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*Rab*3A 低分子量GTP結合蛋白質の標的蛋白質の精製、クローニングと生化学的性状

白 瀧 博 通

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SUMMARY

*Rab*3A small GTP-binding protein is implicated in regulated secretion, particularly in neurotransmitter release from the presynapse. In the present study, I have purified a putative target protein for *Rab*3A from the bovine brain crude membranes, determined its primary structure, and named it Rabphilin-3A. Moreover, since Rabphilin-3A, which has no transmembrane region, is associated with synaptic vesicles, I have here studied how Rabphilin-3A is associated with synaptic vesicles. Rabphilin-3A more preferentially interacted with GTP-*Rab*3A than with GDP-*Rab*3A. Rabphilin-3A was a protein of 704 amino acids with a calculated Mr of 77,976. Structural analysis of Rabphilin-3A revealed that Rabphilin-3A had two copies of an internal repeat which were homologous to the C_2 domain of protein kinase C as described for synaptotagmin, which was thought to be one of Ca^{2+}-sensors in the presynapse. Northern (RNA) blot and Immunoblot analyses showed that Rabphilin-3A was exclusively expressed on the brain but not on the liver, lung, heart, and kidney. Treatment with 1 M NaCl of synaptic vesicles isolated from rat brain completely solubilized Rabphilin-3A from the vesicles. The vesicles deprived of Rabphilin-3A still contained *Rab*3A and synaptophysin. Recombinant Rabphilin-3A bound to the vesicles deprived of endogenous Rabphilin-3A in dose-dependent and saturable manners. The concentration of Rabphilin-3A giving a half maximum binding was about 50 nM and maximally 5 $\pm$ 1 molecules of Rabphilin-3A bound to one vesicle. Addition of exogenous *Rab*3A or removal of endogenous *Rab*3A by the action of *Rab* GDI did not affect the binding of Rabphilin-3A to the vesicles. However,

treatment of the vesicles with trypsin completely abolished the binding of Rabphilin-3A. These results indicate that Rabphilin-3A is a novel protein that has C_2-like domains and selectively interacts with GTP-*Rab*3A, and that Rabphilin-3A is associated with the vesicles at least through a Rabphilin-3A-anchoring protein on synaptic vesicles in a manner independent of *Rab*3A.

INTRODUCTION

*Rab*3A is a member of the *Rab* subfamily of the *Ras*-related small G protein[1] superfamily. The *Rab* family is implicated in intracellular vesicle transport including endocytosis, exocytosis, and transcytosis (for reviews, see Refs. 1-3). *Rab*3A is present in cells with regulated secretion such as neurons, endocrine cells, and exocrine cells. In neurons, *Rab*3A is present in the presynapse, especially enriched on synaptic vesicles (4-7). Although the precise function of *Rab*3A has not yet been clarified, it is suggested from study of mutations in *Rab*3A-knockout mice that *Rab*3A regulates the docking of synaptic vesicles to the presynaptic plasma membrane (8).

*Rab*3A has interconvertible GDP-bound inactive and GTP-bound active forms (3). GTP-*Rab*3A interacts with its target protein and performed its function. The conversion between the GDP- and GTP-bound forms are regulated by GDP/GTP exchange and GTPase reactions (3). The GDP/GTP exchange reaction is regulated by two types of GDP/GTP exchange proteins, stimulatory and inhibitory ones, named GDS and GDI, respectively. The GTPase reaction is regulated by a GTPase activating protein. Recently, Takai's group has purified GDI for *Rab*3A, determined its primary structure, and named it *Rab* GDI (9,10). *Rab* GDI regulates not only the GDP/GTP exchange reaction of *Rab*3A but also the translocation of *Rab*3A between the cytosol and the membrane. On the basis of the available evidence, a tentative model of the mode of action of *Rab*3A in neurotransmitter release has been proposed as follows: (1) GDP-*Rab*3A complexed with *Rab* GDI stays in the cytosol. (2) Once GDP-*Rab*3A is dissociated from *Rab* GDI, *Rab*3A is convert-

ed from the GDP-bound form to the GTP-bound form. (3) GTP-*Rab*3A binds to its target protein on synaptic vesicles. (4) Synaptic vesicles containing *Rab*3A are translocated and docked to the active zone of the presynaptic plasma membrane, where a voltage-dependent Ca^{2+} channels are clustered. (5) After Ca^{2+} is influxed, synaptic vesicles are fused with the presynaptic plasma membrane and neurotransmitter is released. (6) Then, *Rab*3A is converted from the GTP-bound form to the GDP-bound form by GTPase reaction. (7) GDP-*Rab*3A is complexed with *Rab* GDI and translocated to the cytosol. This model suggests that *Rab*3A cyclically regulates neurotransmitter release.

According to this model, I have attempted to search for a target protein for *Rab*3A. I have purified the putative target protein for *Rab*3A, isolated its cDNA, and determined its primary structure. Structural analysis has revealed that this protein has two repeats of C_2-like domain as described for synaptotagmin (11,12). I have tentatively named it Rabphilin-3A (Rab for *Rab*3A, philin for the Greek word for friend).

It has been recently revealed that although Rabphilin-3A has no transmembrane region, Rabphilin-3A is located on synaptic vesicles (13). Therefore, I have attempted here to clarify the mechanism how Rabphilin-3A is associated with synaptic vesicles. There are two possible ways for Rabphilin-3A to bind to synaptic vesicles: (1) There is an anchoring protein for Rabphilin-3A on the vesicles; and (2) Rabphilin-3A is post-translationally modified so that it binds to the vesicles. I have found here that Rabphilin-3A binds to synaptic vesicles at least through its anchoring protein of the vesicles in a manner independent of *Rab*3A.

MATERIAL AND METHODS

Materials and chemicals——Lipid-modified and -unmodified *Rab*3As were purified from the membrane fraction of Sf9 cells overexpressing *Rab*3A and from *E. coli* overexpressing *Rab*3A, respectively (14). *Rab*11 was purified from *E. coli* overexpressing *Rab*11 (14). c-Ki-*Ras*, *Rho*A, and *Rap*1B were purified from the cytosol fraction of Sf9 cells overexpressing c-Ki-*Ras*, *Rho*A, and *Rap*1B, respectively (15). *Rab* GDI was purified from the cytosol fraction of bovine brain (16). Synaptic vesicles were prepared from rat brain (6). Synaptophysin was purified from rat brain (17). An anti-*Rab*3A monoclonal antibody used was the same as that used previously (6). An anti-synaptophysin monoclonal antibody was from Boehringer Mannheim. ^{125}I-Labeled Bolton-Hunter reagent (specific activity, 74 TBq/mmol) was from Amersham Corp. The chemical cross-linker, DSS, was from Pierce Chemical Co. PVDF membrane (Problott), 0.45-μm pore size, was obtained from Applied Biosystems. AP-I was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All materials used in the nucleic acid study were purchased from Takara Shuzo Co., Ltd. (kyoto, Japan). Prokaryotic expression plasmid pGEX-2T was obtained from Pharmacia LKB Biotechnology, Inc.. Other materials and chemicals were obtained from commercial sources.

Preparation of radioiodinated Rab3A——Lipid-unmodified *Rab*3A (6.5 nmol, 500 μl) was dialyzed three times against 50 ml of 20 mM HEPES/NaOH at pH 7.4, 5 mM $MgCl_2$, and 0.6% CHAPS. GTPγS-*Rab*3A was made by incubating lipid-unmodified *Rab*3A (300 pmol) for 2 h at 30°C with 50 μM GTPγS in a mixture (100 μl) containing 20 mM HEPES/NaOH at pH 7.4, 5 mM

$MgCl_2$, 1 mM L-α-dimyristoylphosphatidylcholine, and 0.3% CHAPS. GTPγS-*Rab*3A was then iodinated by ^{125}I-labeled Bolton-Hunter reagent (18). Radioiodinated *Rab*3A was separated from the free reagent by gel filtration on a Sephadex G-25 column equilibrated with 20 mM HEPES/NaOH at pH 7.4, 5 mM $MgCl_2$, 1 μM GTPγS, and 0.5% sodium cholate.

Cross-link assay——Radioiodinated *Rab*3A (1 pmol, 2.2-2.6 x 10^5 cpm) was incubated with the sample to be assayed in a mixture (45 μl) containing 22.2 mM HEPES/NaOH at pH 7.4, 5.56 mM $MgCl_2$, 1.11 μM GTPγS, 222 mM NaCl, and 0.056% sodium cholate for 5 min at 30°C. After the incubation, 20 mM DSS in 20% dimethyl sulfoxide (5 μl) was added, followed by the second incubation for 30 min at 30°C. The reaction was stopped by the addition of an SDS-stopping solution (25 μl) containing 200 mM Tris/HCl at pH 6.7, 9% SDS, 6% 2-mercaptoethanol, and 15% glycerol. An aliquot (60 μl) of the sample was subjected to SDS-PAGE (10% polyacrylamide gel), followed by the autoradiography. The radioactivity of each band was measured by Fujix BAS2000 Bio-imaging Analyzer with radioiodinated *Rab*3A as a standard. During the purification procedures of Rabphilin-3A described below, an aliquot (10 μl) of the indicated fractions of each column chromatography was cross-linked to radioiodinated *Rab*3A to measure the Rabphilin-3A activity.

Molecular cloning and determination of nucleotide sequence of the Rabphilin-3A cDNA——Oligonucleotide probes were synthesized using a DNA synthesizer (Applied Biosystems, Model 380A) according to the partial amino acid sequences determined from purified Rabphilin-3A. A bovine brain cDNA library

was constructed using a cDNA synthesis system and a cDNA cloning system λgt10 (Amersham Corp.) according to the manufacture's manual. The hybridization procedures for screening the library were done as described (19). The obtained cDNA clones in a λgt10 phage vector were recloned using a pUC19 plasmid (19). Several deleted clones were constructed as described (20). The nucleotide sequence was determined as described (21).

Expression of GST-Rabphilin-3A and recombinant Rabphilin-3A——Plasmids for expression of Rabphilin-3A in Sf9 cells and *E. coli* were constructed with the following procedures. The 2.1-kb fragment containing the complete Rabphilin-3A cDNA coding region with the *Bam*H1 and *Kpn*1 sites upstream of the initiator methionine codon and downstream of the termination codon was synthesized by polymerase chain reaction (19). This fragment was digested by *Bam*H1 and inserted into the *Bam*H1-cut pGEX-2T plasmid to express the Rabphilin-3A cDNA under the control of the *tac* promoter in *E. coli* strain JM109 , and into the *Bam*H1-cut pAcYM1 *Autographa californica* baculovirus transfer vector to express the Rabphilin-3A cDNA under the control of the polyhedrin promoter in Sf9 cells. GST-Rabphilin-3A and recombinant Rabphilin-3A were purified as described, respectively (22).

Generation of an anti-Rabphilin-3A polyclonal antibody——A rabbit polyclonal antibody was generated against a 18-mer peptide corresponding to the partial amino acid sequence of Rabphilin-3A (Pro-Ala-Arg-Ala-Pro-Thr-Arg-Gly-Asp-Thr-Glu-Asp-Arg-Arg-Gly-Pro-Gly-Gln) (19). The anti-serum was affinity purified using the same peptide as described (19). Immunoblot analysis was carried out by use of this antibody as described (23).

Complex formation of GST-Rabphilin-3A with Rab3A

——Lipid-unmodified *Rab*3A (100 pmol) was incubated with 0.5 mM GDP or 0.5 mM GTPγS for 2 h at 30°C to make lipid-unmodified GDP-*Rab*3A or lipid-unmodified GTPγS-*Rab*3A (14). After each form of *Rab*3A was incubated with GST-Rabphilin-3A (20 pmol) for 30 min at 25°C in 200 μl of Buffer A (20 mM HEPES/NaOH at pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 200 mM NaCl, and 0.1% CHAPS) containing 300 μM GDP or 300 μM GTPγS, respectively. After then, each mixture was subjected to sucrose density gradient ultracentrifugation. A tube contained a 1ml-40% sucrose bed and a 3.8ml-sucrose density gradient (5-20% sucrose) in Buffer A containing 10 μM GDP or 10 μM GTPγS. Centrifugation was performed at 238,000 x g for 19 h at 4°C. In another set of experiments, either GST-Rabphilin-3A (20 pmol), lipid-unmodified GDP-*Rab*3A (100 pmol), or lipid-unmodified GTPγS-*Rab*3A (100 pmol) was separately subjected to the same sucrose density gradient ultracentrifugation. Fractions of 0.2 ml each were collected. An aliquot (40 μl) of each fraction was subjected to SDS-PAGE followed by immunoblot analysis by use of the anti-Rabphilin-3A polyclonal antibody or anti-*Rab*3A monoclonal antibody.

Immunoblot analysis——Various tissues of adult female rat (about 100 μg of wet weight each) were homogenized as described (6). The homogenates were subjected to SDS-PAGE followed by immunoblot analysis by use of the anti-Rabphilin-3A polyclonal antibody.

Northern (RNA) blot analysis——Preparation and gel transfer hybridization analysis of total RNAs were performed under high-stringency conditions as described previously (10,19). The probe for the Rabphilin-3A mRNA was a 2.7-kb *Eco* RI fragment

of the cDNA (the whole clone cDNA).

Preparation of Synaptic Vesicles Free from Rabphilin-3A——To remove endogenous Rabphilin-3A, synaptic vesicles (40 µg of protein) were incubated with 1 M NaCl for 2 h on ice in 80 µl of 20 mM HEPES/NaOH at pH 7.4, 1 mM EDTA, 10 mM EGTA, and 80 mM sucrose, followed by centrifugation at 230,000 x *g* for 90 min. After the supernatant was removed, the pellet was washed once with 200 µl of the same buffer and resuspended by repeatedly forcing it through a 27-gauge syringe needle in 80 µl of Glycine Buffer (5 mM HEPES and 0.3 M glycine at pH 7.4). This preparation of the vesicles was practically free of Rabphilin-3A (See **Fig. 11A**). The recovery of the vesicles was estimated to be 90-95% by Western blotting with the anti-synaptophysin antibody.

Preparation of Synaptic Vesicles Free from Rab3A——To make endogenous *Rab*3A the GDP-bound form, the vesicles deprived of Rabphilin-3A (100 µg of protein) were first incubated with 6 µM GDP for 15 min at 30°C in 45 µl of 20 mM Tris/HCl at pH 7.4, 2.2 mM HEPES/NaOH at pH 7.4, 10 mM EDTA, 2.5 mM $MgCl_2$, 133 mM glycine, and 10 µM APMSF. After the first incubation, 5 µl of 127.5 mM $MgCl_2$ was added and the mixture was immediately cooled on ice to prevent the dissociation of GDP from *Rab*3A. To solubilize *Rab*3A from the vesicles, the mixture was incubated with *Rab* GDI (2.1 nmol) in 550 µl of 20 mM Tris/HCl at pH 7.4, 0.18 mM EDTA, 4.1 mM $MgCl_2$, 1.0 mM DTT, 10 µM APMSF, and 0.6 µM GDP for 5 min on ice. Then, a 580-µl aliquot was layered on the top of 400 µl of 5% sucrose bed in Glycine Buffer, followed by centrifugation at 230,000 x *g* for 90 min. The supernatant was aspirated and the pellet was resuspended in 50 µl of

Glycine Buffer as described above. This preparation of the vesicles was practically free of *Rab*3A (See **Fig. 13A**). The recovery of the vesicles was estimated to be 25-30% by Western blotting with the anti-synaptophysin antibody.

Preparation of Synaptic Vesicles Associated with Exogenous Rab3A——Lipid-modified GTPγS-*Rab*3A was first made by incubating lipid-modified *Rab*3A (400 pmol) with 50 μM GTPγS for 20 min at 30°C in 39 μl of 20 mM Tris/HCl at pH 7.4, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 0.3% CHAPS. After the incubation, 1 μl of 405 mM $MgCl_2$ was added to give a final concentration of 15 mM and the mixture was immediately cooled on ice to prevent the dissociation of GTPγS from *Rab*3A. The vesicles deprived of Rabphilin-3A (200 μg of protein) were incubated with lipid-modified GTPγS-*Rab*3A (400 pmol) for 10 min on ice in 400 μl of 20 mM Tris/HCl at pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 5 μM GTPγS, and 0.075% CHAPS. After the incubation, a 380-μl aliquot was layered on the top of 400 μl of 5% sucrose bed in Glycine Buffer containing 5 mM $MgCl_2$ and 5 μM GTPγS, followed by centrifugation at 230,000 x *g* for 30 min. The supernatant was aspirated and the pellet was resuspended in 100 μl of Glycine Buffer as described above. This preparation of the vesicles contained exogenous *Rab*3A in an amount ten times more than that of endogenous *Rab*3A (See **Fig. 13A**). The recovery of the vesicles was estimated to be 25-30% by Western blotting with the anti-synaptophysin antibody.

Tryptic Digestion of Synaptic Vesicles——The vesicles deprived of Rabphilin-3A (250 μg of protein) were incubated with or without trypsin (trypsin/vesicle protein weight ratio = 1:250) for 1 h at 37°C in 380 μl of 50 mM HEPES and 0.2 M glycine at pH

7.4. To stop the digestion, 20 µl of 0.5 mg/ml of soybean trypsin inhibitor and 0.2 mM APMSF were added and the sample was further incubated for 30 min at 4°C. The digested vesicles were centrifuged at 230,000 x *g* for 90 min, washed three times with 400 µl of 5 mM HEPES, and 0.5 M glycine, 10 µg/ml of soybean trypsin inhibitor, and 10 µM APMSF at pH 7.4, and incubated overnight at 4°C. After the incubation, the digested vesicles were centrifuged at 230,000 x *g* for 90 min, followed by resuspension in 125 µl of Glycine Buffer as described above. The digestion of the vesicles was confirmed by SDS-PAGE, followed by protein staining with Coomassie brilliant blue.

Assay for the Binding of Rabphilin-3A to Synaptic Vesicles——Synaptic vesicles (5 µg of protein) were incubated with the indicated amounts of recombinant Rabphilin-3A for 10 min on ice in 80 µl of 20 mM HEPES, 1 mM EDTA, 10 mM EGTA, 150 mM NaCl, and 37.5 mM glycine at pH 7.4 or 20 mM HEPES, 1 mM EGTA, 150 mM NaCl, 1.09 mM $CaCl_2$, and 37.5 mM glycine at pH 7.4. After the incubation, a 70-µl aliquot was layered on the top of 400 µl of 5% sucrose bed in Glycine Buffer, followed by centrifugation at 230,000 x *g* for 30 min. The supernatant was aspirated and the pellet was suspended in 120 µl of 66.7 mM Tris/HCl at pH 6.7, 3% SDS, 2% 2-mercaptoethanol, and 5% glycerol. A 60-µl aliquot was subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A antibody. The recovery of the vesicles was estimated to be 25-30% by Western blotting with the anti-synaptophysin antibody.

Other Procedures——SDS-PAGE was performed as described (24). Rabphilin-3A, *Rab*3A, and synaptophysin transferred to ni-

trocellulose sheets were detected by using the ECL immunoblotting detection system (Amersham Corp.). Amounts of Rabphilin-3A, *Rab*3A, and synaptophysin were quantified by densitometric tracing at 420 nm with purified Rabphilin-3A, *Rab*3A, and synaptophysin as standards, respectively. Protein concentrations were determined with bovine serum albumin as a reference protein (25). The computer program IDEAS was used for homology search and hydropathy plot (26,27).

RESULTS

Identification of a Protein Cross-linked with Rab3A——When the radioiodinated GTPγS-*Rab*3A was incubated with or without DSS, and each sample was subjected to SDS-PAGE followed by autoradiography, several radioactive bands in addition to the *Rab*3A band were detected for both samples (**Fig. 1, *lanes 1* and *2***). These bands were derived from the proteins slightly contaminating the *Rab*3A sample used. When the radioiodinated GTPγS-*Rab*3A was cross-linked with the crude membrane fraction of bovine brain, only one distinct radioactive band was detected (**Fig. 1, *lanes 3* and *4***). The Mr of this radioactive band was about 110,000 and appeared to be composed of the radioiodinated *Rab*3A and a molecule with a Mr of about 86,000, because the Mr of *Rab*3A was about 24,000 on SDS-PAGE (14). This molecule is tentatively termed here Rabphilin-3A. Rabphilin-3A was extracted from the crude membrane fraction with sodium cholate (**Fig. 1, *lanes 5* and *6***).

Purification of Rabphilin-3A——The crude membrane fraction was prepared from bovine brain as described (28) except

that 10 mM HEPES/NaOH at pH 7.4 was used instead of 10 mM Tris/HCl at pH 7.4. After the crude membrane fraction (800 ml, 9.6 g of protein) was centrifuged at 20,000 x g for 1 h, the protein was extracted from the pellet suspended in 320 ml of 2% sodium cholate in Buffer B (20 mM HEPES/NaOH at pH 7.4, 5 mM $MgCl_2$, and 1 mM DTT) with stirring for 30 min. After centrifugation at 100,000 x g for 1 h, the supernatant was pooled and used as the extract from the crude membrane fraction (320 ml, 2.9 g of protein). One fourth of the extract from the crude membrane fraction (80 ml, 725 mg of protein) was applied to a HiLoad 26/10 Q Sepharose HP column (2.6 x 10 cm) equilibrated with Buffer B containing 1% sodium cholate. After the column was washed with 148 ml of the same buffer, elution was performed with a 600-ml linear gradient of NaCl (0-1.0 M) in Buffer B containing 1% sodium cholate. Fractions of 12 ml each were collected. Rabphilin-3A appeared as a single peak in Fractions 37-39. The rest of the extract from the crude membrane fraction was treated in the same way. The active fractions of these peaks of the four column chromatographies were combined. To exchange sodium cholate for CHAPS in the buffer, the combined fractions were diluted with 144 ml of Buffer B, concentrated to 14.4 ml by an Amicon concentrator (PM-30), and again diluted to 144 ml with Buffer B containing 0.6% CHAPS. This sample (144 ml, 72 mg of protein) was applied to a Mono Q HR10/10 column (1 x 10 cm) equilibrated with Buffer B containing 0.6% CHAPS. After the column was washed with 56 ml of the same buffer, elution was performed with a 240-ml linear gradient of NaCl (0-0.5 M) in Buffer B containing 0.6% CHAPS. Fractions of 2 ml each were collected. Rabphilin-3A appeared as a single peak in Fractions 128-141. The active fractions (28 ml,

1.4 mg of protein) were applied to a hydroxyapatite column (0.8 x 2.0 cm) equilibrated with Buffer B containing 0.6% CHAPS and 50 mM KH_2PO_4. After the column was washed with 42 ml of Buffer B containing 0.6% CHAPS and 80 mM KH_2PO_4, elution was performed with 9 ml of Buffer B containing 0.6% CHAPS and 120 mM KH_2PO_4. Fractions of 1.5 ml each were collected. Rabphilin-3A appeared as a single peak in Fractions 49-52. The active fractions (6 ml, 10 µg of protein) were concentrated by Centricon-30 to 1.2 ml and subjected to sucrose density gradient ultracentrifugation using six tubes. Each tube contained a 4.8 ml-sucrose density gradient (5-20% sucrose in Buffer B containing 0.6% CHAPS). Centrifugation was performed at 238,000 x g for 19 h. Fractions of 0.25 ml each were collected. Rabphilin-3A appeared as a single peak in Fractions 7-10. **Fig. 2** shows that the profile of Rabphilin-3A activity and the protein staining pattern of the indicated fractions of the ultracentrifugation. The active fractions (6 ml, 5 µg of protein) were used as the highly purified sample of Rabphilin-3A. The highly purified sample of Rabphilin-3A was cross-linked with radioiodinated *Rab*3A (**Fig. 1, *lanes 7 and 8***). This cross-linking was abolished by pretreatment of the highly purified sample of Rabphilin-3A with heat boiling or tryptic digestion (**Fig. 1, *lanes 9 and 10***). About 100 µg of the highly purified sample of Rabphilin-3A was accumulated by the same purification procedures. About 5 µg of the highly purified sample of Rabphilin-3A was resolved by SDS-PAGE and electrophoretically transferred to a PVDF membrane (23). After staining the membrane with Ponceau S, the band corresponding to Rabphilin-3A was cut out with a clear razor for peptide map analysis.

Kinetic Properties of Rabphilin-3A——The cross-linking of radioiodinated *Rab*3A with the highly purified sample of Rabphilin-3A was progressively inhibited by the addition of increasing amounts of nonradioactive lipid-unmodified GTPγS-*Rab*3A (**Fig. 3**). Nonradioactive lipid-unmodified GDP-*Rab*3A also inhibited this cross-linking but much less efficiently. The IC_{50} of nonradioactive lipid-unmodified GTPγS-*Rab*3A and GDP-*Rab*3A were about 8 and 70 nM, respectively. Similar inhibition was observed when lipid-modified *Rab*3A was used instead of lipid-unmodified *Rab*3A. The GTPγS-bound form of other small G proteins, including c-Ki-*Ras*, *Rho*A, *Rap*1B, and *Rab*11, were ineffective in this inhibition.

Peptide map and amino acid sequence analyses of Rabphilin-3A——Each sample of Rabphilin-3A on the PVDF membrane was digested with AP-I at a molar ratio of 1:50 (AP-I:Rabphilin-3A) for 24 h at 37°C in 300 μl of 0.2 M Tris/HCl at pH 9.0 containing 8% acetonitrile (29). When the peptides released after the *in situ* protease digestion of Rabphilin-3A were combined and subjected to Bakerbond WP C_8 column chromatography as described (30), more than thirty peptides were separated. Eleven peaks of them were sequenced using an automated gas-phase sequencer (Applied Biosystems, Model 470A).

Molecular cloning and determination of nucleotide and deduced amino acid sequences of Rabphilin-3A——Two sets of oligonucleotide probes for the cloning of the Rabphilin-3A cDNA were synthesized according to the partial amino acid sequences of Rabphilin-3A, Lys-Met-Glu-Glu-Met-Glu-Gln-Glu and Glu-Phe-Asn-Glu-Glu-Phe-Phe-Tyr. Twenty-four clones were hybridized with both probes out of about 600,000 recombinant phage plaques

from the bovine brain cDNA library. A nucleotide sequence of one cloned cDNA with about 2.7 kb was obtained. The deduced amino acid sequence of Rabphilin-3A is shown in **Fig. 4**. The cDNA contained an open reading frame of 704 amino acids. The neighboring sequence of the first ATG is consistent with the translation initiation start site proposed by Kozak (31). The deduced amino acid sequence was identical to all the amino acid sequences determined from purified Rabphilin-3A. The Mr value was calculated to be 77,976. This value was similar to those of purified Rabphilin-3A estimated by SDS-PAGE, which were about 85,000. These results indicate that Rabphilin-3A is composed of a single polypeptide without a subunit structure.

Of the twenty-four isolated clones, four of them hybridized under high stringency conditions to two fragments: one consisted of the N-terminal half of the coding region of the isolated Rabphilin-3A cDNA and the other consisted of the C-terminal half of the coding region. These results indicate that they were identical to the Rabphilin-3A cDNA clone. Ten of the clones hybridized to only one fragment of them under the same conditions and appeared to be a part of the whole coding region of the isolated Rabphilin-3A cDNA. The other clones did not hybridize to either fragment under both high- and low-stringency conditions. It is likely that they are not other Rabphilin-3A cDNAs, and I did not characterize them further.

Complex formation of GST-Rabphilin-3A with Rab3A——I examined whether GST-Rabphilin-3A makes a complex with lipid-unmodified GTPγS-*Rab*3A on sucrose density gradient ultracentrifugation. For this experiment, I produced GST-Rabphilin-3A as a GST fusion protein in *E. coli*, and purified it. When either GST-Rab-

philin-3A, lipid-unmodified GDP-*Rab*3A, or lipid-unmodified GTPγS-*Rab*3A was separately subjected to the sucrose density gradient ultracentrifugation, the immunoreactive protein corresponding to GST-Rabphilin-3A appeared at the near bottom and the immunoreactive protein corresponding to both forms of *Rab*3A appeared at the position with an Mr value of about 25,000 (**Fig. 5A**). Since GST also appeared at the near bottom under the same conditions (data not shown), GST-Rabphilin-3A was aggregated due to the property of GST. When GST-Rabphilin-3A was mixed with lipid-unmodified GDP-*Rab*3A and the mixture was then subjected to the same sucrose density gradient ultracentrifugation, the immunoreactive proteins corresponding to GST-Rabphilin-3A and *Rab*3A appeared at their respective original positions (**Fig. 5B**). When GST-Rabphilin-3A was mixed with lipid-unmodified GTPγS-*Rab*3A and the mixture was then subjected to the same sucrose density gradient ultracentrifugation, the immunoreactive protein corresponding to GST-Rabphilin-3A appeared at the near bottom (**Fig. 5C**). The immunoreactive protein corresponding to *Rab*3A at the position with an Mr value of about 25,000 shifted to this position (**Fig. 5C**). It may be noted that only a part of *Rab*3A shifted to this position because the molar ratio of *Rab*3A to GST-Rabphilin-3A used in this experiment was five to one. These results indicate that GST-Rabphilin-3A makes a complex with lipid-unmodified GTPγS-*Rab*3A but not with lipid-unmodified GDP-*Rab*3A. Under the same conditions, GST-Rabphilin-3A did not make a complex with either GTPγS- or GDP-*Rab*11 (data not shown).

Structural properties of Rabphilin-3A——Rabphilin-3A contained two copies of an internal repeat that were homologous

to the C_2 domain of protein kinase C and synaptotagmin (**Figs. 6 and 7**) (11,12,32-35). It has been shown that synaptotagmin, which is known to be localized in the membrane of synaptic vesicles and to be implicated in neurotransmitter release from the presynapse, has the C_2 domains in its C-terminal half (36-41). Rabphilin-3A also had the C_2 domains in its C-terminal half and showed the striking sequence homology with synaptotagmin at the C_2 domains, but the N-terminal half of Rabphilin-3A was not homologous to synaptotagmin (**Figs. 6 and 7**). Hydropathy analysis (27) indicated that Rabphilin-3A was overall hydrophilic except two short hydrophobic regions (**Fig. 8**).

Tissue distribution of Rabphilin-3A——Various tissues were obtained from rat and the amount of Rabphilin-3A was quantified in each tissue by immunoblot analysis by use of the anti-Rabphilin-3A polyclonal antibody. The immunoreactive band was detected only in rat brain but not in other rat tissues including pancreas, liver, spleen, kidney, and lung (**Fig. 9**). However, the position of this band was slightly lower than that of Rabphilin-3A purified from bovine brain on SDS-PAGE. This difference might be due to the difference of species, because I have recently found that when ^{125}I-labeled *Rab*3A was cross-linked in the presence of DSS with bovine and rat brain crude membranes, a radioactive band of a Mr value of about 110,000 was detected in both bovine and rat membranes (data not shown).

The Rabphilin-3A mRNA levels in bovine brain and various rat tissues were examined. By Northern blot analysis of the Rabphilin-3A mRNA, one major band and two minor bands were detected in the RNA of bovine brain (**Fig. 10**). The major band and minor bands were calculated to be about 5.1, 7.7, and 15 Kb long, re-

spectively. The 4.8 Kb band was detected in the RNA of rat brain but not in the RNA of other tissues, including pancreas, liver, spleen, kidney, and lung. Although I do not known why three bands were detected in bovine brain, it is conceivable that the three mRNA are derived from three highly conserved genes or result from alternative splicing of the primary transcript of a single gene.

Presence of Rabphilin-3A and Rab3A on Synaptic Vesicle——It was recently revealed that Rabphilin-3A is present on synaptic vesicles (13). Therefore, synaptic vesicles isolated from rat brain were subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A, anti-*Rab*3A, or anti-synaptophysin antibody. All Rabphilin-3A, *Rab*3A, and synaptophysin were detected in the vesicles (**Fig. 11A**). In mammal, each synaptic vesicle has been shown to contain ~1.6 x 10^6 daltons of protein (42). Based on this value, it was calculated that about one Rabphilin-3A, two *Rab*3A, and ten synaptophysin molecules were detected in one vesicle.

Removal of Rabphilin-3A from Synaptic Vesicles——The isolated synaptic vesicles were treated with 1 M NaCl and subjected to centrifugation to separate the soluble and vesicle fractions. Each fraction was then subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A, anti-*Rab*3A, or anti-synaptophysin antibody. After treatment of the vesicles without NaCl, all Rabphilin-3A, *Rab*3A, and synaptophysin were detected in the vesicle fraction but not in the soluble fraction (**Fig. 11A**). However, after treatment of the vesicles with NaCl, Rabphilin-3A was detected in the soluble fraction but not in the vesicle fraction, indicating that Rabphilin-3A is solubi-

lized by the NaCl treatment. In contrast, both *Rab*3A and synaptophysin were detected in the vesicle fraction but not in the soluble fraction. The solubilization of Rabphilin-3A from the vesicles was dependent on the concentrations of NaCl (**Fig. 11B**). The concentrations of NaCl giving half maximal and maximal solubilizations were 0.4 M and 1.0 M, respectively.

Dose-dependent and Saturable Binding of Rabphilin-3A to Synaptic Vesicles in a Manner Sensitive to Tryptic Digestion of the Vesicles——Exogenous Rabphilin-3A bound to synaptic vesicles, which had been deprived of endogenous Rabphilin-3A as described above, in dose-dependent and saturable manners (**Fig. 12A**). The dose giving a half maximal binding was about 50 nM. Five ± one molecules of Rabphilin-3A were calculated to maximally bind to one vesicle. The binding of exogenous Rabphilin-3A to the vesicles was abolished by the tryptic digestion of the vesicles (**Fig. 12, A** and **B**). Exogenous Rabphilin-3A did not bind to human erythrocyte ghosts (data not shown).

No Effect of Rab3A on the Binding of Rabphilin-3A to Synaptic Vesicles——Synaptic vesicles deprived of Rabphilin-3A still contained *Rab*3A as described above. The vesicles were first incubated with GDP to convert all *Rab*3A to the GDP-bound form and then with *Rab* GDI to remove GDP-*Rab*3A from the vesicles by forming a soluble *Rab* GDI-*Rab*3A complex. The vesicles were then centrifuged to separate the soluble and vesicle fractions. The vesicle fraction obtained was subjected to SDS-PAGE, followed by Western blotting by the anti-*Rab*3A and anti-synaptophysin antibodies. *Rab*3A was not detected in the vesicle fraction, although synaptophysin was detected (**Fig. 13A**). Exogenous Rabphilin-3A similarly bound to the vesicles deprived

of *Rab*3A (**Fig. 13B**). Conversely, exogenous GTPγS-*Rab*3A bound to the vesicles, but this binding did not affect the binding of exogenous Rabphilin-3A to the vesicles (**Fig. 13B**).

Effect of Ca^{2+} on the Solubilization of Rabphilin-3A from and its Binding to Synaptic Vesicles——The experiments described above were carried out in the presence of 10 mM EGTA. Even at 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} M Ca^{2+}, most of Rabphilin-3A was solubilized by the treatment of synaptic vesicles with 1 M NaCl (data not shown). However, about 10% of the total amount of Rabphilin-3A was not solubilized from the vesicles in the presence of Ca^{2+}. At 1×10^{-4} M Ca^{2+}, exogenous Rabphilin-3A bound to both the intact synaptic vesicles and the trypsin-digested vesicles in the dose-dependent but non-saturable manners (**Fig. 14**). However, the trypsin-sensitive specific binding curve of exogenous Rabphilin-3A to the vesicles at 1×10^{-4} M Ca^{2+} was the same as that in the absence of Ca^{2+}. At 1×10^{-4} M Ca^{2+}, the dose giving a half-maximal binding was about 50 nM and 5 ± 1 molecules of exogenous Rabphilin-3A were calculated to maximally bind to one vesicle. The essentially similar results were obtained at 1×10^{-6} and 1×10^{-5} M Ca^{2+}.

DISCUSSION

In the present study, I have purified the putative target protein for *Rab*3A (Rabphilin-3A) from the bovine crude membrane fractions. Rabphilin-3A more preferentially interacted with GTP-*Rab*3A than with GDP-*Rab*3A. Moreover, Rabphilin-3A did not interact with c-Ki-*Ras*, *Rho*A, *Rap*1B, and *Rab*11. Subsequently, I

have isolated its cDNA from a bovine brain cDNA library and determined its whole primary nucleotide sequence. The amino acid sequence deduced from the nucleotide sequence of the isolated cDNA was identical to all of the amino acid sequences determined from purified Rabphilin-3A. The analysis by sucrose density ultracentrifugation has revealed that GST-Rabphilin-3A makes a complex with GTPγS-*Rab*3A but not with GDP-*Rab*3A. It is most likely from these results that the isolated cDNA encodes the putative target protein for *Rab*3A. To my knowledge, Rabphilin-3A is the first identified target protein for the *Ras*-related small GTP-binding protein in mammals.

Homology search analysis has revealed that the C-terminal half of Rabphilin-3A contains two copies of an internal repeat that are homologous to the C_2 domain of protein kinase C and synaptotagmin, both of which are known to bind to membrane phospholipid in a Ca^{2+}-dependent manner (11,12,32-35). Consistently, it was revealed that Rabphilin-3A binds to membrane phospholipid in a Ca^{2+}-dependent manner (43). Furthermore, Northern (RNA) blot and immunoblot blot analyses have revealed that Rabphilin-3A is highly expressed on the brain but not on other tissues such as pancreas, liver, spleen, kidney, and lung. It was revealed that *Rab*3A is expressed on the brain and pancreas, but not on the liver, spleen, kidney, and lung (6). It may be noted that although *Rab*3A is expressed on the pancreas, expression of Rabphilin-3A is not detected on the pancreas. Thus, it is possible that the Rabphilin-3A levels in pancreas is too low to be detected by immunoblot blot analysis or that pancreas has another target protein for *Rab*3A which is similar to but different from Rabphilin-3A.

It has been recently revealed that Rabphilin-3A is located on synaptic vesicles (13). The hydropathy profile of Rabphilin-3A has shown that Rabphilin-3A is overall hydrophilic except for two short hydrophobic regions. Rabphilin-3A contained neither a signal peptide sequence nor a sufficient transmembrane domain. Therefore, I have examined how Rabphilin-3A is associated with synaptic vesicles. I have shown here (1) that endogenous Rabphilin-3A is solubilized from synaptic vesicles by NaCl treatment; (2) that exogenous Rabphilin-3A binds to the vesicles deprived of endogenous Rabphilin-3A in dose-dependent and saturable manners; (3) that the binding of exogenous Rabphilin-3A to the vesicles deprived of endogenous Rabphilin-3A is abolished by prior treatment of the vesicle with trypsin; (4) that exogenous Rabphilin-3A binds to the vesicles deprived of endogenous Rabphilin-3A irrespective of the presence and absence of *Rab*3A; and (5) that exogenous Rabphilin-3A dose not bind to human erythrocyte ghosts. Taken together, these results indicate that Rabphilin-3A is a peripheral membrane protein associated with synaptic vesicles at least through a vesicle protein in a manner independent of *Rab*3A. It is likely that this protein serves as an anchoring protein for Rabphilin-3A. I have not yet identified this anchoring protein and it is not known whether this protein is a single species or a mixture of different protein species. However, this protein may be present on synaptic vesicles at a ratio of 5 ± 1 molecules to one vesicle, because maximally 5 ± 1 molecules of Rabphilin-3A specifically bind to one vesicle.

I have shown here the effects of Ca^{2+} on the solubilization of Rabphilin-3A from and its binding to synaptic vesicles. Even in the presence of Ca^{2+}, Rabphilin-3A is solubilized from synap-

tic vesicles by treatment with 1 M NaCl and exogenous Rabphilin-3A binds to the vesicles deprived of endogenous Rabphilin-3A. However, in the presence of Ca^{2+}, the solubilization of Rabphilin-3A is incomplete and the binding of the exogenous protein is non-saturable and is observed even for the trypsin-digested vesicles. These results suggest that Rabphilin-3A is associated with synaptic vesicles both specifically and non-specifically in the presence of Ca^{2+}. This non-specific binding of Rabphilin-3A may be attributed to protein-phospholipid interactions through the C_2-like domains, because it interacts with phospholipid through the C_2-like domains in the presence of Ca^{2+} (43). It remains to be clarified whether the association of Rabphilin-3A with synaptic vesicles is also regulated by its post-translational modifications.

It remains to be clarified how *Rab*3A is associated with synaptic vesicles. It has been previously shown that *Rab*3A, associated with synaptic vesicles is almost GTP-bound form (45). Therefore, it is tempting to speculate that *Rab*3A is associated with the vesicles through a protein molecule, which interacts with GTP-bound form of *Rab*3A but not with GDP-bound form of *Rab*3A. Rabphilin-3A interacts with GTP-*Rab*3A but not with GDP-*Rab*3A. Therefore, It was possible that *Rab*3A may bind to the vesicles through Rabphilin-3A. However, further study is necessary to clarify it.

Neurotransmitters are released from the presynapse by Ca^{2+}-regulated exocytosis of synaptic vesicles (for review, see Ref 44). Synaptic vesicles store high concentrations of neurotransmitters. When Ca^{2+} is influxed through a voltage-dependent Ca^{2+} channel on the synaptic plasma membrane, Ca^{2+} appears to trigger

the fusion of synaptic vesicles with the synaptic plasma membrane, resulting in neurotransmitter release (44). It has been recently revealed that the fusion of synaptic vesicles with the synaptic plasma membrane is mediated by the general fusion machinery, so called NSF-SNAP-SNAREs system (46,47). Moreover, it has been previously shown that synaptotagmin located on synaptic vesicles inhibits the fusion of synaptic vesicles with the synaptic plasma membrane mediated by NSF-SNAP-SNAREs system, and that influx of Ca^{2+} into the presynapse attenuates the inhibitory action of synaptotagmin on NSF-SNAP-SNAREs system, resulting in the fusion of synaptic vesicles with the synaptic plasma membrane (45). Since Rabphilin-3A has C_2 domains such as synaptotagmin, it is possible that Rabphilin-3A may act the same function as synaptotagmin. Moreover, the genetic analyses of yeast have recently shown that the *Rab* family regulates the assembly of v-SNAREs (48). Therefore, it is possible that *Rab*3A may regulate the assembly of v-SNAREs on synaptic vesicles through Rabphilin-3A. Further study is necessary to clarify the function of *Rab*3A and Rabphilin-3A on the neurotransmitter release.

CONCLUSION

I have purified a putative target protein for *Rab*3A, determined its primary structure, and named it Rabphilin-3A. Rabphilin-3A more preferentially interacted with GTP-*Rab*3A than with GDP-*Rab*3A. Rabphilin-3A was a protein of 704 amino acids with a calculated Mr of 77,976 and had two copies of an internal repeat which were homologous to the C_2 domain of protein kinase C. Rabphilin-3A was exclusively expressed on the brain but not on the

liver, lung, heart, and kidney. Rabphilin-3A was a peripheral membrane protein associated with synaptic vesicles at least through a vesicle protein in a manner independent of *Rab*3A.

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FOOTNOTES

The abbreviations used are: G protein, GTP-binding protein; GDS, GDP dissociation stimulator; GDI, GDP dissociation inhibitor; Sf9 cells, *Spodoptera frugiperda* cells; *E. coli*, *Escherichia coli*; DSS, disuccinimidyl suberate; PVDF membrane, polyvinylidene difluoride membrane; *Achromobacter* protease I, API; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NFS attachment protein; SNAREs, SNAP receptors.

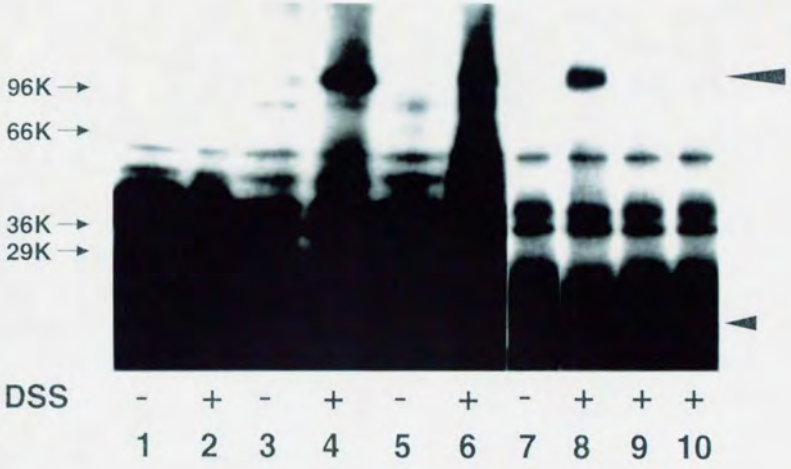


Fig. 1. Cross-linking of radioiodinated *Rab*3A with a bovine brain membrane protein by use of DSS. Radioiodinated *Rab*3A was cross-linked with the crude membrane fraction, the extract from this fraction, or the highly purified Rabphilin-3A before or after treatment with heat boiling or tryptic digestion. ***Lanes 1*** and ***2***, none; ***lanes 3*** and ***4***, the crude membrane fraction; ***lanes 5*** and ***6***, the extract from this fraction; ***lanes 7*** and ***8***, the highly purified sample of Rabphilin-3A; ***lane 9***; the boiled highly purified sample of Rabphilin-3A; ***lane 10*** the tryptic-digested highly purified sample of Rabphilin-3A. The protein makers used were phosphorylase *b* (*Mr* = 96,000), bovine serum albumin (*Mr* = 66,000), glyceraldehyde-3-phosphate dehydrogenase (*Mr* = 36,000), and carbonic anhydrase (*Mr* = 29,000). The *small* and *large* arrowheads show the position of radioiodinated *Rab*3A and the band which appeared after cross-linking, respectively.

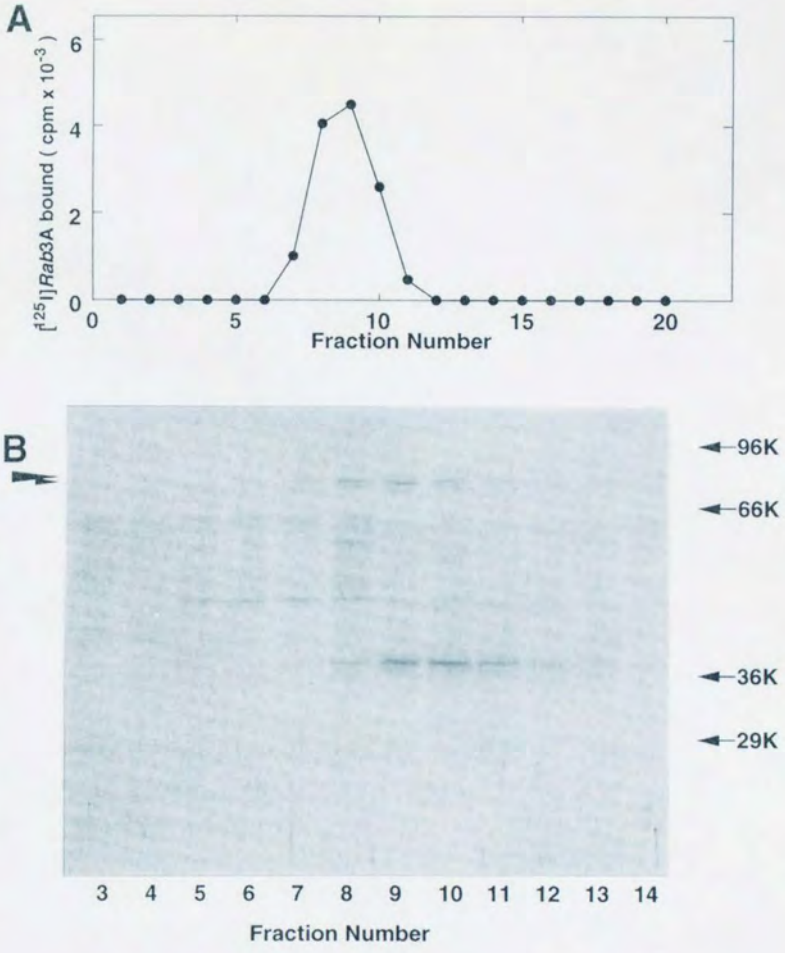


Fig. 2. Sucrose density gradient ultracentrifugation of Rabphilin-3A. Aliquots (10 and 40 µl) of the inducated fractions were used for the cross-linking assay and SDS-PAGE, respectively. (**A**) Profile of the Rabphilin-3A activity. (●), ^{125}I-labeled *Rab*3A bound. (**B**) Protein staining patterns visualized with silver. The protein makers used were the same as those used in **Fig. 1**. The *small* or *large arrowhead* shows the position of Rabphilin-3A.

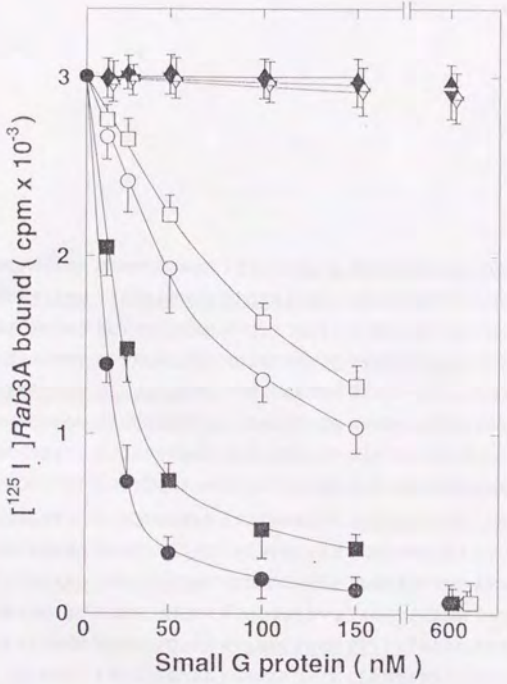


Fig. 3. Effect of various nonradioactive small G proteins on the cross-linking of radioiodinated *Rab*3A with Rabphilin-3A. Radioiodinated *Rab*3A was cross-linked with the highly purified Rabphilin-3A in the presence of various nonradioiodinated small G proteins. (●), in the presence of lipid-unmodified GTPγS-*Rab*3A; (○), in the presence of lipid-unmodified GDP-*Rab*3A; (■) in the presence of lipid modified GTPγS-*Rab*3A; (□), in the presence of lipid modified GDP-*Rab*3A; (△), in the presence of GTPγS-c-Ki-*Ras*; (▲), in the presence of GTPγS-*Rho*A; (▼), in the presence of GTPγS-*Rap*1B; (▽), in the presence of GTPγS-*Rab*11. The values were means ± S.E. of three independent experiments.

MTDTVFSSSS SRWMCPSDRP LQSNDKEQLQ TGWSVHPSGQ PDRQRKQEEL 50

TDEEKEIINR VIARAEKMEE MEQERIGRLV DRLENMRKNV AGDGVNRCIL 100

CGEQLGMLGS ACVVCEDCKK NVCTKCGVET SNNRPHPVWL CKICIEQREV 150

WKRSGAWFFK GFPKQVLPQP MPIKKNKPQQ PVSEPVPAAP EPATPEPKHP 200

ARAPTRGDTE DRRGPGQKTG PDMTSAPGRG SYGPPVRRAS EARMSSSGRD 250

SDSWDQGHGM AAGDPSQSPA GLRRANSVQA SRPAPASMQS PAPPQPGQPG 300

PPGGSRPSPG PTGRFPDQRP EVAPSDPDYT GAAAQPREER TGGIGGYSAA 350

GTREDRAGHP PGSYTQASAA APQPVVASAR QPPPPEEDEE EANSYDSDEA 400

TTLGALEFSL LYDQDNSSLH CTIIKAKGLK PMDSNGLADP YVKLHLLPGA 450

SKSNKLRTKT LRNTRNPIWN ETLVYHGITD EDMQRKTLRI SVCDEDKFGH 500

NEFIGETRFS LKKLKPNQRK NFNICLERVI PMKRAGTTGS ARGMALYEEE 550

QVERIGDIEE RGKILVSLMY STQQGGLIVG IIRCVHLAAM DANGYSDPFV 600

KLWLKPDMGK KAKHKTQIKK KTLNPEFNEE FFYDIKHSDL AKKSLDISVW 650

DYDIGKSNDY IGGCQLGISA KGERLKHWYE CLKNKDKKIE RWHQLQNENH 700

VSSD 704

Fig. 4. The deduced amino acid sequence of Rabphilin-3A. The amino acid sequences determined from purified rabphilin-3A are indicated by solid underlines. The amino acid sequence which is used for the production of the anti-Rabphilin-3A polyclonal antibody is indicated by a broken underline.

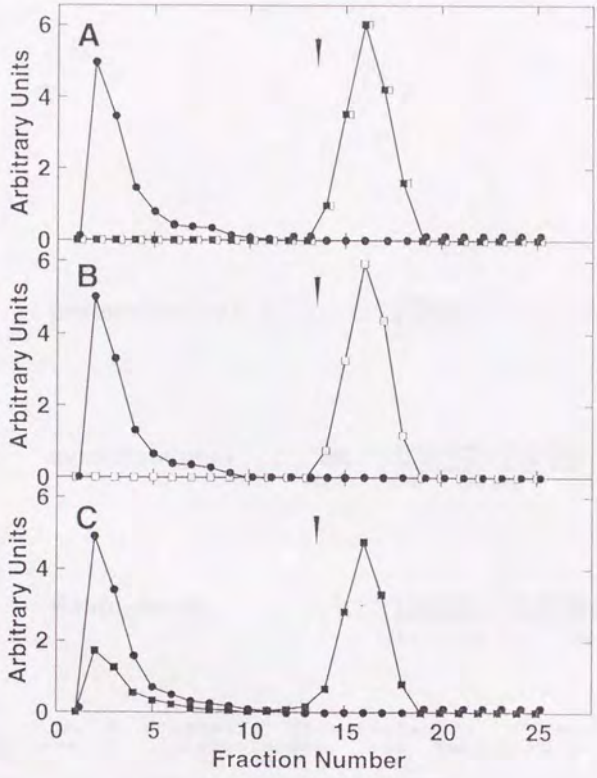


Fig. 5. Complex formation of GST-Rabphilin-3A with *Rab*3A. (**A**) Either GST-Rabphilin-3A, lipid-unmodified GDP-*Rab*3A, or lipid-unmodified GTPγS-*Rab*3A was separately subjected to the sucrose density gradient ultracentrifugation. (**B**) After GST-Rabphilin-3A was incubated with lipid-unmodified GDP-*Rab*3A, the mixture was subjected to the same sucrose density gradient ultracentrifugation. (**C**) After GST-Rabphilin-3A was incubated with lipid-unmodified GTPγS-*Rab*3A, the mixture was subjected to the same sucrose density gradient ultracentrifugation. Symbols: ●—●, GST-Rabphilin-3A; □—□, lipid-unmodified GDP-*Rab*3A; ■—■, lipid-unmodified GTPγS-*Rab*3A. The arrow head indicates the position of human hemoglobin (4.5S, Mr = 64,500). The results shown are representative of three independent experiment.

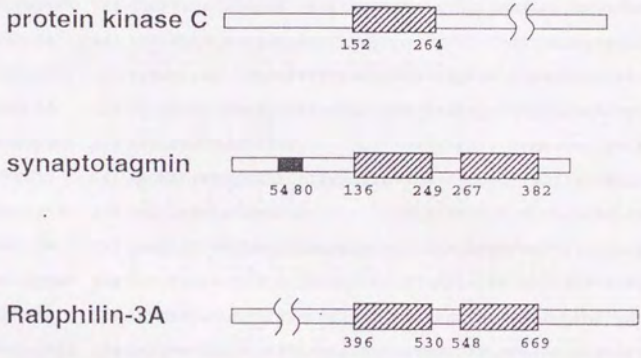


Fig. 6. Schematic representation of protein kinase C, synaptotagmin, and Rabphilin-3A. Shaded boxes indicate the homologous regions that are designated as the C_2 domain of protein kinase C (11). A black box indicates a transmembrane region.

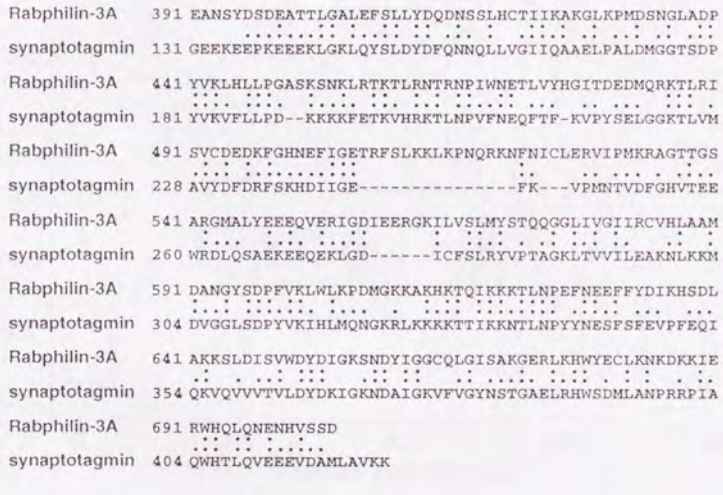


Fig. 7. Sequence homology of Rabphilin-3A with synaptotagmin. Colons between amino acids designate identities. Dots indicate conserved amino acid changes. Dashes indicate gaps imposed to maximize alignment. Conservative amino acid substitutions are grouped as follows; C; S, T ,P, A, G; N, D, E, Q; H, R, K; M, I, L, V; F, Y, W (49).

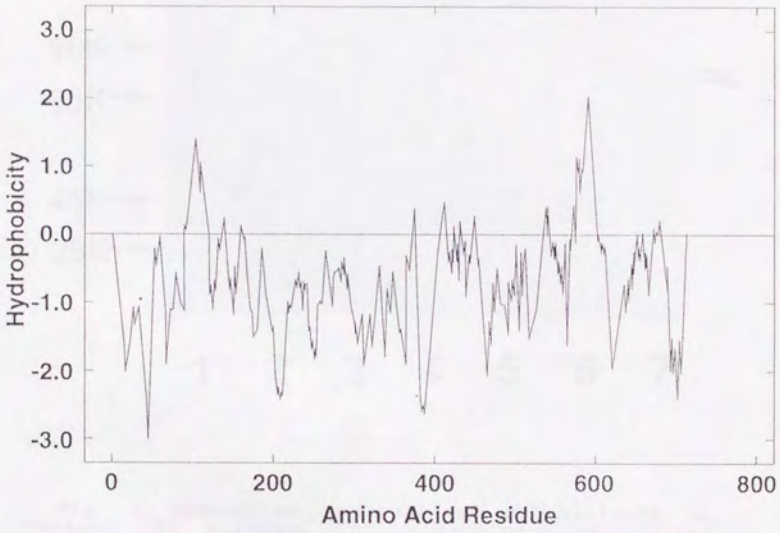
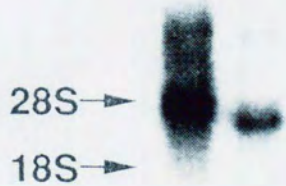


Fig. 8. Hydrophilicity plot of Rabphilin-3A. The plot was performed according to Kyte and Doolittle with a window of 15 amino acids (27). Hydrophilicity is indicated by negative value and hydrophobicity is indicated by positive value.

Fig. 9. Immunoblot analysis of Rabphilin-3A in various rat tissues. The homogenates of various rat tissues (100 μg each of protein) were subjected to SDS-PAGE followed by immunoblot analysis by use of the anti-Rabphilin-3A polyclonal antibody. ***Lanes*** ***1***, Rabphilin-3A purified from bovine brain (100 ng of protein); ***2***, rat brain; ***3***, rat pancreas; ***4***, rat liver; ***5***, rat spleen; ***6***, rat kidney; ***7***, rat lung. The protein markers used were the same as those used in **Fig. 1**. The *large* and *small arrow* heads indicate the positions of Rabphilin-3A of rat and bovine, respectively. The results shown are representative of three independent experiments.



1 2 3 4 5 6 7

Fig. 10. Northern blot analysis of the Rabphilin-3A mRNA of bovine brain and various rat tissues. Total RNAs (20 µg each) were electrophoresed on a 1% agarose-3% formaldehyde gel, transferred to a nitrocellulose sheet, and hybridized with the radiolabeled Rabphilin-3A cDNA. ***Lanes***; ***1***, bovine brain; ***2***, rat brain; ***3***, rat pancreas; ***4***, rat liver; ***5***, rat spleen; ***6***, rat kidney; ***7***, rat lung. rRNAs (18S and 28S) were used as internal size markers. The results shown are representative of three independent experiments.

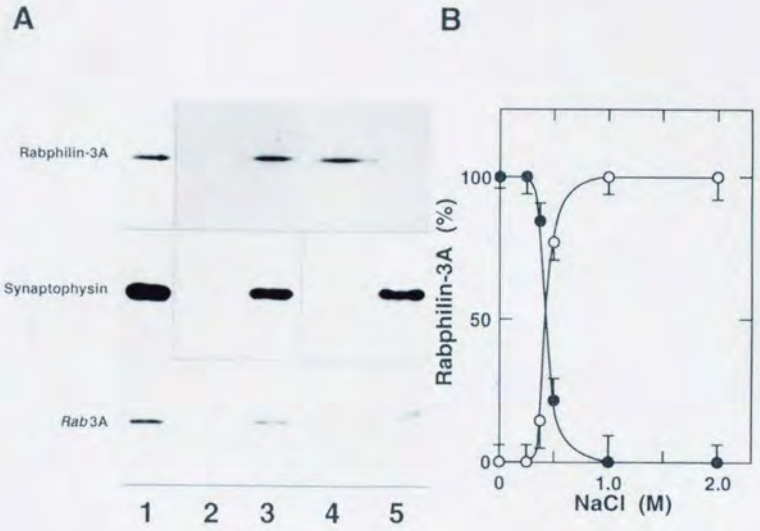


Fig. 11. Removal of Rabphilin-3A from synaptic vesicles by NaCl. (**A**) Western blot analysis of synaptic vesicles treated with or without NaCl. In one set of experiments (***lane* 1**), the vesicles (10 μg of protein for Rabphilin-3A, 1 μg of protein for synaptophysin, or 2 μg of protein for *Rab*3A) were subjected to SDS-PAGE, followed by Western blotting with the corresponding antibody. In another set of experiments (***lanes* 2-5**), the vesicles (40 μg of protein) were treated with or without 1 M NaCl and centrifuged. Twenty-μl aliquots for Rabphilin-3A, 2-μl aliquots for synaptophysin, or 4-μl aliquots for *Rab*3A of the soluble and vesicle fractions were subjected to SDS-PAGE, followed by Western blotting with the corresponding antibody. Since the amounts of the sample used for each experiment were different, SDS-PAGE and Western blotting for each experiment were separately performed, and the protein bands corresponding to the respective proteins are cut out and aligned. ***Lane* 1**, the control vesicles before the treatment; ***lanes* 2** and **3**, the vesicles treated without NaCl; ***lanes* 4** and **5**, the vesicles treated with NaCl. ***Lanes* 2** and **4**, the soluble fraction; ***lanes* 3** and **5**, the vesicle fraction. The results shown are representative of three independent experiments. (**B**) Dose-dependent effect of NaCl. Synaptic vesicles (40 μg of protein) were treated with the indicated concentrations of NaCl and centrifuged. Twenty-μl aliquots of the soluble and vesicle fractions were subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A antibody. The amount of Rabphilin-3A of the vesicle fraction was expressed as per cent of that of the vesicle fraction in the absence of NaCl and the values were expressed as means ± standard errors of three independent experiments. (○), the soluble fraction; (●), the vesicle fraction.

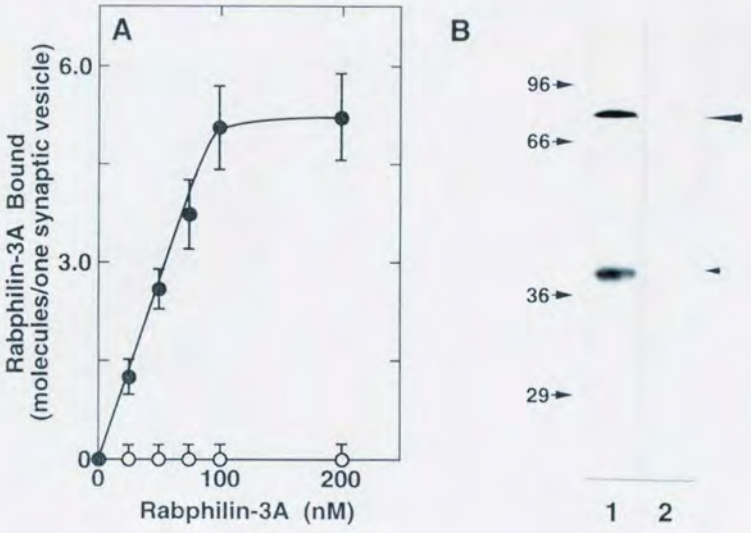


Fig. 12. Dose-dependent and saturable binding of Rabphilin-3A to synaptic vesicles in a manner sensitive to tryptic digestion of the vesicles. (**A**) Dose-dependent and saturable binding of Rabphilin-3A to synaptic vesicles treated with or without tryptic digestion. In one set of experiments, the vesicles deprived of endogenous Rabphilin-3A were used. In another set of experiments, the vesicles deprived of endogenous Rabphilin-3A were digested with trypsin and was used after the digestion was stopped by trypsin inhibitor and APMSF. The indicated amounts of Rabphilin-3A were incubated with the vesicles treated with or without tryptic digestion and centrifuged. A sixty-µl aliquot of each vesicle fraction was subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A antibody. Rabphilin-3A more than 200 nM aggregated and sedimented to the tube bottom even in the absence of the vesicle. This amount was subtracted from the amount found at the tube bottom of the tube containing the vesicles. (●), the intact vesicles; (○), the digested vesicles. The values were expressed as means ± standard errors of three independent experiments. (**B**) Western blot analysis of the binding of Rabphilin-3A to synaptic vesicles treated with or without tryptic digestion. Rabphilin-3A (8 pmol) was incubated with the vesicles treated with or without tryptic digestion and centrifuged. A sixty-µl aliquot of each vesicle fraction was subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A antibody and anti-synaptophysin. ***Lane 1***, the intact vesicles; ***lane 2***, the digested vesicles. The small and large arrowheads show the positions of synaptophysin and Rabphilin-3A, respectively. The protein markers used were the same as those used in **Fig. 1**. The results shown are representative of three independent experiments.

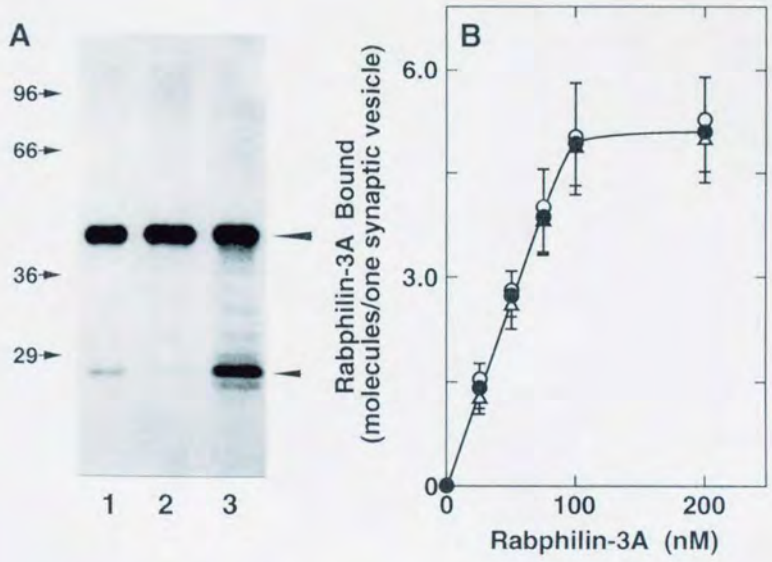


Fig. 13. Effect of *Rab*3A on the binding of Rabphilin-3A to synaptic vesicles. (**A**) Western blot analysis of the vesicles deprived of endogenous *Rab*3A or supplemented with exogenous *Rab*3A. The vesicles deprived of *Rab*3A by the action of *Rab* GDI and the vesicles supplemented with exogenous *Rab*3A were prepared. Each vesicles (2 μg of protein) were subjected to SDS-PAGE, followed by Western blotting with the anti-*Rab*3A and anti-synaptophysin antibody. ***Lane 1***, the intact vesicles; ***lane 2***, the vesicles deprived of *Rab*3A; ***lane 3***, the vesicles supplemented with exogenous *Rab*3A. The protein markers used were the same as those used in **Fig. 1**. The small and large arrowheads show the positions of *Rab*3A and synaptophysin, respectively. The results shown are representative of three independent experiments. (**B**) Binding of Rabphilin-3A to various vesicle samples. Various vesicle samples prepared in **A** were incubated with the indicated amounts of Rabphilin-3A and centrifuged. A sixty-μl aliquot of each vesicle fraction was subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A antibody. (●), the intact vesicles; (○), the vesicles deprived of *Rab*3A; (△), the vesicles supplemented with exogenous *Rab*3A. The values were expressed as means ± standard errors of three independent experiments.

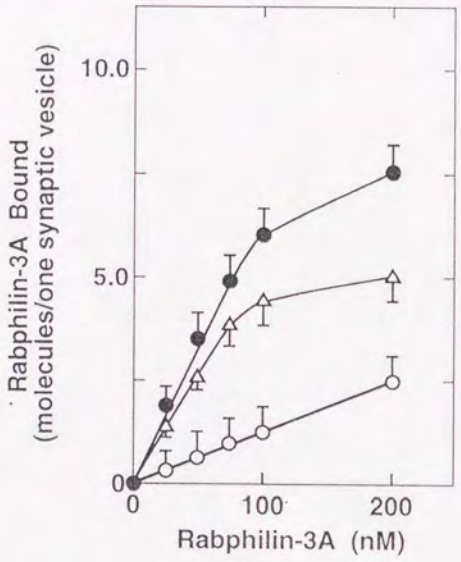
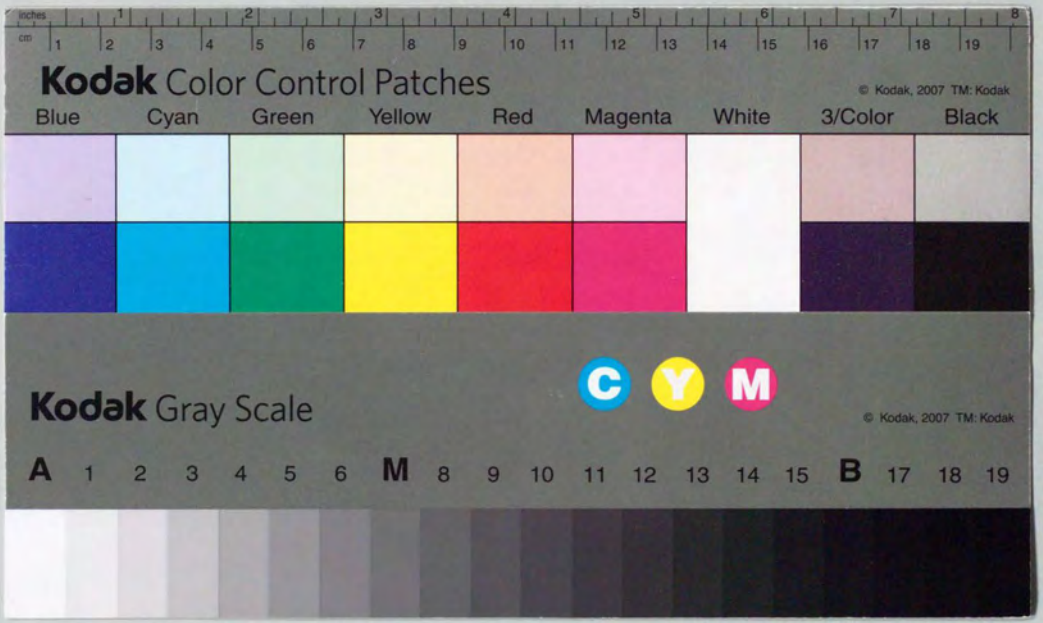


Fig. 14. Effect of Ca^{2+} on the binding of Rabphilin-3A to Synaptic Vesicles. Effect of Ca^{2+} on the binding of Rabphilin-3A to synaptic vesicles treated with or without tryptic digestion in the presence of Ca^{2+}. The vesicles treated with or without tryptic digestion were prepared as described in Fig. 12, respectively. The indicated amounts of Rabphilin-3A were incubated with the vesicles treated with or without tryptic digestion at 1 x 10^{-4} M Ca^{2+} and centrifuged. A sixty-µl aliquot of each vesicle fraction was subjected to SDS-PAGE, followed by Western blotting with the anti-Rabphilin-3A antibody. (●), the intact vesicles; (○), the digested vesicles. (△), the trypsin-sensitive binding of Rabphilin-3A to the vesicles at 1 x 10^{-4} M Ca^{2+}. The values were expressed as means ± standard errors of three independent experiments.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