Isolation and Characterization of a GDP/GTP Exchange Protein Specific for the Rab3 Subfamily Small G Proteins

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The Rab small G protein family, consisting of nearly 30 members, is implicated in intracellular vesicle trafficking. They cycle between the GDP-bound inactive and GTP-bound active forms, and the former is converted to the latter by the action of a GDP/GTP exchange protein (GEP). No GEP specific for each Rab family member or Rab subfamily (5–10). No GEP specific for Rab3 subfamily has been isolated. Here we purified a GEP from rat brain with lipid-modified Rab3A as a substrate. The purified protein was specifically active on Rab3A, Rab3C, and Rab3D of the Rab3 subfamily. Of these subfamily members, Rab3A and Rab3C are implicated in Ca2+-dependent exocytosis, particularly in neurotransmitter release. This GEP (Rab3 GEP) was active on the lipid-modified form, but not on the lipid-unmodified form. Rab3GEP showed a minimum molecular mass of about 200 kDa on SDS-polyacrylamide gel electrophoresis. We cloned its cDNA from a rat brain cDNA library and determined its primary structure. The isolated cDNA encoded a protein with a Mr of 177,982 and 1,602 amino acids, which showed no homology to any known protein. The recombinant protein exhibited GEP activity toward Rab3A, Rab3C, and Rab3D. Northern blot and Western blot analyses indicated that Rab3 GEP was expressed in all the rat tissues examined with the highest expression in brain.

The Rab small G protein family consists of nearly 30 members and implicated in intracellular vesicle trafficking, such as exocytosis, endocytosis, and transcytosis (for reviews, see Refs. 1–6). All the Rab family members have unique C-terminal structures, which undergo posttranslational modifications with geranylgeranyl moieties in most cases. The Rab family members cycle between the GDP-bound inactive and GTP-bound active forms and between the cytosol and membrane fractions. These two types of cycling are essential for their action in vesicle trafficking. The conversion from the GDP-bound form to the GTP-bound form is regulated by two types of regulatory proteins; one is Rab GAP, which stimulates this conversion, and the other is Rab GDI, which inhibits this conversion. The conversion of the GTP-bound form to the GDP-bound form is regulated by Rab GAP. Rab GDI has been isolated and well characterized (6). Rab GDI interacts specifically with the GDP-bound form of all the Rab family members thus far examined and keeps them both in the GDP-bound form and in the cytosol or releases them from the membranes. In contrast to Rab GDI, little is known about Rab GEP and Rab GAP. As for Rab GEP, a yeast GEP, named DSS4, and its mammalian counterpart, named MSS4, have thus far been reported, but MSS4 is not specific for a Rab family member or Rab subfamily (7–10). No GEP specific for each Rab family member or Rab subfamily has been isolated.

The Rab3 subfamily consists of four members, Rab3A, Rab3B, Rab3C, and Rab3D (6). Of these members, Rab3A and Rab3C are implicated in Ca2+-dependent exocytosis, particularly in neurotransmitter release. A GEP active on Rab3A has been partially purified from rat brain, but neither its primary structure nor its precise property has been studied (10–12). Therefore, we have attempted here to isolate a GEP specific for Rab3A or the Rab3 subfamily.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Lipid-modified Rab3A, Rab3B, Rab3C, Rab3D, Rab2, Rab6A, Rab10, and Rab11 were purified from the membrane fraction of Sf9 cells expressing each cDNA (13, 14). Lipid-unmodified Rab3A was purified from Rab3A-overexpressing Escherichia coli as a fusion protein with N-terminal glutathione S-transferase, of which the glutathione-S-transferase carrier was cleaved off from Rab3A by digestion with thrombin (15). MSS4 was purified from MSS4-overexpressing E. coli, as described (10). Rab GDI was purified from bovine brain cytosol (16).

Activity for Rab3 GEP Activity—The Rab3 GEP activity was assayed by measuring the dissociation of [3H]GDP from lipid-modified Rab3A as follows: Rab3A (3 pmol) was incubated for 20 min at 30°C with 3 μM [3H]GDP in a reaction mixture (5 μl) containing 50 mM Tris/Cl at pH 8.0, 5 mM MgCl2, 10 mM EDTA, 0.5 mM DTT, and 0.12% CHAPS. The reaction was stopped by adding 2 μl of 100 mM MgCl2 and 5 μl of a solution containing 50 mM Tris/Cl at pH 8.0, 5 mM MgCl2, 0.5 mM EDTA, and 1 mM DTT. The sample was incubated for 10 min at 30°C with [3H]GDP bound to Rab3A in a reaction mixture (50 μl) containing 50 mM Tris/Cl at pH 8.0, 12 mM MgCl2, 2 mM EDTA, 0.2 mg/ml bovine serum albumin, 12 μM GTPyS, and 0.06% CHAPS. The mixture was applied to a nitrocellulose filter, and the radioactivity retained on the filter was determined by counting. The Rab3 GEP activity to stimulate the binding of [3H]GDP to lipid-modified Rab3A was assayed as described above, except that [3H]GDP and GTPyS were replaced with GDP and [33]GTPyS, respectively.

Purification of Rab3 GEP—All the purification procedures were performed at 0–4°C. The synaptic soluble fraction was prepared from rat brains (17). A half of the fraction (500 ml, 455 mg of protein) was applied to a nitrocellulose filter, and the radioactivity retained on the filter was determined by counting. The Rab3 GEP activity to stimulate the binding of [3H]GDP to lipid-modified Rab3A was assayed as described above, except that [3H]GDP and GTPyS were replaced with GDP and [33]GTPyS, respectively.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) U72995.
adjusted to 0.2 M NaCl and applied to a Q-Sepharose FF column (2.6 × 10 cm) equilibrated with Buffer A (20 mM Tris/Cl at pH 7.5 and 1 mM DTT, 0.6% CHAPS, and 10% glycerol), and centrifuged at 100,000 × g for 1 h. The supernatant was also subjected to the successive column chromatographies in the same manner as described above. The active fractions of the two successive column chromatographies, Rab3 GEP, appeared in a single peak. The active fractions were collected and used as recombinant Rab3 GEP.

RESULTS

Rab3 GEP was purified from the synaptic soluble fraction of rat brain with lipid-modified (geranylgeranylated and methylated) Rab3A as a substrate by column chromatographies, including Q-Sepharose, phenyl-Sepharose, hydroxyapatite, Mono Q, and Superdex 200 column chromatographies. On these column chromatographies, Rab3 GEP appeared in a single peak. The GEP activity well coincided with one protein with a molecular mass of about 200 kDa on the last column chromatography (Fig. 1A). When this sample was further subjected to re-hydroxyapatite column chromatography, Rab3 GEP appeared in two peaks (Rab3 GEPI and GEPII), but its activity of

![A GEP Specific for the Rab3 Subfamily](image)

**FIG. 1. Purification of Rab3 GEP.** A, Superdex 200 column chromatography. The dissociation of [3H]GDP from Rab3A (3 pmol) was assayed by incubating with an aliquot (4 µl) of each fraction. ●, [3H]GDP bound; ––––, absorbance at 280 nm. An aliquot (20 µl) of each fraction was subjected to SDS-PAGE (6.5% polyacrylamide gel), followed by silver staining. B, second hydroxyapatite column chromatography. The dissociation of [3H]GDP from Rab3A (3 pmol) was assayed by incubating with an aliquot (4 µl) of each fraction. ●, [3H]GDP bound; ––––, absorbance at 280 nm. An aliquot (20 µl) of each fraction was subjected to SDS-PAGE (6.5% polyacrylamide gel), followed by silver staining.

![Graphs showing purification](graphs)

29–33 and 34–38 (see Fig. 1B). The first (5 ml, 15.5 µg of protein) and second (5 ml, 7.5 µg of protein) peaks were separately collected as Rab3 GEPI and GEPII, respectively, and stored at −80 °C.

Peptide Mapping of Rab3 GEP and Molecular Cloning of the Rab3 GEP cDNA—Purified Rab3 GEPI (20 µg of protein) and GEPII (10 µg of protein) were separately subjected to SDS-PAGE (6.5% polyacrylamide gel). Each protein band corresponding to a protein with a molecular mass of about 200 kDa was cut out from the gel, digested completely with a lysyl endopeptidase, and subjected to C18 reverse phase high pressure liquid column chromatography (18). The amino acid sequences of the peptides were determined with a peptide sequencer. To determine the N-terminal amino acid sequence of Rab3 GEPII, purified GEPII (4 µg of protein) was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The protein band was cut from the membrane and directly subjected to the peptide sequencer. A rat brain cDNA library in AZAP II (Stratagene) was screened using the oligonucleotide probes designed from the partial amino acid sequences (19). DNA sequencing was performed by the dideoxy nucleotide termination method using an ABI373 DNA sequencer.

Expression of Recombinant Rab3 GEP—The cDNA of Rab3 GEP was cloned into the pcMV vector and the construct was transfected to COS7 cells with the DEAE-dextran method (20). The COS7 cells were homogenized with a buffer containing 20 mM Tris/Cl at pH 7.5, 1 mM DTT, 0.6% CHAPS, 0.45% sodium cholate, 10% glycerol, and 0.15 M NaCl. Elution was performed with the same buffer. Fractions of 2 ml each were collected. The Rab3 GEP activity appeared in Fractions 20–29, 30–33, and 34–38 (see Fig. 1B). The first (5 ml, 15.5 µg of protein) and second (5 ml, 7.5 µg of protein) peaks were separately collected as Rab3 GEPI and GEPII, respectively, and stored at −80 °C.
both peaks well coincided with proteins with a molecular mass of about 200 kDa (Fig. 1B).

Rab3 GEP II was inactive on other Rab subfamily members, including Rab2, Rab5A, Rab10, and Rab11 (Fig. 2A). Rab3 GEP II was active on Rab3A and Rab3C, and partially active on Rab3D, but was nearly inactive on Rab3B (Fig. 2B). Rab3 GEP II was active on lipid-modified Rab3A, but not on the lipid-unmodified form (Fig. 3A). These properties of Rab3 GEP II were different from those of MSS4 which was equally active on lipid-modified and -unmodified Rab3A and active on many other Rab family members, including Rab3A, Rab3C, Rab3D, Rab10, and Rab11 (Figs. 2 and 3A). Rab3 GEP II as well as MSS4 was inactive on Rab3A complexed with Rab GDI (Fig. 3B). The properties of Rab3 GEP II, including the requirement for lipid modifications of Rab3A, the substrate specificity, and the sensitivity to Rab GDI, were similar to those of Rab3 GEPI described above (data not shown).

Both the Rab3 GEPI and GEPII proteins with molecular masses of about 200 kDa were accumulated from 800 rat brains by the same series of column chromatographies as described above, and their peptide maps were determined. The peptide maps of these proteins were apparently identical (data not shown). Therefore, the amino acid sequences of the nine peptides of Rab3 GEPII were determined. The N-terminal amino acid sequence of Rab3 GEPII was further determined. On the basis of these amino acid sequences, we cloned a cDNA from a rat brain cDNA library and determined its nucleotide sequence (accession number U72995). The deduced amino acid sequence included all the amino acid sequences of the peptides (Fig. 4). The initial methionine residue appears to be cleaved off after the translation. The encoded protein consisted of 1,602 amino acids and showed a calculated Mr of 177,982. Computer homology search revealed homology to proteins encoded by Caenorhabditis elegans cDNA yk26 g7.5 (accession number U498945) and by human DENN (accession number U44953), of which functions are unknown. The deduced amino acid sequence of yk26 g7.5 protein showed 35% identity over the entire sequence to that of Rab3 GEP. The deduced amino acid sequence of human DENN protein is almost identical over the entire sequence to that of Rab3 GEP, while human DENN protein lacks about 300 C-terminal amino acids. The exact relationship between Rab3 GEPI and GEPII is not known, but it is most likely that Rab3 GEPI is a splicing isoform of Rab3 GEPII, since several splicing isoforms were isolated.

The recombinant protein was prepared from the Rab3 GEP cDNA-transfected COS7 cells. The properties of the recombinant Rab3 GEP, including the requirement for lipid modifications of Rab3A, the substrate specificity, and the sensitivity to Rab GDI, were similar to those of the native Rab3 GEPII described above (data not shown). Northern blot and Western blot analyses indicated that Rab3 GEP was expressed in all the rat tissues examined, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis, with the highest expression in brain (data not shown).
DISCUSSION

We have isolated here for the first time a GEP specific for one Rab subfamily, the Rab3 subfamily, determined its primary structure, and characterized it. Our Rab3 GEP is the most active on Rab3A and Rab3C and partially on Rab3D, but inactive on Rab3B. This substrate specificity is apparently consistent with the similar properties of Rab3A and Rab3C concerning their tissue and subcellular distributions and functions (6, 21). Rab3A and Rab3C are present in cells with a regulated secretion pathway and abundant in brain where both are highly concentrated on synaptic vesicles. Both Rab3A and Rab3C have been implicated in Ca2+-dependent exocytosis, particularly in neurotransmitter release. The tissue distributions of Rab3B and Rab3D are different from those of Rab3A and Rab3C, and their functions remain to be clarified. Because Rab3 GEP is inactive on Rab3B, Rab3B may have its own specific GEP which may be different from Rab3A GEP. GEPs for other small G proteins, including the Ras and Rho family members, have been isolated and characterized (for a review, see Ref. 22). GEPs for the Ras and Rho family members share the common catalytic domains specific for each family. Our Rab3 GEP does not have any homologous region to these GEPs. Our present result that Rab3 GEP is specific for the Rab3 subfamily members suggests that each Rab family member or Rab subfamily has its own specific GEP. It is important to isolate GEPs specific for each Rab family member or Rab subfamily and to know whether they have a common catalytic domain or their own specific catalytic domain.

Rab GDI functions as a regulatory protein for the two types of cycling of the Rab family members between the GDP-bound and GTP-bound forms and between the cytosol and membrane fractions (6). We have shown here that Rab3 GEP is inactive on Rab3A complexed with Rab GDI, suggesting that another factor is further necessary for the conversion. A factor, named GDF, has been shown to be necessary for the dissociation of GDP-Rab5 and GDP-Rab9 from Rab GDI (23, 24). Although Rab5 or Rab9 GDF has not been identified, it has been suggested to be located on the membrane fraction (23, 24). We have shown previously that GDP-Rab3A complexed with Rab GDI stays in the cytosol of nerve terminals, and that rabphilin3 is associated with synaptic vesicles (6). We have recently detected a GDF activity to Rab3A in isolated synaptic vesicles.2 Taken together, the conversion from the GDP-bound form to the GTP-bound form occurs in the proximity of synaptic vesicles where once the GTP-bound form is produced, it is immediately transferred to rabphilin3. It may be noted that Rab3 GEP as well as Rab GDI requires the posttranslational lipid-modifications of Rab3A, whereas rabphilin3 does not. The lipid moieties of Rab3A may be masked by both Rab GDI and Rab3 GEP until it is converted to the GTP-bound form. Once the GTP-bound form is produced, Rab3A interacts with synaptic vesicles through both protein-protein (Rab3A-rabphilin3) and lipid-lipid (geranylgeranyl-vesicle phospholipid) interactions. Thus, the lipid modifications of Rab3A are important to determine the intracellular compartment of Rab3A, its regulators, and target.

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