Rabconnectin-3, a Novel Protein That Binds Both GDP/GTP Exchange Protein and GTPase-activating Protein for Rab3 Small G Protein Family*

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Rab3A, a member of the Rab3 small G protein family, regulates Ca2+-dependent exocytosis of neurotransmitter. The cyclical activation and inactivation of Rab3A are essential for the Rab3A action in exocytosis. GDP-Rab3A is activated to GTP-Rab3A by Rab3 GDP/GTP exchange protein (Rab3 GEP), and GTP-Rab3A is inactivated to GDP-Rab3A by Rab3 GTPase-activating protein (Rab3 GAP). It remains unknown how or in which step of the multiple exocytosis steps these regulators are activated and inactivated. We isolated here a novel protein that was co-immunoprecipitated with Rab3 GEP and GAP by their respective antibodies from the crude synaptic vesicle fraction of rat brain. The protein, named rabconnectin-3, bound both Rab3 GEP and GAP. The cDNA of rabconnectin-3 was cloned from a human cDNA library and its primary structure was determined. Human rabconnectin-3 consisted of 3,036 amino acids and showed a calculated Mr of 339,753. It had 12 WD domains. Tissue and subcellular distribution analyses in rat indicated that rabconnectin-3 was abundantly expressed in the brain where it was enriched in the synaptic vesicle fraction. Immunofluorescence and immunoelectron microscopy revealed that rabconnectin-3 was concentrated on synaptic vesicles at synapses.

These results indicate that rabconnectin-3 serves as a scaffold molecule for both Rab3 GEP and GAP on synaptic vesicles.

Rab3A is a member of the Rab3 family, along with Rab3B, -3C, and -3D, and plays a key regulatory role in Ca2+-dependent exocytosis of neurotransmitter (for reviews, see Refs. 1–3). The process of Ca2+-dependent exocytosis of neurotransmitter consists of several steps: translocation of synaptic vesicles from the reserve pool to the active zone of the presynaptic plasma membrane where a Ca2+ channel localizes, docking of the vesicles to the active zone, transition from the docking to the priming of the vesicles in the readily releasable pool, and fusion of the vesicles with the membrane induced by Ca2+ influx (1–3). The Rab3A gene knockout mouse analysis has revealed two actions of Rab3A: it facilitates the translocation and docking of synaptic vesicles to the presynaptic plasma membrane (4), and it prevents Ca2+-triggered fusion of the vesicles with the plasma membrane (5).

The Rab3 family members are regulated by three regulators: Rab GDI1 and Rab3 GAP and GAP (1–3). Rab3 GAP and GAP are specific for the Rab3 family members, but Rab GDI is active on all the Rab family members. The cyclical activation and inactivation of Rab3A by the action of these regulators are essential for Ca2+-dependent exocytosis of neurotransmitter. A current model for the mode of action of these regulators is as follows: GDP-Rab3A is kept in the cytosol in a complex with Rab GDI. This complex is recruited to synaptic vesicles where GDP-Rab3A is activated to GTP-Rab3A by the action of Rab3 GAP with the help of another unidentified molecule, such as GDI displacement factor (6) or Rab recycling factor (7) proposed for other vesicle trafficking systems. GTP-Rab3A bind to its two downstream effectors: rapsyn-3 and Rim localized on the vesicles and the active zone, respectively. Before or after the fusion step, GTP-Rab3A in a complex with the effectors is inactivated to GDP-Rab3A by the action of Rab3 GAP. GDP-Rab3A forms a complex with Rab GDI, resulting in the translocation of the vesicles to the cytosol. Rat Rab3 GAP consists of 1,602 aa and shows a calculated Mr of 177,982 (8). Human Rab3 GAP consists of one catalytic subunit (p130) and one noncatalytic subunit (p150): the catalytic and noncatalytic subunits consist of 981 and 1,393 aa and show calculated Mr values of 110,521 and 156,081, respectively (9, 10). In this model, however, it still remains unknown how or in which step of the multiple exocytosis steps these regulators are activated and inactivated.

To address these issues, we attempted here to isolate a protein(s) that was co-immunoprecipitated with Rab3 GEP and/or GAP by their respective Abs from CSV of rat brain and identified a novel protein, named rabconnectin-3, which bound both Rab3 GEP and GAP. We describe here the isolation and characterization of rabconnectin-3 and discuss its possible function in Ca2+-dependent exocytosis of neurotransmitter.

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The abbreviations used are: GDI, GDP dissociation inhibitor; GEP, GDP/GTP exchange protein; GAP, GTPase-activating protein; Ab, antibody; CSV, the crude synaptic vesicle fraction; GST, glutathione S-transferase; aa, amino acid(s).
**EXPERIMENTAL PROCEDURES**

**Material and Chemicals—**GST fusion proteins of rabconnectin-3 were expressed in *Escherichia coli* and purified by use of glutathione-Sepharose beads (Amersham Biosciences, Inc.). The fusion proteins contained the following aa: GST-rabconnectin-3, aa 704–1033; and GST-rabconnectin-3-2, aa 1780–1971. Recombinant Rab3 GEP was expressed in SF9 cells transfected with baculovirus carrying the full-length cDNA of Rab3 GEP and purified by use of Mono Q and Superdex 200 columns (Amersham Biosciences, Inc.) as described previously (8). His$_6$-tagged Rab3 GAP p130 and p150 were expressed in *E. coli* and purified by use of Ni$_2^+$-nitrilotriacetic acid-agarose beads (Qiagen) (9, 10). Rabbit polyclonal anti-Rab3 GEP and anti-Rab3 GAP p130 and p150 Abs were prepared as described previously (11). Rat polyclonal anti-rabconnectin-3 Abs 1 and Abs 2 were raised against GST-rabconnectin-3-1 and -2, respectively, and affinity-purified with each antigen covalently coupled to N-hydroxysuccinimide-activated Sepharose beads (Amersham Biosciences, Inc.). Mouse monoclonal anti-Rab3A and rabbit polyclonal anti-raphillin-3 Abs were prepared as described previously (12, 13). A mouse monoclonal anti-synaptophysin Ab was from Roche Molecular Biochemicals. Primary cultured rat hippocampal neurons were prepared as described previously (14). CSV of rat brain was prepared as described previously (15).

**Immunoprecipitation and Determination of the aa Sequences of p340 (rabconnectin-3)—**To identify a Rab3 GEP- and/or GAP-binding protein(s), CSV was incubated at 4°C for 90 min in Buffer A (20 mM Tris/Cl at pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 0.8% NaCl), and the supernatant was centrifuged at 100,000 × g for 60 min. The supernatant in Buffer A or B (4 mg of protein each) was incubated at 4°C overnight with the anti-Rab3 GEP or anti-Rab3 GAP Ab p150. The sample mixed with Rab3 GEP was incubated at 4°C overnight with the anti-Rab3 GAP or anti-Rab3 GAP Ab p150 immobilized on protein A-Sepharose beads (40 µl of wet volume), respectively. After the beads were extensively washed with the respective buffers, the bound proteins were eluted by boiling the beads in an SDS sample buffer (60 mM Tris/Cl at pH 6.7, 3% SDS, 2% (v/v) 2-mercaptoethanol, and 5% glycerol). The sample was subjected to SDS-PAGE, followed by protein staining with silver.

The protein band corresponding to p340 (rabconnectin-3) was cut out from the gel and digested with a lysyl endopeptidase, and the digested peptides were separated by C18 reverse phase high pressure liquid chromatography as described previously (13). The aa sequences of the peptides were determined with a peptide sequencer (Hewlett-Packard G1005A protein sequencing system).

**Molecular Cloning of p340 (rabconnectin-3)—**Three sets of oligonucleotide primers were designed as follows: AGA TTA CAT CCT GAT CTT TTC CTG CGT/TTC ATC TAA GGC ACT GTC GTG ATC TTC, and CAA AAG ACA GTC TTC TGG TAA. Three cDNA fragments were amplified with these primers from a human brain cDNA (CLONTECH). A human brain cDNA library in λZAPII (Stratagene) was screened by use of these cDNA fragments as probes. DNA sequencing was performed by the dyeodeoxynucleotide termination method using a DNA sequencer (Applied Biosystems PRISM 3100 Genetic Analyzer, PE Biosystems).

**Binding of rabconnectin-3 to Rab3 GEP and GAP—**The extract of CSV (0.5 mg of protein) was prepared with Buffer A containing 150 mM NaCl and mixed with recombinant Rab3 GEP, His$_6$-tagged Rab3 GAP p150, or p150 (10 pmol each). The sample mixed with Rab3 GEP was then incubated at 4°C for 120 min with the anti-rabconnectin-3 Ab2 immobilized on protein A-Sepharose beads (20 µl of wet volume) through a goat anti-rat IgG Fe Ab (Chemicon) in Buffer A containing 150 mM NaCl. The sample mixed with Rab3 GAP p130 or p150 was similarly incubated with the anti-rabconnectin-3 Ab2 immobilized on protein G-Sepharose beads (20 µl of wet volume). After the beads were extensively washed with the same buffer, the bound proteins were eluted by boiling the beads in the SDS sample buffer. The sample was subjected to SDS-PAGE, followed by Western blotting.

**Miscellaneous Procedures—**SDS-PAGE (16), subcellular fractionation of rat brain (15), immunofluorescence microscopy of frozen sections (17) and cultured cells (18), and immunoelectron microscopy (19) were performed as described. Protein concentrations were determined with bovine serum albumin as a reference protein as described previously (20).

**RESULTS**

**A Protein Co-immunoprecipitated with Rab3 GEP and GAP by Their Abs**—To first identify a Rab3 GEP-binding protein(s), Rab3 GEP was immunoprecipitated by its Ab from the extract of CSV of rat brain and the immunoprecipitate was subjected to SDS-PAGE, followed by protein staining. Four major proteins with molecular mass values of 340 kDa (p340), 200 kDa (p200), 160 kDa (p160), and 60 kDa (p60) were immunoprecipitated (Fig. 1Aa). Western blot analysis showed that of the four proteins, p200 was Rab3 GEP. The protein staining did not reveal the presence of Rab3A in the immunoprecipitate, because the IgG light chain disturbed the detection of Rab3A (data not shown). However, Western blot analysis showed that Rab3A was faintly co-immunoprecipitated with Rab3 GEP. The amount of Rab3A immunoprecipitated was estimated to be less than 10% of that of Rab3 GEP immunoprecipitated.

To then identify a Rab3 GAP-binding protein(s), Rab3 GAP was immunoprecipitated by the anti-Rab3 GAP p150 Ab from the same fraction, and the immunoprecipitate was subjected to SDS-PAGE, followed by protein staining. Four major proteins with molecular mass values of 340 kDa (p340), 150 kDa (p150), 130 kDa (p130), and 90 kDa (p90) were immunoprecipitated (Fig. 1Ab). Western blot analysis showed that of the four proteins, p150 and p130 were the noncatalytic and catalytic subunits of Rab3 GAP, respectively. Rab3A was not detected in the immunoprecipitate (data not shown). The p340 protein co-immunoprecipitated with Rab3 GEP and the p40 co-immunoprecipitated with Rab3 GAP showed apparently a similar molecular mass. The bands of these two proteins were separately cut out from the gel and digested with a lysyl endopeptidase. The peptides of each protein were separated by C18 reverse phase high pressure liquid column chromatography. The same peptide peaks were observed for both the p340 proteins, and the aa sequences of the seven peptides of each p340 protein were determined. The same aa sequences were obtained. These results indicated that the same protein...
A Rab3 GEP- and GAP-binding Protein

**FIG. 2. Structure of rabconnectin-3.** A, deduced aa sequence. Underlines, aa sequences of the seven peptide peaks; and double underlines, WD domains. B, schematic structure.

was co-immunoprecipitated with Rab3 GEP and GAP.

**Molecular Cloning of p340 (Rabconnectin-3)—** Computer search with these aa sequences of p340 against GenBank™ data bases revealed that two of the seven peptides were included in the aa sequence deduced from a human brain cDNA fragment (KIAA0856, GenBank™ accession number AB020663). All of them were included in the aa sequence deduced from two overlapping BAC clones of the human genome (GenBank™ accession numbers AC020892 and AC066613). On the basis of this information, we screened a human brain cDNA library and isolated overlapping clones 1–8 (see Fig. 2B). These three clones with the KIAA0856 cDNA were aligned, and the complete nucleotide sequence was determined. The sequence contained an initiation codon downstream of an in-frame stop codon in the 5' region and an in-frame stop codon in the 3' region, indicating that this sequence encoded the entire coding region. All the aa sequences of the peptides were included in this sequence. The complete nucleotide sequence encoded a protein with 3,036 aa and a calculated Mr of 339,753 (GenBank™ accession number AF389880) (Fig. 2A).

**Binding of rabconnectin-3 to Both Rab3 GEP and GAP—** When rabconnectin-3 was immunoprecipitated from the extract of CSV by the anti-rabconnectin-3 Ab, neither Rab3 GEP, Rab3 GAP p130, p150, nor Rab3A was detected in the immunoprecipitate (data not shown). This might be just due to the low amounts of Rab3 GEP and GAP in this fraction (see Fig. 3B). We then mixed the recombinant sample of Rab3 GEP, Rab3 GAP p130, or p150 with the extract of CSV, and rabconnectin-3 was immunoprecipitated. Rab3 GEP and Rab3 GAP p150, but not Rab3 GAP p130, were co-immunoprecipitated with rabconnectin-3 (Fig. 1B). These results indicate that rabconnectin-3 binds both Rab3 GEP and Rab3 GAP p150.

**Tissue and Subcellular Distribution of rabconnectin-3—** Western blot analysis of various rat tissues revealed that rabconnectin-3 was abundantly expressed in the brain (Fig. 3A). The subcellular distribution analysis in rat brain revealed that rabconnectin-3 was highly concentrated in CSV (Fig. 3B). On the other hand, both Rab3 GEP and GAP were mainly recovered in the synaptic soluble fraction, whereas rabphilin-3 was highly concentrated in CSV. This subcellular distribution of these proteins is consistent with our previous results (10, 11, 19). Immunofluorescence microscopy revealed that rabconnectin-3 was concentrated in the synaptic regions of mouse hippocampus and primary cultured rat hippocampal neurons (Fig. 4, Aa and Ab). Immunoelectron microscopy revealed that rabconnectin-3 was concentrated on synaptic vesicles of primary cultured rat hippocampal neurons (Fig. 4B). These results indicate that rabconnectin-3 is associated with synaptic vesicles at synapses.

**DISCUSSION**

We have identified here a novel protein that binds both Rab3 GEP and GAP and named it rabconnectin-3. Tissue distribution analysis of rabconnectin-3 in rat has revealed that it is abundantly expressed in the brain. Subcellular distribution analysis of rabconnectin-3 in rat brain and immunofluorescence and immunoelectron microscopy of mouse hippocampus and primary cultured rat hippocampal neurons have revealed that it is associated with synaptic vesicles at synapses. Rabconnectin-3 has no transmembrane segment and is extracted from the vesicles by a detergent (data not shown), suggesting that it is a peripheral membrane protein of synaptic vesicles. Taken together, rabconnectin-3 may serve as a scaffold protein...
of Rab3 GEP and GAP on synaptic vesicles.

In contrast to rabconnectin-3, subcellular distribution analysis indicates that Rab3 GEP and GAP are mainly recovered in the synaptic soluble fraction and partly associated with synaptic vesicles. The exact reason for the different distribution of these proteins is currently unknown, but when Rab3 GEP and GAP activate and inactivate Rab3A, respectively, in the process of Ca"++-dependent exocytosis of neurotransmitter, these regulators may be translocated from the cytosol to synaptic vesicles through their binding to rabconnectin-3. After Rab3 GEP and GAP finish their functions, these regulators may be released from rabconnectin-3, resulting in the translocation of some of these three proteins, such as phosphorylation.

It is of crucial importance to know whether rabconnectin-3 affects Rab3 GEP or GAP activity. To address this issue, we attempted to make recombinant proteins of the full length of rabconnectin-3 in E. coli, Sf9 cells, and COS7 cells, but we have not yet succeeded in preparing any recombinant protein. We cannot conclude from the present immunoprecipitation analysis whether Rab3 GEP and GAP directly or indirectly bind to rabconnectin-3, because we do not have the pure sample of rabconnectin-3.

We have shown here that Rab3A is co-immunoprecipitated by the anti-Rab3 GEP Ab, but not by the anti-Rab3 GAP p150 or anti-rabconnectin-3 Ab, suggesting that Rab3A binds to Rab3 GEP. Thus, rabconnectin-3 directly or indirectly binds Rab3A and its regulators, raising a possibility that rabconnectin-3 additionally binds Rab GDI, rabphilin-3, and Rim directly or indirectly and functions as a core protein scaffolding Rab3A and its related molecules. Rabconnectin-3 may also bind a putative GDI displacement or Rab recycling factor for Rab3A. It is important to clarify the molecular linkage among rabconnectin-3, Rab3A, and its related molecules in more detail for our understanding of Ca"++-dependent exocytosis of neurotransmitter.

REFERENCES
1. Takai, Y., Sasaki, T., Shirataki, H., and Nakanishi, H. (1996) Genes Cells 1, 615–632
2. Darchen, F., and Goud, B. (2000) Biochimie (Paris) 82, 375–384
3. Takai, Y., Sasaki, T., and Motozaki, T. (2001) Physiol. Res. 81, 153–208
4. Geppert, M., Bolskakov, V. Y., Siegelbaum, S. A., Takei, K., De Camilli, P., Hammer, R. E., and Sudhof, T. C. (1989) Nature 339, 493–497
5. Geppert, M., Guda, Y., Stevens, C. P., and Sudhof, T. C. (1997) Nature 387, 810–814
6. Solati, T., Shapiro, A. D., Svejstrup, A. B., and Pfeffer, S. R. (1994) Nature 369, 76–78
7. Luan, P., Balch, W. E., Emm, S. D., and Burd, C. G. (1999) J. Biol. Chem. 274, 14806–14817
8. Wada, M., Nakanishi, H., Satoh, A., Hirano, H., Obaishi, H., Matsuura, Y., and Takai, Y. (1997) J. Biol. Chem. 272, 3875–3878
9. Fukui, K., Sasaki, T., Imaizumi, K., Matsuura, Y., Nakanishi, H., and Takai, Y. (1997) J. Biol. Chem. 272, 4655–4658
10. Nagano, F., Sasaki, T., Fukui, K., Asakura, T., Imaizumi, K., and Takai, Y. (1998) J. Biol. Chem. 273, 24781–24785
11. Oishi, H., Sasaki, T., Nagano, F., Ikeeda, W., Ohyama, T., Wada, M., Ide, N., Nakanishi, H., and Takai, Y. (1998) J. Biol. Chem. 273, 34586–34585
12. Mizoguchi, A., Kim, S., Ueda, T., Kikuchi, A., Yorifuji, H., Hirokawa, N., and Takai, Y. (1990) J. Biol. Chem. 265, 11872–11879
13. Imaizumi, K., Sasaki, T., Takahashi, K., and Takai, Y. (1994) J. Biol. Chem. 269, 1409–1416
14. Brewer, G. J., Torricelli, J. R., Eype, E. K., and Price, P. J. (1993) J. Neuroscience. Res. 35, 567–576
15. Mizoguchi, A., Ueda, T., Ikeeda, K., Shiku, H., Mizoguti, H., and Takai, Y. (1989) Brain Res. Mol. Brain Res. 5, 31–44
16. Laemmli, U. K. (1970) Nature 227, 680–685
17. Kawabe, H., Hata, Y., Takeuchi, M., Ide, N., Mizoguchi, A., and Takai, Y. (1999) J. Biol. Chem. 274, 30914–30918
18. Takeuchi, M., Hata, Y., Hiraoka, K., Toyoda, A., Irie, M., and Takai, Y. (1997) J. Biol. Chem. 272, 11943–11951
19. Mizoguchi, A., Yano, Y., Hamaguchi, H., Yagida, H., Ide, C., Zährlein, A., Shirataki, H., Sasaki, T., and Takai, Y. (1994) Biochem. Biophys. Res. Commun. 202, 1235–1243
20. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
21. Neer, E. J., Schmidt, C. J., Nanbudrup, R., and Smith, T. F. (1994) Nature 371, 297–300
22. Kraemer, C., Enkehaar, T., Zabel, B., and Schmidt, E. R. (2000) Genomics 64, 97–101