Localization of the Rab3 Small G Protein Regulators in Nerve Terminals and Their Involvement in Ca\textsuperscript{2+}-dependent Exocytosis*

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The Rab3 small G protein subfamily (Rab3) consists of four members, Rab3A, -B, -C, and -D. We have recently isolated and characterized the Rab3 regulators, GDP/GTP exchange protein (GEP) and GTPase activating protein (GAP), both of which are specific for the Rab3 subfamily. Rab3 GEP stimulates the conversion of the GDP-bound inactive form to the GTP-bound active form, whereas Rab3 GAP stimulates the reverse reaction. Of the four members of the Rab3 subfamily, evidence is accumulating that Rab3A is involved in Ca\textsuperscript{2+}-dependent exocytosis, particularly in neurotransmitter release. We first analyzed the subcellular localization of Rab3 GEP and GAP in rat brain. Subcellular fractionation analysis showed that both Rab3 GEP and GAP were enriched in the synaptic soluble fraction. Immunocytochemical analysis in primary cultured rat hippocampal neurons showed that both Rab3 GEP and GAP were concentrated at the presynaptic nerve terminals. We then examined whether Rab3 GEP and GAP were involved in Ca\textsuperscript{2+}-dependent exocytosis by use of human growth hormone (GH) co-expression assay system of cultured PC12 cells. Overexpression of the deletion mutant of Rab3 GEP possessing the catalytic activity reduced the high K\textsuperscript{+}-induced GH release without affecting the basal GH release, whereas that of the deletion mutant lacking the catalytic activity showed no effect on the high K\textsuperscript{+}-induced GH release. In contrast, overexpression of Rab3 GAP or its deletion mutant possessing the catalytic activity did not affect the high K\textsuperscript{+}-induced GH release or the basal GH release. These results indicate that Rab3 GEP and GAP are colocalized with Rab3A at the synaptic release sites and suggest that they regulate the activity of Rab3A and are involved in Ca\textsuperscript{2+}-dependent exocytosis.

The Rab family belongs to the small G protein superfamily and consists of over thirty members (for reviews, see Refs. 1–3). The Rab3 subfamily belongs to this Rab family and consists of four members, Rab3A, -B, -C, and -D (for reviews, see Refs. 4 and 5). Of these members, Rab3A and -C are present in cells with Ca\textsuperscript{2+}-dependent exocytosis, particularly abundant in neuron, although Rab3B and -C are also expressed in other types of cells. Evidence is accumulating that Rab3A is involved in Ca\textsuperscript{2+}-dependent exocytosis, particularly in neurotransmitter release. Recent knockout mice results indicate that Rab3A is not essential for Ca\textsuperscript{2+}-dependent neurotransmitter release, but it plays two different roles: one is to efficiently dock synaptic vesicles to the presynaptic plasma membrane (6), and the other is to regulate the efficiency of the fusion process (7). It has also been reported that Rab3A is involved in the formation of long term potentiation in hippocampus (8).

The precise mechanisms of Rab3A in the regulation of these docking and fusion processes remain unknown, but Rab3A is regulated by at least three types of regulators: Rab GDI, Rab3 GEP, and Rab3 GAP (for reviews, see Refs. 5 and 6). Of these regulators, Rab GDI is a general regulator of all the Rab family members including Rab3A, whereas the other two are specific for the Rab3 subfamily. Rab3 GEP is a regulator that stimulates the GDP/GTP exchange reaction and thereby the conversion from the GDP-bound inactive form to the GTP-bound active form (10), and Rab3 GAP stimulates the GTPase activity and thereby the conversion from the GDP-bound form to the GDP-bound form (11). Rab GDI has three activities. 1) Rab GDI preferentially interacts with the GDP-bound form of Rab family members and keeps them in the GDP-bound form in the cytosol by preventing them from being converted to the GTP-bound form by the action of each Rab GEP and from being associated with each of their target membranes; 2) Rab GDI transports its complexed Rab family members to each of their target membranes where the GDP-bound form dissociates from Rab GDI by the action of each putative Rab GDI displacement factor, followed by conversion to the GDP-bound form by the action of each Rab GEP; and 3) after the GDP-bound form accomplishes its function, it is converted to the GDP-bound form by the action of each Rab GAP. Once the GDP-bound form is produced, Rab GDI forms a complex with it and translocates it to the cytosol. All the three regulators are abundant in brain, but they are ubiquitously expressed (10–12).

Several lines of evidence have shown that Rab GDI actually shows these activities in intact cells (5). Recent genetic analysis in Caenorhabditis elegans has shown that Aex-3, a C. elegans homologue of mammalian Rab3 GEP, is involved in Rab3A localization to synaptic vesicles (13), but it has not been shown
whether Rab3 GEP and GAP indeed function in intact cells in mammals. In this study, we first analyzed the localization of Rab3 GEP and GAP in rat brain and primary cultured rat hippocampal neurons. We then examined whether Rab3 GEP and GAP are involved in Ca\(^{2+}\)-dependent exocytosis by use of the GH co-expression assay system in cultured PC12 cells. In this system, expressed GH is known to be stored in dense core vesicles and to be released in response to high K\(^+\) in the presence of extracellular Ca\(^{2+}\) (14, 15). Evidence has been obtained by use of this system that many proteins, including Rab3A (16), rabphilin3 (17, 18), and Doc2 (19), are involved in Ca\(^{2+}\)-dependent exocytosis.

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals—** Recombinant rat Rab3 GEP and human Rab3A GAP were purified from the cytosol fraction of Spodoptera frugiperda (Sf9) cells overexpressing the respective proteins.\(^2\) PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 5% horse serum at 37 °C in 10% CO\(_2\) as described (20). Rabbit polyclonal antibodies were generated against glutathione S-transferase-fusion proteins of the middle region of rat Rab3 GEP (amino acid residues 863–1431) and the N-terminal region of human Rab3A GAP (amino acid residues 1–303). A mouse anti-Rab3A monoclonal antibody, SG11–7, was prepared as described (21). The antibodies against NMDA receptor 2A/B and synaptophysin were purchased from Chemicon and Boehringer Mannheim, respectively. Hybridoma cells expressing the anti-MYC mouse antibody 9E10) were purchased from American Type Culture Collection (Manassas, VA). Human GH was expressed using pXGH5, in which GH expression is driven by the mouse metallothionein-I promoter (22).

**Construction of Expression Vectors—** Mammalian expression plasmids pCMV-MYC and pEFBOS-MYC were generated to express fusion proteins with the N-terminal MYC epitope (19, 23). The cDNA fragments encoding rat Rab3 GEP and its deletion mutants were inserted into pCMV-MYC, and the cDNA fragments of human Rab3A GAP, its deletion mutants, and Rab3AAS1L were inserted into pEFBOS-MYC.

**Subcellular Fractionation of Rat Brain—** Subcellular fractionation of rat brain was carried out as described (21). Aliquots of each subcellular fraction were separated by SDS-PAGE, followed by immunoblotting with the anti-Rab3 GEP and anti-Rab3A GAP polyclonal antibodies and the anti-Rab3A and anti-NMDA receptor 2A/B monoclonal antibodies.

**Hippocampal Cell Culture and Immunocytochemistry—** Embryos were obtained from Wistar rats on generation day 20. Hippocampi were isolated, dissociated, plated on poly-L-lysine-coated glass coverslips (Matsumani Glass, Ind. Ltd., Kishiwada, Japan), and cultured in minimal essential medium with 10% horse serum. After 4 days, the medium was replaced with minimal essential medium supplemented with N2 supplement (24), 1 mg/ml ovalbumin, 1 mM proprionate, and 5 mM cytosine arabinoside. For localization of either Rab3 GEP, Rab3A GAP, Rab3A, or synaptophysin, hippocampal cells were fixed with 4% paraformaldehyde in PBS for 20 min. The fixed cells were incubated for 10 min with 50 mM ammonium chloride in PBS and permeabilized with PBS containing 0.2% Triton X-100 for 10 min. After being washed with 10% FBS/PBS for 1 h, the cells were treated with the first antibodies in 10% FBS/PBS for 1 h. The cells were then washed with PBS three times, followed by incubation with the fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse IgG antibody. A, Rab3B GEP; B, Rab3A GAP, C, Rab3A; D, synaptophysin. The results shown are representative of three independent experiments.

**Transfection of Various cDNA Constructs, Measurement of Release of**

**Fig. 1. Subcellular distribution of Rab3 GEP and GAP in rat brain.** Aliquots of each fraction, containing equal amounts of protein (50 \(\mu\)g for Rab3 GAP and 10 \(\mu\)g for other proteins), were subjected to SDS-PAGE, followed by immunoblotting using the anti-Rab3 GEP, anti-Rab3 GAP, anti-Rab3A, and anti-NMDA receptor (NMDAR) 2A/B antibodies. H0, homogenate; P1, nuclei and cell debris; P2, crude synaptosomes; P3, microsomes; S, soluble cytosol; P2A, myelin and some contamination of membrane components in P2B, P2B, a mixture of endoplasmic reticula, Golgi complex, and plasma membrane; P2C, mainly synaptosomes; P2D, mainly mitochondria; SS, synaptic soluble; CSV, crude synaptic vesicles; CSM, crude synaptic membranes. The protein markers used were myosin (\(M_r = 205,000\)), \(\beta\)-galactosidase (\(M_r = 120,000\)), soybean trypsin inhibitor (\(M_r = 28,600\)), and lysozyme (\(M_r = 19,400\)). The results shown are representative of three independent experiments.

**Fig. 2. Immunostaining of Rab3 GEP and GAP in primary cultured hippocampal neurons.** Rat primary cultured hippocampal neurons (16-day-old cultured) were fixed, stained with the anti-Rab3 GEP, anti-Rab3A GAP, anti-Rab3A, and anti-synaptophysin antibodies, and visualized with the fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse IgG antibody. A, Rab3B GEP; B, Rab3A GAP, C, Rab3A; D, synaptophysin. The results shown are representative of three independent experiments.

\(^2\) Y. Takai, manuscript in preparation.
NaCl) or a low K\textsuperscript{+} solution (PSS containing 4.7 mM KCl and 140 mM NaCl). The amounts of GH released into the medium and retained in the cells were measured using a radioimmunoassay kit (Nichols Institute, San Juan Capistrano, CA).

Immunocytochemistry was performed 48 h after transfection. The cells were fixed and stained as described above. Human GH was detected with a rabbit anti-human GH polyclonal antibody (Dako Co., Carpinteria, CA) and a donkey anti-rabbit antibody conjugated to rhodamine (Chemicon International, Inc., Temecula, CA). The MYC epitope was detected with a mouse anti-MYC monoclonal antibody and a donkey anti-mouse antibody conjugated to fluorescein (Chemicon). The cells were examined using a confocal imaging system (LSM 410; Carl Zeiss, Oberkochen, Germany). To estimate the expression levels of Rab3 GEP and GAP in PC12 cells, both the proteins were detected with the respective antibodies and a donkey anti-rabbit antibody conjugated to fluorescein (Chemicon). The cells were analyzed using a photometry system (P102; Nikon, Tokyo, Japan).

Other Procedures—SDS-PAGE and immunoblotting were performed as described (25, 26). Protein concentrations were determined with bovine serum albumin as a reference protein as described (27).

RESULTS

Subcellular Localization of Rab3 GEP and GAP in Rat Brain—Our previous Northern blot analysis showed that Rab3 GEP and GAP were expressed in all the tissues examined (10, 11). Western blot analysis of the subcellular fractions of rat brain indicated that the proteins detected with antibodies against Rab3 GEP and GAP were enriched in the synaptic soluble fraction (Fig. 1). In contrast, Rab3A was concentrated in the crude synaptic vesicle fraction and the NMDA receptor 2A/B was concentrated in the crude synaptic membrane fraction, consistent with earlier observations (21, 23).

Subcellular Localization in Primary Cultured Rat Hippocampal Neurons—We stained Rab3 GEP and GAP in primary cultured hippocampal neurons from rat embryo using the...
Rab3 GAP with a M system (Fig. 3). Rab3 GAP, which was expressed using the insect/baculovirus system, was recognized by the anti-Rab3 GAP antibody specifically expressed recombinant human Rab3 GAP from the synaptic soluble fraction of rat brain (10, 11). This antibody recognized only Rab3 GAP with a M of about 100,000 in the rat brain synaptic soluble fraction and the PC12 cell lysate (Fig. 3A, lanes 2 and 3). The anti-Rab3 GAP antibody specifically recognized recombinant human Rab3 GAP, which was expressed using the insect/baculovirus system (Fig. 3B, lane 1). This antibody specifically recognized Rab3 GAP with a M of about 100,000 in the rat brain synaptic soluble fraction and the PC12 cell lysate and also Rab3 GAP purified from rat brain (Fig. 3B, lanes 2–4).

Inhibition of the High K+-induced GH Release by Rab3 GEP but Not by Rab3 GAP—We then examined whether Rab3 GEP and GAP are involved in Ca2+-dependent exocytosis by use of a GH co-expression assay system in cultured PC12 cells. When the dominant active mutant of Rab3A (pEFBOS-MYC-Rab3A3Q6L1) was expressed in PC12 cells, the high K+-induced GH release was inhibited about 47% (Fig. 4). Under the same conditions, overexpression of the deletion mutant of Rab3 GAP lacking the C-terminal 108 amino acid residues (pCMV-MYC-Rab3 GAPC1) inhibited the high K+-induced GH release about 32%. However, the deletion mutant lacking the C-terminal 242 amino acids residues (pCMV-MYC-Rab3 GEPAC2) did not affect the high K+-induced GH release. Similarly, the deletion mutant lacking the C-terminal 392 amino acids residues (pCMV-MYC-Rab3 GAPC3), the N-terminal 607 amino acids residues (pCMV-MYC-Rab3 GEPAN1), or the N-terminal 1029 amino acids residues (pCMV-MYC-Rab3 GEPAN2) did not inhibit the high K+-induced GH release. None of these constructs affected the basal GH release. When these MYC-tagged deletion mutants were expressed in COS-7 cells and assayed for the Rab3 GAP activity, the deletion mutant lacking the C-terminal 108 amino acid residues showed GAP activity as full-length Rab3 GAP, whereas other deletion mutants did not show GAP activity, indicating that the GAP activity of Rab3 GAP is necessary for the inhibition of the high K+-induced GH release.

In contrast, full-length Rab3 GAP (pEFBOS-MYC-Rab3 GAP) did not affect the high K+-induced GH release (Fig. 4). We have previously shown that the catalytic domain of Rab3 GAP resided in at least 601–981 amino acid residues (11). The deletion mutant containing the catalytic domain (pEFBOS-MYC-Rab3 GAP-C) did not inhibit the high K+-induced GH release. The deletion mutant lacking the catalytic domain (pEFBOS-MYC-Rab3 GAP-N) did not inhibit the high K+-induced GH release, either. These constructs did not affect the basal GH release. These results indicate that exogenously expressed Rab3 GAP does not inhibit the high K+-induced GH release irrespective of the presence and absence of the GAP activity.

The failure of Rab3 GAP to inhibit the high K+-induced GH release was not simply because of its expression level being lower than that of Rab3 GAP in PC12 cells. As shown in Fig. 3, Rab3 GAP and GAP were indeed expressed in PC12 cells, and the contents of Rab3 GAP and GAP were 0.05 and 0.01% of the total protein, respectively. We then examined how much exogenous Rab3 GAP and GAP were expressed in the transfected cells. An epitope tag (MYC) was attached to the N termini of Rab3 GAP and GAP (pCMV-MYC-Rab3 GAPC1; pEFBOS-MYC-Rab3 GAP) to detect the transfected cells. Immunocytochemical analyses of the transfected and untransfected cells showed that the expression levels of both of the exogenous proteins were about 20–40 times as much as those of the endogenous proteins (Fig. 5). We furthermore confirmed whether GH and a sample to be co-expressed in the same cells. A plasmid encoding Rab3 GAP, GEP, or each mutant was co-transfected with a plasmid encoding human GH (pXGH5) into PC12 cells. A MYC tag was attached to the N termini of these proteins to detect them in the cells. When MYC-tagged proteins and GH were analyzed by immunocytochemistry using each antibody, both the proteins were detected in the same cells (Fig. 6). About 80% of the GH-expressing cells expressed MYC-tagged proteins. Only full-length Rab3 GAP (pCMV-MYC-Rab3 GAP) and the deletion mutant containing the middle portion of Rab3 GAP (pEFBOS-MYC-Rab3 GAP-M) showed very low expression levels (data not shown), but the expression levels of other proteins were found to be similar (Fig. 6).

**DISCUSSION**

In the preceding papers, we have isolated Rab3 GEP and GAP from the synaptic soluble fraction of rat brain (10, 11). Consistently, we have shown here that Rab3 GEP and GAP are enriched in the synaptic soluble fraction of rat brain. Moreover, in primary cultured hippocampal neurons, Rab3 GEP and GAP are localized at the synaptic release sites. These results indicate that Rab3 GEP and GAP are colocalized with Rab3A at the synaptic release sites and suggest that they regulate the activity of Rab3A and play a role in neurotransmitter release.

To obtain evidence supporting this role of Rab3 GEP and...
GAP, we have taken advantage of the GH co-expression assay system of PC12 cells. By use of this assay system, we have shown here that overexpression of the Rab3 GEP deletion mutant possessing the catalytic activity, but not overexpression of the deletion mutants of Rab3 GEP lacking the catalytic activity, inhibits Ca\textsuperscript{2+}-dependent release of GH. Several previous works have shown that the dominant active mutants of Rab3A, Rab3AN135I and Rab3AQ81L, inhibit Ca\textsuperscript{2+}-dependent exocytosis from PC12 cells and chromaffin cells (16, 30). Rab3AN135I has a very high dissociation rate for GDP and GTP, and by analogy with the equivalent Ras mutant, Rab3AN135I may be predominantly the GTP-bound form in the cell (31). Rab3AQ81L has low GTPase activity and should stabilize the GTP-bound form (31). Rab3 GEP may show the same effect on the GDP/GTP cycle of Rab3A and accumulate the GTP-bound form in the cell the same as that of these dominant active mutants. The reason why the effect of overexpressed Rab3 GEP was slightly weaker than that of overexpressed Rab3AQ81L might be because overexpressed Rab3 GEP accumulated the GTP-bound form to a lesser extent. Taken together, the present results suggest that Rab3 GEP is involved in Ca\textsuperscript{2+}-dependent exocytosis by regulating the GDP/GTP exchange reaction of Rab3A. It is currently unknown how the accumulation of the GTP-bound form of Rab3A inhibits Ca\textsuperscript{2+}-dependent exocytosis, but it is likely that the GDP/GTP cycle of Rab3A is essential for the function of Rab3A in the docking and fusion processes (5, 9).

We have previously shown that the GTP-bound form of Rab3A is associated with synaptic vesicles through interaction with rabphilin3 (32), although other groups have shown that Rab3A is associated with synaptic vesicles in a manner independent of rabphilin3 (33, 34). Rabphilin3 is one of the Rab3 downstream targets that are involved in the docking and/or fusion of synaptic vesicles with the presynaptic plasma membrane (17, 18, 35, 36). We have shown that Rab3 GAP is not active on the GTP-bound form of Rab3A that is complexed with rabphilin3 (37). We have shown here that overexpression of full-length Rab3 GAP or its deletion mutant containing the catalytic domain does not act on the GTP-bound form of Rab3A associated with rabphilin3 in PC12 cells. It is likely that the inhibitory effect of rabphilin3 on Rab3 GAP is released by some modifications of Rab3A, rabphilin3, and/or Rab3 GAP, or another factor. It is currently unknown at which stage of the neurotransmitter release processes Rab3 GAP shows its activity, but Rab3 GAP should not be activated at least until Rab3A accomplishes its function in the docking and fusion processes. It is important to clarify at which stage of the neurotransmitter release processes Rab3 GAP shows its activity and how Rab3 GAP is activated at that stage.

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