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Different Rab GTPases Associate Preferentially with α or β GDP-Dissociation Inhibitors

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GDIs (GDP-dissociation inhibitors) bind to Rab GTPases and mediate their membrane targeting and recycling. *In vitro,* most Rabs can bind to either of the major isoforms of GDI, leading to the assumption that the proportion of each specific Rab/GDI complex *in vivo* reflects the relative abundance of the α versus β forms of GDI. Here we show that when human teratocarcinoma cells (Ntera2) are induced to differentiate into postmitotic neurons (NT2N), there is a major change in the proportion of GDIα relative to GDIβ. Under these conditions, certain Rab GTPases associate preferentially with either GDIα or GDIβ, irrespective of the relative abundance of the GDI isoform. These findings suggest that heretofore unrecognized functional specificity may exist between the two major forms of GDI.

Key Words: **Rab; GDP-dissociation inhibitor; GDI; GTPase.**

In mammalian cells different Rab GTPases modulate vesicular transport between discrete intracellular membrane compartments (1–4). It remains to be determined exactly how Rab proteins control vesicle budding and/or fusion. However, accumulated evidence suggests that the mechanism involves the active cycling of the GTPases on and off membranes in a manner that depends on their guanine nucleotide state (5). Cytosolic proteins termed Rab GDP-dissociation inhibitors (GDIs), which form 1:1 complexes with Rab proteins in the GDP-bound state (6–8), play a key role in targeting Rab proteins to budding vesicles and recycling inactive Rabs after vesicle fusion at the acceptor membrane (9–12).

Cells are generally thought to contain between 30 and 40 different Rab proteins, but only a few distinct GDIs have been identified (13, 14). The mammalian GDIs can be subdivided into two general classes (α and β), based on their mobilities on SDS polyacrylamide gels and sequence similarities. The α GDIs migrate at 50–55 kDa and include the original bovine Rab3A-GDI (7, 14), rat GDI-α (13), human GDI-α (15), and mouse GDI-1 (16). The β GDIs migrate at 45–50 kDa and include rat GDI-β (13), human GDI-β (13) and two mouse proteins termed GDI-β (17) and GDI-2 (16). The relative expression of the α and β forms of GDI varies in different tissues. For example, GDIα predominates in brain and insulin-secreting cells, whereas GDIβ is the major form in liver and CHO cells (18). Most Rabs are capable of associating with either the α or β form of GDI (19), although it remains unclear whether the resulting complexes are functionally equivalent. It has been proposed that the proportion of each specific Rab-GDI complex in a particular tissue reflects the relative abundance of the α versus β forms of GDI expressed in that tissue (18). In the present study, we tested this hypothesis in the Ntera2 cell culture model, where differentiation of the teratocarcinoma stem cells into mature neurons is accompanied by a marked increase in the proportion of GDIα relative to GDIβ. Surprisingly, we find that some Rabs associate preferentially with either GDIα or GDIβ, even when the particular GDI is not the predominant form expressed in the cells. These findings contradict the prevailing notion that Rab GTPases associate indiscriminately with GDIs and raise the possibility that important functional differences may exist between the GDI isoforms.

EXPERIMENTAL PROCEDURES

Antibodies. An affinity-purified rabbit IgG was generated against a peptide corresponding to amino acids 1–17 of bovine brain GDIα as described (20). This antibody is currently available from Zymed Laboratories (South San Francisco, CA). Polyclonal antibodies against the following Rab proteins were obtained from the indicated sources: Rab1B and Rab3A (Zymed Laboratories), Rab5 and Rab6 (Santa Cruz Biotechnology, Santa Cruz, CA).

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Cell culture and fractionation. Ntera2 teratocarcinoma cells were maintained in Dulbecco's modified Eagle medium containing 10% (v/v) fetal bovine serum. The cells were induced to differentiate into mature post-mitotic neurons by treatment with retinoic acid and mitotic inhibitors as described (21). For immunoblot analysis of total cellular GDI or Rab proteins, cells were harvested, collected by centrifugation at 500*g* for 10 min, and disrupted directly in SDS–PAGE sample buffer (22). Protein concentration was determined by the method of Bradford (23), using the BioRad kit (Hercules, CA). To obtain soluble and particulate fractions, pelleted cells were disrupted in an equal volume of 100 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 0.1 mM GDP with complete mini-EDTA free protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and the lysate was centrifuged at 100,000*g* for 30 min. Soluble and particulate fractions were then mixed with SDS sample buffer and assayed for protein concentration.

Immunoblot analyses. Aliquots of whole-cell lysate, or soluble or particulate fractions containing 30 μg of protein, were subjected to SDS–PAGE and immunoblot analysis on Immobilon membranes (Millipore Corp., Bedford, MA) using established procedures described previously (24). In all cases, horseradish peroxidase-conjugated goat anti-rabbit IgG and ECL reagent (Amersham Pharmacia Biotech, Arlington Heights, IL) were used to detect bound primary antibody on the blots.

Gel filtration chromatography. For size-exclusion chromatography studies, soluble fractions derived from six 100-mm cultures of NT2N neurons or three confluent cultures of Ntera2 cells were prepared as described above. For each preparation, 100 μl of cytosol was loaded on a 30 cm × 7.8 mm G2000SWXL column (TosoHass Inc., Montgomeryville, PA) on a Beckman System Gold HPLC system, and proteins were eluted at flow rate of 1 ml/min. The elution buffer was the same as the cell lysis buffer, except that it was supplemented with 0.15 M NaCl. Fractions (0.5 ml) were collected and mixed with 0.1 ml of 5× SDS sample buffer. For each set of immunoblots, one quarter of each fraction was subjected to SDS–PAGE and the proteins were transferred to Immobilon membrane. The top portion of each membrane, containing proteins between 40 and 100 kDa, was incubated with the antibody against GDI. The lower portion of the same membrane was immunoblotted with antibody against Rab1B, Rab3A, Rab5, or Rab6. Chemiluminescent signals were quantified with a Lumi-Imager system (Roche).

RESULTS AND DISCUSSION

The bovine brain GDI, first isolated by Takai and coworkers (7, 14, 25) binds specifically to Rab3A and several other Rab proteins, but not to other Ras-related GTPases (i.e., Rho, Cdc42) (9, 10, 19, 26). This GDI is now regarded as the prototype for a family of related proteins which are conserved among yeast, insects, and mammalian cells (27). As mentioned earlier, the mammalian GDIs can be subdivided into two major forms (α and β), based on their mobilities on SDS polyacrylamide gels and their amino acid sequences.

To test the possibility that some Rabs may associate preferentially with a specific form of GDI, we sought a cell culture system where major physiological changes occur in the relative abundance of GDIα versus GDIβ. We chose to study the Ntera2 human teratocarcinoma cell line, which can be induced to undergo neuronal differentiation by treatment with retinoic acid (28, 29). The differentiated cells (NT2N) extend arrays of axonal and dendritic processes (Fig. 1A) (30) and exhibit

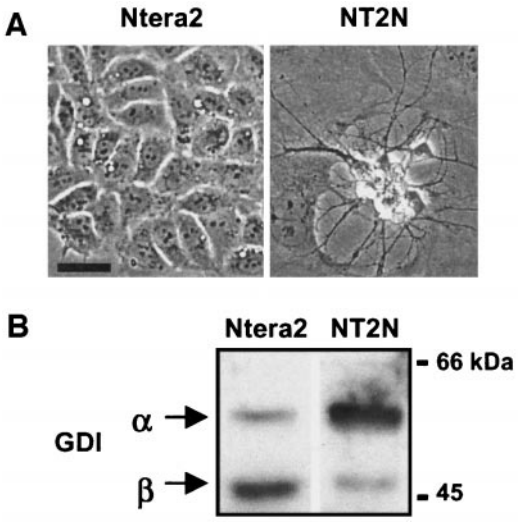

FIG. 1. (A) Phase contrast micrographs showing the typical morphology of Ntera2 teratocarcinoma cells and the mature NT2N neurons after induction of differentiation by retinoic acid and selection with mitotic inhibitors (see Experimental Procedures). The bar equals 10 microns. (B) Relative expression of GDIα and GDIβ determined by immunoblot analysis of 50 μg protein derived from Ntera2 or NT2N cell lysates, using an antibody that recognizes an amino acid sequence found in both forms of GDI. Positions of the molecular mass standards are indicated at the right of the panel.

many properties of mature postmitotic neurons (29, 31, 32). Unlike the Ntera2 stem cells, NT2N neurons also express Rab3A and several other proteins involved in synaptic function (synaptobrevin, SNAP-25, synaptophysin and synapsin) (33).

To facilitate comparison of the major forms of GDI in these cells by Western blot analysis, we generated an antibody against an N-terminal peptide sequence that is common to both GDIα and GDIβ. Using this antibody, we found that the ratio of GDIα to GDIβ undergoes a remarkable change when the undifferentiated Ntera2 cells are induced to differentiate into neurons (NT2N). As shown in Fig. 1B, GDIβ was clearly the predominant isoform in the undifferentiated teratocarcinoma cells. However, when the cells were treated with retinoic acid and mitotic inhibitors, the resulting neurons showed a four- to fivefold increase in GDIα and a two- to threefold decrease in GDIβ. Thus, GDIα became the predominant isoform in the NT2N cells, just as it is in the brain.

We next compared the expression and subcellular distribution patterns for several widely-studied Rab proteins in the Ntera2 cells and the NT2N neurons. Three of the selected Rab proteins function in trafficking pathways that occur in all cell types: Rab1B controls ER → Golgi trafficking (34), Rab5 mediates early steps of the endocytic pathway (35) and Rab6 appears to function in retrograde transport within the *trans*-Golgi network and medial Golgi (36). On the other hand, Rab3A has a specialized function in the regu-

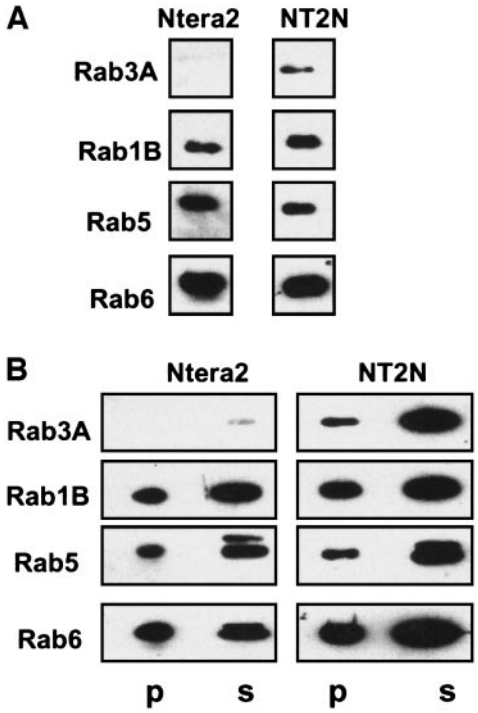

FIG. 2. Expression and subcellular distribution of Rab proteins in Ntera2 cells and NT2N neurons. (A) Equal aliquots of whole-cell lysate were immunoblotted with monospecific antibodies against the indicated Rab proteins as described under Materials and methods. (B) Cells were partitioned by centrifugation into soluble (s) and particulate (p) components, and Rab distribution was determined by immunoblot analysis of 30 μg protein from each fraction. All of the panels in the illustration are taken from the region of the blot between the 21- and 30-kDa markers.

lated exocytosis of neurotransmitters in nerve terminals (37). As expected, Rab3A was readily detected in the NT2N neurons but was nearly absent from the Ntera2 stem cells (Fig. 2A). Each of the other Rabs was expressed at a similar level in the undifferentiated cells compared with the differentiated neurons (Fig. 2A). In every case, at least half of the total Rab protein was localized in the soluble fraction (Fig. 2B), consistent with the typical presence of a large cytoplasmic Rab/GDI pool in mammalian cells (7, 8, 38).

Previous studies have used membrane extraction assays (19) and ion-exchange chromatography (18) to show that several different Rab proteins can associate with either GDIα or GDIβ. However, it is difficult to establish quantitative recovery of different endogenous Rab/GDI complexes by these methods. The use of co-immunoprecipitation assays to detect different Rab proteins bound to endogenous GDIs (39) is problematic because the available antibodies against GDI do not recover endogenous Rab/GDI complexes quantitatively, and the Rab/GDI complexes can be disrupted by non-ionic detergents added to the immunoprecipitation buffers to minimize non-specific protein interactions. As an alternative approach, size-exclusion chromatography has been used to distinguish GDI-bound Rabs, which elute as a broad peak around 80 kDa, from monomeric Rab GTPases, which elute around 25 kDa (10, 40). We recently developed a modified size-exclusion method that can partially resolve the Rab/GDIα and Rab/GDIβ complexes, based on their distinct elution profiles (20). Therefore, we decided to use this method to determine whether the partitioning of different Rabs between the GDIα and GDIβ pools might change in relation to the marked alterations in GDI isoform expression that occur in the Ntera2 versus NT2N cells.

Figure 3 illustrates the chromatography assay, using cytosol derived from NT2N neurons. Immunoblots performed on individual fractions with the common GDI antibody revealed two well-separated bands corre-

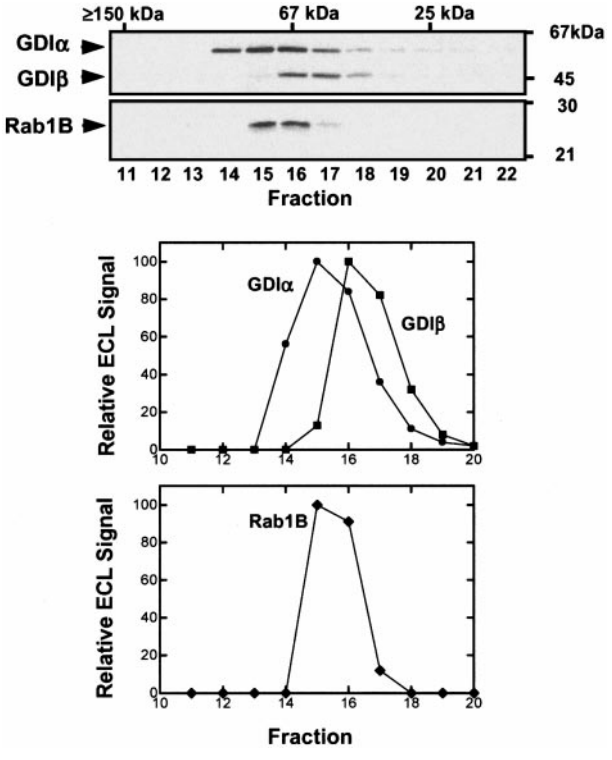

FIG. 3. Size exclusion chromatography assay to detect endogenous Rab/GDI complexes in NT2N cells. A 100-μl aliquot of cytosol was subjected to chromatography as described under Materials and Methods. Individual fractions were subjected to SDS–PAGE and immunoblot analysis. The upper portion of the blot was incubated with an antibody that recognizes the α and β forms of GDI. The lower portion of the blots were probed with a monospecific antibody to Rab1B. Elution positions of chymotrypsinogen (25 kDa), bovine serum albumin (67 kDa), and alcohol dehydrogenase (150 kDa) or dextran blue (≥150 kDa) are indicated at the top of the blot. Early fractions above 150 kDa did not contain immunoreactive proteins. The relative elution positions of each protein are represented graphically in the lower panels: The peak heights do not reflect the actual quantities of the proteins, since the scale for each protein was normalized by setting the maximum chemiluminescent signal for that protein to 100, and relating the signals in all other fractions to the maximum value. Symbols are as follows: GDIα (●), GDIβ (■), Rab1B (◆).

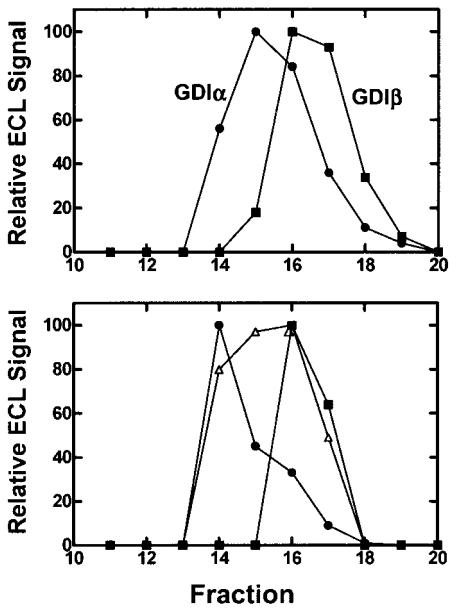

FIG. 4. Chromatographic distributions of Rab3A, Rab5, and Rab6 in differentiated NT2N neurons. Cytosol was subjected to size-exclusion chromatography as described in the legend to Fig. 3. Equal aliquots were removed from each fraction and subjected to SDS–PAGE and immunoblot analysis, using antibodies against Rab3A (△), Rab5 (■), and Rab6 (●). The upper panel shows the elution profiles of GDIα (●) and GDIβ (■) from the same column fractions.

sponding to GDIα (55 kDa) and GDIβ (46 kDa). Although the two forms of GDI were not completely separated by on the column, their elution profiles were staggered, with the α isoform eluting earlier than the β isoform. When the same column fractions were immunoblotted with an antibody against Rab1B, no monomeric protein was observed; i.e., all of the Rab1B in the cytosol was eluted in fractions containing GDI. The similar elution patterns of Rab1B and GDIα suggested that most of the Rab1B was associated with the α isoform (fractions 15 and 16). Although GDIβ is also present in fraction 16, it does not appear that much Rab1B is associated with the β isoform, since fraction 17 contains nearly the same amount of GDIβ, but very little Rab1B.

Using the same method, we also examined the distributions of Rab5, Rab6 and Rab3A in the NT2N neurons (Fig. 4). As in the case of Rab1B, Rab6 was found predominantly in the earlier fractions containing GDIα. In contrast, the shifted elution profile of Rab5 suggested that it was associated mainly with GDIβ, despite the fact that the β isoform is far less abundant than the α isoform in the neurons (see Fig. 1B). The gel filtration profile of Rab3A differed from that of the other Rabs insofar as the GTPase was eluted as a broad peak that spanned fractions containing both GDIα and GDIβ. Thus, Rab3A may exist in complexes with both GDI isoforms.

We next asked whether Rab1B, Rab5 and Rab6 might be distributed differently between the two GDI pools in the undifferentiated Ntera2 cells, where the ratio of GDIα to GDIβ is essentially inverted in comparison to the NT2N neurons (see Fig. 1B). The results shown in Fig. 5 underscore the following points: First, in contrast to the NT2N neurons, where Rab1B was co-eluted with GDIα, Rab1B in the Ntera2 cells was found predominantly in fractions containing GDIβ. Second, unlike the distribution of Rab1B, which shifted with the change in the relative abundance of GDIβ versus GDIα, the distribution of Rab6 was unchanged. That is, Rab6 still eluted with GDIα, despite the fact that GDIβ was the most prevalent isoform in the Ntera2 cells. Finally, the elution profile of Rab5, which paralleled that of the minor GDIβ isoform in the NT2N cells, was the same in the Ntera2 cells, where GDIβ was the major isoform.

Although the preceding gel filtration studies cannot completely rule out the possibility that the Rab proteins eluting in the fractions with GDIα and GDIβ are physically associated with proteins other than GDI, we believe that this is unlikely. Such unidentified proteins would need to be sufficiently abundant to bind the majority of cytosolic Rab proteins and would have to form a complex that is coincidentally the same size as Rab/GDI. The only other endogenous soluble proteins known to form cytosolic complexes with Rabs are the Rab Escort Proteins (REP), which function transiently

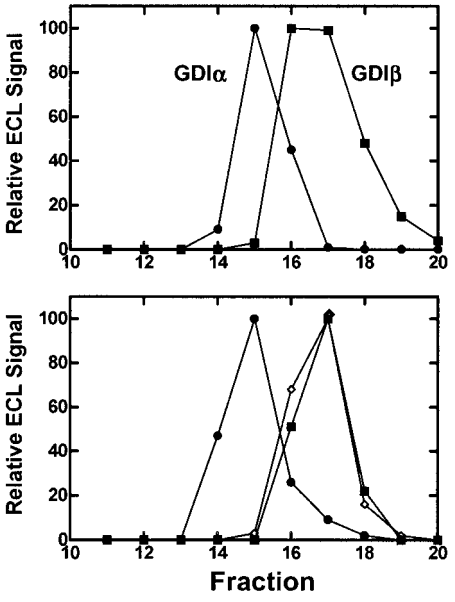

FIG. 5. Chromatographic distributions of Rab1B, Rab5, and Rab6 in undifferentiated Ntera2 teratocarcinoma cells. Cytosol was subjected to size-exclusion chromatography as described in the legend to Fig. 3. Equal aliquots were removed from each fraction and subjected to SDS–PAGE and immunoblot analysis, using antibodies against Rab1B (□), Rab5 (■), and Rab6 (●). The upper panel shows the elution profiles of GDIα (●) and GDIβ (■) from the same column fractions.

in the prenylation reaction (41). However, REP is larger than GDI, and the Rab/REP complex typically elutes around 150 kDa on size exclusion columns (24, 41).

Based on the preceding observations, we conclude that contrary to the commonly accepted view, certain Rab proteins may interact preferentially with either GDIα or GDIβ, even when the particular isoform is not the predominant GDI species expressed in a given tissue or cell type. Rab5 represents a good example insofar as it co-migrates on the size exclusion column with GDIβ, regardless of whether GDIβ is the major isoform (as in the Ntera2 cells) or is only a small fraction of the total GDI (as in the NT2N neurons). Rab6 shows the opposite specificity, associating mainly with GDIα, irrespective of large differences in the relative abundance of the α isoform in NT2N versus Ntera2 cells. Nevertheless, it is important to note that preferential association of specific Rabs with specific GDIs is not a general rule. This is clearly illustrated by the behavior of Rab1B, which seems able to associate with either GDIα (in differentiated NT2N cells) or GDIβ (in undifferentiated Ntera2 cells), depending on which isoform is most abundant.

Although various Rab proteins have been successfully reconstituted in GDIα complexes that can deliver the Rabs to appropriate membranes *in vitro* (9–11, 19), GDIβ complexes have not been widely used for this purpose. The present findings suggest that preferential association of certain Rabs with specific forms of GDI occurs in intact cells, raising the possibility that α and β GDI/Rab complexes may have distinct roles in Rab targeting and/or recycling *in vivo*.

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