffer A, proteins were eluted with 150 ml of a linear gradient of NaCl (0-400 mM). 5-ml fractions were collected. 50 μl of each fraction were used to test their ability to extract myc epitope-tagged Rab6 from membranes (see above).

**Release of myc Epitope-tagged Rab6 from Membranes**—Membranes were prepared from CHO cells expressing myc epitope-tagged Rab6 as described above and washed once in Hepes buffer to eliminate contaminant cytosolic proteins. 7 μg of membrane proteins were then loaded onto a 15-ml Mono Q HR 5/5 column equilibrated in buffer A (30 mM Hepes, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 0.5% CHAPS). After washing with 30 ml of buffer A, proteins were eluted with 150 ml of a linear gradient of NaCl (0-400 mM). 2-ml fractions were collected and analyzed for GDI activity by the GTP overlay assay as described previously (28). Rab3A GDI, GDI β, and Rab proteins were detected using specific antibodies and peroxidase-labeled anti-rabbit IgG antibody (ECL protocol), alkaline phosphatase-labeled anti-rabbit or anti-mouse IgG antibody (Promega), or 125I-Protein A as described previously (25). Specific bands were quantitated by scanning immunoblots and autoradiograms on a Master Scan (Biorad).

**Release of myc Epitope-tagged Rab6 from Membranes**—Membranes were prepared from CHO cells expressing myc epitope-tagged Rab6 as described above and washed once in Hepes buffer to eliminate contaminant cytosolic proteins. 7 μg of membrane proteins were then incubated with aliquots of the different fractions collected throughout the purification procedure of GDI β for 45 min at 30 °C in 25 mM Hepes, pH 7.5, 5 mM MgCl₂, 0.3 mM sucrose, 0.2 mM GDP, and protease inhibitors. The samples were centrifuged at 4 °C for 15 min at 150,000 × g in a TL100 Beckman centrifuge. Supernatants were then boiled in Laemmli's sample buffer (29), electrophoresed, and blotted onto nitrocellulose. myc epitope-tagged Rab6 was revealed using the 9E10 antibody.
Rub Proteins Complexed with GDI Isoforms

**RESULTS**

Rab proteins occur as complexes in cytosol, as detected by gel filtration or sucrose gradient sedimentation (16, 21). It is generally thought that these complexes consist of Rab and Rab3A GDI proteins in a 1:1 ratio. Since cDNAs encoding potential candidates for Rab3A GDI isoforms have been recently isolated (22, 23), we decided to characterize in further detail these complexes in order to determine whether cytosolic Rab proteins are complexed with Rab3A GDI or with isoforms of this protein. For this purpose, we chose to analyze various cytosols prepared from different cell and tissue extracts by Mono Q chromatography. Small GTP-binding proteins present in the eluted fractions were detected by the GTP overlay assay. The presence in these fractions of several Rab proteins (Rab1A, Rab2, Rab3A, Rab4A, and Rab6) and that of Rab3A GDI was also monitored by using specific antibodies raised against these proteins. Under the experimental conditions used in this study, the majority (>95%) of the small GTP-binding proteins were retained on the Mono Q column (data not shown).

The Majority of Rab Proteins Are Complexed with Rab3A GDI in Brain Cytosol and to Another Protein in CHO Cytosol—

The chromatography of brain cytosol (Fig. 1A) showed that the majority of small GTP-binding proteins, as well as most of Rab1A, Rab3A, Rab4A (data not shown), and Rab6 co-eluted with Rab3A GDI at an ionic strength of 250–350 mM NaCl. To test whether GTP-binding proteins and Rab proteins were complexed with Rab2A GDI in these fractions, they were cross-linked with EGS. After cross-linking, the majority of small GTP-binding proteins migrated with an apparent molecular mass of around 80 kDa (Fig. 1B). The presence of Rab3A GDI in this band was revealed by immunoblot analysis (data not shown). The GTP overlay assay preferentially reveals proteins of the Rab and Ral families (32). In addition, Ral proteins do not interact with Rab3A GDI. The ~80-kDa species then most likely correspond to cross-linked 1:1 complexes formed between Rab3A GDI (which migrates on SDS gels with an apparent molecular mass of 55 kDa, see Fig. 3B) and Rab proteins (21–28 kDa). It should be pointed out that Rab proteins eluted from the Mono Q column in two peaks (Fig. 1A). Most of Rab1A and Rab6, as well as of Rab4A (data not shown), were detected in the first one (fractions 11–13), whereas the majority of Rab3A was detected in the second peak (fractions 15–17). This elution profile can be explained by the fact that Rab3A is one of the most acidic Rab proteins (pI 4.7) as determined by two-dimensional gel electrophoresis (33). Complexes formed between Rab3A and Rab3A GDI are therefore expected to elute at a higher ionic strength than Rab3A GDI complexed with other members of the Rab family.

This experiment confirms that Rab proteins are complexed with Rab3A GDI in brain cytosol, as has been previously documented (16). It also indicates that Rab proteins do not dissociate from Rab3A GDI at the ionic strength allowing the elution of complexes from the Mono Q column. This was further supported by *in vitro* studies showing that Rab3A GDI-Rab complexes are stable up to 0.5 M NaCl concentration (data not shown). Mono Q chromatography thus provided an accurate...
method to analyze cytosolic Rab complexes.

The chromatography on the same Mono Q column of a cytosol prepared from CHO cells gave a very different profile (Fig. 2A). In order to facilitate the detection of Rab complexes, we used a CHO cell line stably expressing myc epitope-tagged Rab6 at about 5-fold the endogenous level of Rab6. The majority of small GTP-binding proteins present in CHO cytosol was eluted from the Mono Q column at lower ionic strength (around 100-150 mM NaCl, corresponding to fractions 6-9) than in brain cytosol (Fig. 2A). Only a minor fraction of GTP-binding proteins (around 10%) was found to co-elute with Rab3A GDI (fractions 11-12). To demonstrate the presence of complexes in the different fractions, they were cross-linked with EGS and analyzed by the GTP overlay assay (Fig. 2B). Cross-linking of fraction 12 generated complexes of the same size as those detected in cross-linked brain cytosol and as such probably correspond to Rab proteins complexed with Rab3A GDI. In contrast, cross-linking of fraction 8, in which the majority of the small GTP-binding proteins were recovered, generated complexes of smaller size migrating with an apparent molecular mass around 70 kDa. Similar size complexes were obtained after cross-linking unfractionated cytosol (Fig. 2B). This suggests that the majority of Rab proteins were not complexed with Rab3A GDI but to another protein with a smaller molecular weight. This was confirmed by using specific antibodies against several Rab proteins. As shown in Fig. 2A, Rab1A as well as endogenous Rab6, eluted with the bulk of small GTP-binding proteins. Rab2 and Rab4A were also found in fractions 6-9 (data not shown). myc epitope-tagged Rab6, which contains 4 additional acidic residues, was eluted from the Mono Q column at a higher NaCl concentration than endogenous Rab6 (fraction 10). After cross-linking of proteins present in fraction 10 with EGS, the majority of myc epitope-tagged Rab6 was detected in a band corresponding to a ~70-kDa complex (Fig. 2C). In contrast, cross-linking of fraction 12, in which only a minor amount of myc epitope-tagged Rab6 was present, but which contains Rab3A GDI, revealed the presence of Rab6 in a ~80-kDa complex. This result demonstrates that the majority of myc epitope-tagged Rab6 was complexed to a protein with a smaller apparent molecular mass than Rab3A GDI.

**Purification of the Protein Complexed with Cytosolic Rab Proteins in CHO Cells**—The above result prompted us to purify the protein which forms cytosolic complexes with Rab proteins in CHO cells. About 250 mg of cytosolic proteins prepared from wild-type CHO cells were loaded onto a QFF anion exchange column and eluted with a linear gradient of NaCl. Since one of the properties of Rab3A GDI is to induce the dissociation of Rab proteins from membranes (20), we tested each fraction for its ability to remove membrane-bound myc epitope-tagged Rab6. As expected, fractions which contained Rab3A GDI were able to dissociate Rab6 from membranes. However, a significant release of myc epitope-tagged Rab6 was observed in fractions which did not contain detectable levels of Rab3A GDI, suggest-
Rab Proteins Complexed with GDI Isoforms

Fig. 3. Partial purification from CHO cytosol of a ~47-kDa protein that displays a GDI-like activity. A, 1 pl of fractions 13–18 collected at the final step of the purification procedure (Mono Q column, see “Experimental Procedures”) were tested for their ability to remove myc epitope-tagged Rab6 from membranes as described under “Experimental Procedures” (upper panel). The GDI-like activity correlates with the presence in the fractions of a protein (arrowhead) migrating above the 45-kDa marker (lower panel). B, comparison of the electrophoretic mobility of the ~47-kDa protein (fraction 14, arrowhead) with that of Rab3A GDI purified from bovine brain.

The ~47-kDa protein was then excised, digested with the Lys-specific endoproteinase, and the resulting peptides were purified by high pressure liquid chromatography. The chromatographic profile of the digested protein was found to be close to that of Rab3A GDI (data not shown). However, several peptides were different and some of them were microsequenced. We show in Fig. 4 the amino acid sequences of three of these peptides. Of the 32 amino acids sequenced, no difference was found with the predicted sequence of GDI whose cDNA has been recently cloned from a rat brain library (22). Only two differences were found with GDI-2, a novel GDI isoform characterized from mouse skeletal muscle (22). On the other hand, we found more differences (7 out of 32) between the sequenced peptides and those corresponding to Rab3A GDI (bovine) or GDI α and GDI-1, the rat and mouse counterparts of Rab3A GDI, respectively (17, 22, 23). The protein which forms cytosolic complexes in CHO cells is then most likely GDI β.

GDI β from CHO cells migrates with a slightly faster mobility on SDS-PAGE than Rab3A GDI (Fig. 3B). On the other hand, rat GDI β has the same predicted molecular weight as Rab3A GDI (51,000) (22). We found, however, that *Escherichia coli* expressed mouse GDI β, whose cDNA has been recently cloned in our laboratory, migrates ~at 47 kDa (data not shown). This indicates that GDI β and Rab3A GDI do not have the same apparent masses as determined by SDS-PAGE. This observation can explain why cross-linked cytosolic Rab protein complexes were found to be of smaller size in CHO cells (~70 kDa) as compared with complexes formed with Rab3A GDI in brain cytosol (~80 kDa). In addition, the predicted isoelectric point of rat GDI β (pI 5.7) is more basic than that of bovine Rab3A GDI (pI 4.8), as calculated by the Genetics Computer Group software. Comparable values were obtained by two-dimensional gel electrophoresis (see Fig. 7). This likely explains why Rab-GDI complexes were eluted from the Mono Q column at lower ionic strength than Rab/2 Rab3A GDI complexes (Figs. 1 and 2).

Depending on Cell Types, a Variable Fraction of Rab Proteins Are Complexed with Either GDI β or Rab3A GDI—In order to extend the above observations, we analyzed various cytosols by Mono Q chromatography. Fig. 5 illustrates the chromatographic profile of a cytosol prepared from insulin-secreting RINm5F cells. As in the case of brain cytosol, the majority of small GTP-binding proteins co-eluted with Rab3A GDI at an ionic strength around 250 mM. Especially, Rab3A, which is expressed in this cell line, was recovered in the same fraction (fraction 16) as that in which most of Rab3A present in brain cytosol was collected (Fig. 1A). These results confirm a previous report showing that the majority of cytosolic Rab proteins are complexed with Rab3A GDI in RINm5F cells (16). However, a minor amount of small GTP-binding proteins including Rab1A, Rab6, and Rab4A (data not shown), did not co-elute with Rab3A

2 I. Janoueix-Lerosey, F. Jollivet, and B. Goud, manuscript in preparation.
Rub Proteins Complexed with GDI Isoforms

peptide 1 | peptide 2 | peptide 3
---|---|---
~47 kDa protein | FLVYVANFDEK | EIRPALELLE |
GDI β (rat) | (143) FLVYVANFDEK | (365) EIRPALELLE | (380) FVSISDLFVXP
GDI-2 (mouse) | (143) FLVYVANFDEK | (365) EIRPALELLE | (380) FVSISDLFVXP
GDI α (rat) | (143) FLVYVANFDEK | (365) EIRPALELLE | (380) FVSISDLFVXP
GDI-1 (mouse) | (143) FLVYVANFDEK | (365) EIRPALELLE | (380) FVSISDLFVXP
rab3A GDI (bovine) | (143) FLVYVANFDEK | (365) EIRPALELLE | (380) FVSISDLFVXP

Fig. 4. Comparison of amino acid sequences of three peptides derived from the ~47-kDa protein with those of other GDI proteins. Peptide sequences were compared with those available in data banks. Amino acids which differ between the sequenced peptides and the corresponding ones in the known GDI proteins are underlined. Numbers in parentheses indicate positions of amino acid residues from the NH₂ terminus of GDI proteins. (X) indicates the unknown residue.

![Graph](image1.png)

FIG. 5. Analysis of Rab protein complexes in RINm5F cytosol by Mono Q chromatography. 1.2 mg of RINm5F cytosol was eluted from a Mono Q column and analyzed as described in the legends to Figs. 1 and 2. Most of GTP-binding proteins (GBPs) as well as most Rab1A, Rab3A, and Rab6 co-eluted with Rab3A GDI (fractions 12 and 13). Rab3A was found in the same fraction (fraction 16) after chromatography of brain cytosol (Fig. 1A). However, a minor amount of small GTP-binding proteins eluted at the same ionic strength as the majority of them in CHO cytosol (fractions 7–9).

GDI, but eluted at about the same NaCl concentration as the majority of small GTP-binding proteins present in CHO cytosol (fractions 8 and 9).

The elution profile of small GTP-binding proteins present in rat liver cytosol was found to be intermediate between the one obtained for CHO cytosol and the one found for brain cytosol (Fig. 6). Indeed, about half of the total small GTP-binding proteins, including about half Rab1A and Rab6 as well as Rab2 and Rab4A (data not shown), eluted at the same ionic strength as the majority of these proteins in CHO cytosol (fractions 6–9).

The other half of the GTP-binding proteins co-eluted with Rab3A GDI and was recovered in the same fractions in which small GTP-binding proteins eluted at an ionic strength of 150 mM (fractions 6–9) and the other half co-eluted with Rab3A GDI at a higher ionic strength (fractions 11–13).

![Graph](image2.png)

FIG. 6. Analysis of Rab protein complexes in rat liver cytosol by Mono Q chromatography. 7.8 mg of rat liver cytosol was analyzed by Mono Q chromatography as described in the legends to Figs. 1, 2, and 5. Around half of the total small GTP-binding proteins eluted at an ionic strength of ~70 kDa (fractions 6–9) and the other half co-eluted with Rab3A GDI at a higher ionic strength (fractions 11–13). As the majority of these proteins in CHO cytosol (fractions 6–9).

These results indicate that a minority of Rab proteins in RINm5F cells and about half of the total in rat liver cytosol were not complexed with Rab3A GDI. The fact that these complexes have the same apparent molecular mass (~70 kDa) and eluted at the same ionic strength as in CHO cytosol strongly
suggests that Rab proteins were interacting with GDI β. To confirm this point, fractions 7–9 and 11–12 obtained after chromatography of rat liver cytosol were analyzed by high resolution two-dimensional gel electrophoresis and immunoblotting using an antibody reacting against both Rab3A GDI and GDI β (see Fig. 8 for the characterization of this antibody). As shown in Fig. 7, a protein displaying the same electrophoretic mobility and isoelectric point as GDI β purified from CHO cells was detected in fractions 7–9. On the other hand, the protein that forms complexes with Rab proteins in fractions 11–12 was found to be, as expected, Rab3A GDI.

A Correlation Exists between the Level of Expression of Rab3A GDI and GDI β and the Proportion of Rab Proteins Complexed with Them.—In an attempt to understand why Rab proteins are complexed with either Rab3A GDI or GDI β, we next estimated the relative proportion of these two proteins in the different cytosols that were tested above. These experiments were performed with a polyclonal antibody generated against Rab3A GDI which also recognizes GDI β, as illustrated in Fig. 8 A. This antibody allowed the detection of both proteins in CHO extracts (Fig. 8B). In brain, liver, and RINm5F cytosols, the antibody identified two bands migrating with the same electrophoretic mobility as Rab3A GDI and GDI β. The level of expression of Rab3A GDI was found to be much lower in liver and CHO cytosols as compared with brain and RINm5F cells. In contrast, GDI β appeared to be expressed at the same levels in brain, RINm5F, and liver cytosols (Fig. 8, B and C), confirming previous studies showing that GDI β mRNA is expressed at a similar level in a broad range of tissues (22). However, GDI β was about 4-fold more abundant than Rab3A GDI in CHO cytosol. Interestingly, the above observations point out that a correlation exists between the relative amount of both GDI isoforms and the proportion of Rab proteins complexed with them. When Rab3A GDI is far more abundant than GDI β, such as in brain or RINm5F cells, the majority of Rab proteins form complexes with Rab3A GDI. In contrast, they appear to be complexed with either GDI β or Rab3A GDI when both proteins are expressed at about the same levels, such as in rat liver cytosol. Remarkably, when GDI β is the more abundant GDI, such as in CHO cytosol, it can complex the majority of Rab proteins. Therefore, the simplest interpretation of the above results is that the majority of Rab proteins can form complexes with either GDI isoform and do so in proportion to their relative abundance.
DISCUSSION

Recent studies have illustrated that the function of GDI could be to chaperone Rab proteins throughout their cycle between cytosol and membranes. For instance, Rab3A GDI has been shown to mediate in vitro specific membrane association of Rab5 and Rab9 proteins (34, 35). It is also likely that another important role of GDI is to remove the GDP-bound form of Rab proteins from membranes when they have performed their function, thus completing the Rab cycle (35). These studies have, however, been performed with Rab3A GDI. In this study, we provide the first evidence that Rab proteins form, in fact, in vivo complexes with at least another GDI isoform, GDI β. Interestingly, the proportion of Rab proteins found as cytosolic complexes with either GDI isoform depends on the tissue and cell type. In rat brain and insulin-secreting RINm5F cells, cytosolic Rab proteins are mostly complexed with Rab3A GDI, in good agreement with previous findings (16). In contrast, Rab proteins are mainly complexed with GDI β in CHO cells. An intermediate situation was found for rat liver cytosol, in which roughly half of the small GTP-binding proteins, including Rab1A, Rab2, Rab4A, and Rab6, are complexed with Rab3A GDI and the remaining with GDI β (18). These observations raise the possibility that GDI isoforms might have different affinities for Rab proteins. It will then be of interest to determine whether GDI β is phosphorylated in vivo, as previously shown for Rab3A GDI (39).

In conclusion, the recent discovery of one or two new GDI isoforms has rendered the understanding of GDI function more complicated than previously thought. Clearly, at least two GDI isoforms can regulate Rab function. Further experiments will be required to test whether GDI isoforms are redundant or whether they fulfill different functions.

Acknowledgments—We are grateful to Dr. Marino Zerial for providing us with the antibody against Rab3A GDI and to Dr. François Darchen for the antibody against Rab3A. We thank Dr. Yoshimi Takai for the gift of purified bovine brain Rab3A GDI. We thank Dws. Gordon Langley, Bernard Hofack, Isabelle Janoux-Lerson, and Mary McCaffrey for critical reading of this manuscript. We thank Lucien Cabané (Institut Pasteur) for his help in cell culture and Jacques d’Ailly (Institut Pasteur) for microsequencing.

REFERENCES

1. Valenica, A., Chardin, P., Wittinghofer, A., and Sander, C. (1991) Biochemistry 30, 4657–4664
2. Takai, Y., Kaibuchi, K., Kikuchi, A., and Kawata, M. (1992) Int. Rev. Cytol. 135, 187–220
3. Goud, B. (1992) Semin. Cell Biol. 3, 301–307
4. Novick, P., and Brenwald, N. (1993) Cell 75, 597–601
5. Zerial, M., and Stenmark, H. (1993) Curr. Opin. Cell Biol. 5, 613–620
6. Stüller, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Grompe, M., Tempe, S., and Rothman, J. E. (1993) Nature 365, 518–524
7. Robinson, J. E., and Warren, G. (1994) J. Cell Biol. 123, 229–233
8. Burton, J., Roberts, D., Mestald, M., Novick, P., and De Camilli, P. (1993) Nature 361, 464–467
9. Moya, M., Roberts, D., and Novick, P. (1993) Nature 361, 460–463
10. Burstein, E., and Mecare, I. (1993) Proc. Natl. Acad. Sci. U. S. A. 89, 1154–1158
11. Walworth, N. C., Brenwald, N., Kabacsi, A. K., Garrett, M., and Novick, P. (1992) Mol. Biol. Cell 12, 2017–2028
12. Strom, M., Vollmer, P., Tan, T. S., and Gallwitz, D. (1993) Nature 361, 736–739
13. Kabacsi, A. K., and Gallwitz, D. (1993) J. Cell Biol. 123, 225–235
14. Sasaki, T., Kikuchi, A., Araki, S., Hata, Y., Isomura, M., and Takai, Y. (1990) J. Biol. Chem. 265, 22259–22264
15. Araki, S., Kikuchi, A., Hata, Y., Isomura, M., and Takai, Y. (1990) J. Biol. Chem. 265, 13007–13015
16. Regazzi, R., Kikuchi, A., Takai, Y., and Wollheim, C. B. (1992) J. Biol. Chem. 267, 17342–17349
17. Matsumura, Y., Kikuchi, A., Araki, S., Hata, Y., Kondo, J., Teranishi, Y., and Takai, Y. (1990) Mol. Cell. Biol. 10, 4116–4122
18. Sasaki, T., Takai, K., Kabacsi, A. K., Novick, P. J., and Takai, Y. (1991) Mol. Cell. Biol. 11, 2909–2912

3 M. Asada, K. Kaibuchi, and Y. Takai, unpublished results.
Rub Proteins Complexed with GDI Isoforms

19. Ueda, T., Takayama, Y., Ohmori, T., Ohyama, H., Suitoh, Y., and Takai, Y. (1991) Biochemistry 30, 909-917
20. Ulrich, O., Stenmark, H., Alexandrov, K., Huber, L. A., Kubusch, K., Sasaki, T., Takai, Y., and Zerial, M. (1993) J. Biol. Chem. 268, 18143-18159
21. Soldati, T., Riederer, M. A., and Pfeffer, S. R. (1993) Mol. Biol. Cell 4, 625-634
22. Nishimura, N., Nakamura, H., Takai, Y., and Sano, K. (1994) J. Biol. Chem. 269, 14191-14198
23. Shishowa, A., Södhof, T., and Czech, M. P. (1994) Mol. Cell. Biol. 14, 3459-3468
24. Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lütcke, A., Gruenburg, J., and Zerial, M. (1994) EMBO J. 13, 1287-1296
25. Goud, B., Zahraoui, A., Tavitian, A., and Saraste, J. (1990) Nature 345, 553-556
26. Maridonneau-Parini, I., Yang, C. Z., Bonne, M., and Goud, B. (1991) J. Clin. Invest. 87, 901-907
27. Evan, G., Lewis, G., Ramsay, G., and Bishop, J. (1985) Mol. Cell. Biol. 5, 3610-3616
28. Yang, C.-Z., Mayau, V., Godeau, F., and Goud, B. (1992) Biochem. Biophys. Res. Commun. 182, 1499-1505
29. Laemmli, U. K. (1970) Nature 227, 680-685
30. O'Farrell, P. (1975) J. Biol. Chem. 250, 4007-4021
31. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
32. Bhullar, R. (1992) FEBS Lett. 298, 61-64
33. Huber, L. A., Ulrich, O., Takai, Y., Lütcke, A., Dupree, P., Olkkonen, V., Virta, H., de Hoop, M. J., Alexandrov, K., Peter, M., Zerial, M., and Simons, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7874-7878
34. Soldati, T., Shapiro, A. D., Dirac Svejstrup, A. B., and Pfeffer, S. R. (1994) Nature 369, 76-78
35. Ulrich, O., Horiuchi, H., Bucci, C., and Zerial, M. (1994) Nature 368, 157-160
36. Darchen, F., Zahraoui, H., Hammel, F., Monteils, M.-P., Tavitian, A., and Scherman, D. (1994) EMBO J. 13, 1287-1296
37. Fischer von Mollard, G., Mignery, G., Baumert, M., Perin, M., Hanson, J., Burger, P., Jahn, R., and Sudhof, T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5692-5696
38. Matteoli, M., Takei, K., Cameron, R., Hurlbut, P., Johnston, P. A., Südhof, T. C., Jahn, R., and De Camilli, P. (1991) J. Cell Biol. 115, 625-633
39. Steege-Mortimer, O., Gruenburg, J., and Clague, M. J. (1995) FEBS Lett. 326, 313-318