Physical and Functional Interaction of Rabphilin-3A with α-Actinin*

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Rabphilin-3A is a downstream target molecule of Rab3A small GTP-binding protein and implicated in Ca2+-dependent neurotransmitter release. Here we have isolated a rabphilin-3A-interacting molecule from a human brain cDNA library by the yeast two-hybrid method and identified it to be α-actinin, known to cross-link actin filaments into a bundle. α-Actinin interacts with the N-terminal region of rabphilin-3A, with which GTP-Rab3A interacts, and this interaction stimulates the activity of α-actinin to cross-link actin filaments into a bundle. The interaction of rabphilin-3A with α-actinin is inhibited by guanosine 5′-O-(3-thio)triphosphate–Rab3A. These results suggest that the Rab3A-rabphilin-3A system regulates the α-actinin-regulated reorganization of actin filaments. It has been shown that reorganization of actin filaments is also involved in Ca2+-dependent exocytosis. Therefore, rabphilin-3A may serve as a linker for Rab3A and cytoskeleton.

The Rab small GTP-binding protein family, consisting of more than 30 members, is implicated in intracellular vesicle trafficking (for reviews, see Refs. 1–3). The Rab3 subfamily consists of four members, Rab3A, -B, -C, and -D. Of these members, Rab3A and -C have been shown to be implicated in Ca2+-dependent exocytosis, particularly in neurotransmitter release (for a review, see Ref. 4). We have identified a downstream target molecule of the Rab3 subfamily members that specifically interacts with their GTP-bound form and named it rabphilin-3A (5, 6). Structural and biochemical analyses indicate that rabphilin-3A has at least two functionally different domains: the N-terminal GTP-Rab3-binding domain and the C-terminal two C2-like domains that interact with Ca2+ and phospholipid (6, 7). Tissue and subcellular distribution analyses of rabphilin-3A indicate that it is specifically expressed in neuronal cells where it is highly concentrated on synaptic vesicles (6, 8, 9). Overexpression of the N-terminal or C-terminal fragment of rabphilin-3A in bovine adrenal chromaffin cells and PC12 cells (10, 11) or microinjection of these fragments into the presynaptic nerve terminal of squid giant axon has shown to inhibit Ca2+-dependent exocytosis, suggesting that rabphilin-3A as well as Rab3A is involved in Ca2+-dependent exocytosis. However, it still remains to be clarified how Rab3A and rabphilin-3A regulate Ca2+-dependent exocytosis.

Synaptotagmin has been shown to have two C2-like domains that interact with Ca2+ and phospholipid and to serve as a Ca2+ sensor for neurotransmitter release (for reviews, see Refs. 12 and 13). The mode of action of this protein in neurotransmitter release has not definitely been clarified, but it has been shown to interact with syntaxin, one plasma membrane component of the SNARE system. Recently, the binding of Ca2+ to synaptotagmin has been shown to induce its tighter interaction with syntaxin (12). The assembly of the SNARE components is known to be followed by the assembly of the NSF/SNAP system, which finally causes the fusion of the vesicle with the plasma membrane (for a review, see Ref. 14). It has been suggested that synaptotagmin regulates these SNARE and NSF/SNAP system in response to Ca2+. Moreover, synaptotagmin has been shown to interact with many substances, such as AP2, neurexin, IP4, and β-SNAP (12, 15, 16). AP2 is an adapter protein for clathrin, known to be involved in endocytosis (for a review, see Ref. 17); neurexin is the receptor for α-latrotoxin, known to induce massive neurotransmitter release (18); IP4 is a potential second messenger, known to regulate the intracellular Ca2+ concentration and to be a blocker of neurotransmitter release (19); and β-SNAP is a brain-specific SNAP, but its role is still unclear (14).

In contrast to synaptotagmin, any protein directly interacting with rabphilin-3A, except the Rab3 subfamily members, has not been identified. In this study, we attempted to isolate a rabphilin-3A-interacting protein by the use of the yeast two-hybrid system and identified it to be α-actinin, known to interact with actin filaments and to cross-link them into a bundle.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Recombinant rabphilin-3A and Rab3A were purified from the membrane fraction of overexpressing Spodoptera frugiperda cells (Sf9 insect cells) (20, 21). Rab3A used for measuring low shear viscosity was further purified by hydroxyapatite column chromatography. GTP-S-Rab3A was prepared as described (22). Chicken gizzard α-actinin was purified as described (23). Recombinant chicken lung type α-actinin was purified from overexpressing Ercherichia coli. An anti-rabphilin-3A polyclonal antibody and an anti-α-actinin monoclonal antibody (CP2–1) were prepared.

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1 M. E. Burns, T. Sasaki, Y. Takai, and G. Augustine, manuscript in preparation.

2 The abbreviations used are: SNARE, SNAP receptor; NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; AP2, clathrin adaptor complex-2; IP4, inositol 1,3,4,5-tetrakisphosphate; GTP-S, guanosine 5′-O-(3-thio)triphosphate; aa, amino acids; HA, hemagglutinin; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

3 M. Imamura, manuscript in preparation.
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as described (8, 24).

Strain and Media—Yeast strain L40 (MATa trp1 leu2 his3 lys2 lexA ura3 lexA-lacZ) was grown on YPD medium that contained 2% glucose, 2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Difco), 0.04% adenine sulfate, and 0.02% uracil. Yeast transformations were performed by the lithium acetate methods (25). Transformants were selected on SD medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids, and amino acids (Difco) were supplemented to SD medium when required. Standard yeast genetic manipulations were performed as described (26). An E. coli strain DH5α was used for construction and propagation of plasmids.

Screening for Rabphilin-3A-interacting Molecule by the Two-hybrid Method—A strain L40 was transformed with a derivative of pBTM116 bearing the N-terminal fragment (1–280 amino acids (aa)) of rabphilin-3A fused to the LexA DNA-binding domain: pBTM116-rabphilin-3A-N. A strain L40 carrying pBTM116-rabphilin-3A-N was transformed with the library DNA (MATCHMAKER human brain oligo(dT)-primed library in pGAD10, Clontech). Approximately 2.4 × 10⁸ transformants were screened for the growth on SD plate media lacking tryptophan, leucine, and histidine, but containing 0.5 mM 3-amino-1,2,4-triazole, which is a specific inhibitor of the HIS3 gene product. His+ colonies were then placed on the nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity as described (27). From the 36 His+ and lacZ+ positive clones obtained with this screening, library plasmids were recovered through E. coli transformation. The recovered plasmids were transformed into L40 containing pBTM116-rabphilin-3A-N, and it was found that 11 clones conferred the His+ and lacZ+ phenotypes on L40 containing pBTM116-rabphilin-3A-N. The nucleotide sequences of the insert DNA of these 11 clones were determined.

In Vitro Binding of Chicken α-Actinin to Recombinant Rabphilin-3A—Purified α-actinin (100 pmol) was incubated at 4 °C for 90 min with HA-tagged full-length rabphilin-3A, the HA-tagged N-terminal fragment, or the HA-tagged C-terminal fragment (20 pmol each), which was immobilized on protein A-Sepharose (Pharmacia Biotech Inc.) through the anti-HA antibody, in 20 mM Tris/HCl at pH 8.0, 1 mM dithiothreitol, 1 mM EGTA, and 150 mM NaCl. After washing three times with the same buffer, α-actinin associated with the beads was detected by SDS-PAGE followed by immunoblotting with the anti-α-actinin monoclonal antibody (CP2–1).

Immunoprecipitation of Rabphilin-3A from PC12 Cells—Confluent PC12 cells were washed with serum-free Dulbecco’s modified Eagle’s medium and lysed in 0.25 M d/l dish of TNE buffer (10 mM Tris/Cl at pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 10 μM (p-amidinophenyl)methanesulfonfluryde). The extract was centrifuged at 15,000 g for 20 min, and the supernatant was incubated at 4 °C for 90 min with either the anti-rabphilin-3A polyclonal antibody or rabbit IgG, which was immobilized on protein A-Sepharose (Pharmacia Biotech Inc.) (28). The immunoprecipitate was washed three times with TNE buffer and subjected to SDS-PAGE followed by immunoblotting with either the anti-rabphilin-3A polyclonal antibody or the anti-α-actinin monoclonal antibody (CP2–1).

Low Shear Viscometry—Effect of rabphilin-3A on the actin filament bundling activity of recombinant chicken lung type α-actinin was examined by measuring low shear viscosity using a falling ball viscometer (29). Recombinant chicken lung type α-actinin (120 nm) was incubated at 4 °C for 1 h with the indicated amounts of HA-tagged full-length rabphilin-3A, the HA-tagged N-terminal fragment, or the HA-tagged C-terminal fragment in 20 mM Tris/Cl at pH 7.2, 4 mM EDTA, 8 mM MgCl₂, 0.12% CHAPS, 0.6 mM dithiothreitol, and 100 mM KCl. After the incubation, each sample was mixed with F-actin (100 μg/ml) in 20 mM Tris/Cl at pH 7.2, 2 mM EDTA, 6 mM MgCl₂, 0.06% CHAPS, 100 mM KCl, 180 μM ATP, and 0.65 mM EGTA, and the solution was sucked into a 0.1-ml micropipette. After incubation at 25 °C for 1 h, the time for a stainless steel ball to fall a fixed distance in the pipette was measured and converted into viscosity in centipoise using various concentrations of sucrose solution at 20 °C as a standard.

Electron Microscopy—A part of each sample prepared for measuring low shear viscosity was negatively stained with 2% uranyl acetate and viewed with a Hitachi electron microscope (model H-7100) (30, 31).

Other Procedures—SDS-PAGE was performed as described (32). Rabphilin-3A and α-actinin transferred to nitrocellulose sheets were detected using the ECL immunoblotting detection system (Amersham Corp.). Protein concentrations were determined with bovine serum albumin as a standard protein (33).
interaction of recombinant rabphilin-3A with α-actinin in rabphilin-3A from intact PC12 cells. Confluent PC12 cells were lysed in TNE buffer, and α-actinin was coimmunoprecipitated with rabphilin-3A in intact cells, or the HA-tagged C-terminal fragment (20 pmol each), which was immobilized on protein A-Sepharose through the anti-HA antibody. After washing, α-actinin associated with the beads was detected by SDS-PAGE followed by immunoblotting with the anti-α-actinin monoclonal antibody (CP2–1). Lane 1, protein A-Sepharose alone; lane 2, full-length rabphilin-3A; lane 3, the N-terminal fragment; lane 4, the C-terminal fragment. B, coimmunoprecipitation of α-actinin with rabphilin-3A from intact PC12 cells. Confluent PC12 cells were washed with serum-free Dulbecco’s modified Eagle’s medium and lysed in 0.25 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40 and 0.25% sodium deoxycholate, and the solution was sucked into a 0.1-ml microfuge. After incubation at 25 °C for 1 h, the time for a stainless steel ball to fall a fixed distance in the pipette was measured and converted into viscosity in centipoise using various concentrations of sucrose solution at 20 °C as a standard. •, with full-length rabphilin-3A; •, with the N-terminal fragment; •, with the C-terminal fragment. Rabphilin-3A (Fig. 1A). This clone also interacted with full-length rabphilin-3A, but not with its C-terminal fragment (Fig. 1B). The region of rabphilin-3A interacting with α-actinin was overlapped with that interacting with GTP-γ-S-Rab3A (Fig. 1C). Interaction of Recombinant Rabphilin-3A with Purified α-Actinin—The interaction of recombinant rabphilin-3A with α-actinin purified from chicken gizzard was then examined. The HA-tagged full-length rabphilin-3A, its N-terminal fragment, and its C-terminal fragment were separately immobilized on protein A-Sepharose, and the binding of α-actinin to these immobilized proteins were analyzed. α-Actinin indeed interacted with both full-length rabphilin-3A and its N-terminal fragment, but to a little extent with its C-terminal fragment (Fig. 2A). The stoichiometry of binding between α-actinin and full-length rabphilin-3A was at least 0.1 under the assay conditions used here. Coimmunoprecipitation of α-Actinin with Rabphilin-3A from Intact PC12 Cells—To further confirm the rabphilin-3A-α-actinin interactions in intact cells, it was examined whether α-actinin is coimmunoprecipitated with rabphilin-3A in intact PC12 cells. Confluent PC12 cells were lysed in TNE buffer, and the cell lysate was incubated with protein A-Sepharose con-

Fig. 2. In vitro and in vivo binding of α-actinin to rabphilin-3A. A, interaction of recombinant rabphilin-3A with purified α-actinin. Purified α-actinin (100 pmol) was incubated at 4 °C for 90 min with HA-tagged full-length rabphilin-3A, the HA-tagged N-terminal fragment, or the HA-tagged C-terminal fragment (20 pmol each), which was immobilized on protein A-Sepharose through the anti-HA antibody. After washing, α-actinin associated with the beads was detected by SDS-PAGE followed by immunoblotting with the anti-α-actinin monoclonal antibody (CP2–1). Lane 1, protein A-Sepharose alone; lane 2, full-length rabphilin-3A; lane 3, the N-terminal fragment; lane 4, the C-terminal fragment. B, coimmunoprecipitation of α-actinin with rabphilin-3A from intact PC12 cells. Confluent PC12 cells were washed with serum-free Dulbecco’s modified Eagle’s medium and lysed in 0.25 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40 and 0.25% sodium deoxycholate, and the solution was sucked into a 0.1-ml microfuge. After incubation at 25 °C for 1 h, the time for a stainless steel ball to fall a fixed distance in the pipette was measured and converted into viscosity in centipoise using various concentrations of sucrose solution at 20 °C as a standard. •, with full-length rabphilin-3A; •, with the N-terminal fragment; •, with the C-terminal fragment. Rabphilin-3A (Fig. 1A). This clone also interacted with full-length rabphilin-3A, but not with its C-terminal fragment (Fig. 1B). The region of rabphilin-3A interacting with α-actinin was overlapped with that interacting with GTP-γ-S-Rab3A (Fig. 1C). Interaction of Recombinant Rabphilin-3A with Purified α-Actinin—The interaction of recombinant rabphilin-3A with α-actinin purified from chicken gizzard was then examined. The HA-tagged full-length rabphilin-3A, its N-terminal fragment, and its C-terminal fragment were separately immobilized on protein A-Sepharose, and the binding of α-actinin to these immobilized proteins were analyzed. α-Actinin indeed interacted with both full-length rabphilin-3A and its N-terminal fragment, but to a little extent with its C-terminal fragment (Fig. 2A). The stoichiometry of binding between α-actinin and full-length rabphilin-3A was at least 0.1 under the assay conditions used here. Coimmunoprecipitation of α-Actinin with Rabphilin-3A from Intact PC12 Cells—To further confirm the rabphilin-3A-α-actinin interactions in intact cells, it was examined whether α-actinin is coimmunoprecipitated with rabphilin-3A in intact PC12 cells. Confluent PC12 cells were lysed in TNE buffer, and the cell lysate was incubated with protein A-Sepharose con-

Fig. 3. Enhancement of the actin filament bundling activity of α-actinin by rabphilin-3A estimated by low shear viscometry. Recombinant chicken lung type α-actinin (120 nM) was incubated at 4 °C for 1 h with the indicated amounts of HA-tagged full-length rabphilin-3A, the HA-tagged N-terminal fragment, or the HA-tagged C-terminal fragment. After the incubation, each sample was mixed with F-actin (1.0 mg/ml), and the solution was sucked into a 0.1-ml microfuge. After incubation at 25 °C for 1 h, the time for a stainless steel ball to fall a fixed distance in the pipette was measured and converted into viscosity in centipoise using various concentrations of sucrose solution at 20 °C as a standard. •, with full-length rabphilin-3A; •, with the N-terminal fragment; •, with the C-terminal fragment.
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We have shown here that Rabphilin-3A directly interacts with α-actinin and stimulates its actin filament bundling activity. We have moreover shown here that GTP-Rab3A inhibits this interaction of Rabphilin-3A with α-actinin. Both Rab3A and Rabphilin-3A have been shown to be associated with synaptic vesicles (8, 9, 22, 39). It has not yet been reported that Rabphilin-3A is involved in the docking and fusion processes. We have shown previously that Rabphilin-3A interacts with β-adducin through the C2-like domain in the presence of Ca2+- and phospholipid (44, 45). β-Adducin has been implicated in the assembly of spectrin-actin complexes (for a review, see Ref. 46). The function of this interaction of Rabphilin-3A with β-adducin remains to be clarified, but the present results together with these earlier observations suggest that Rabphilin-3A may be an important linker for Rab3A and various cytoskeletal proteins.

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