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Binding of Rab3A to Synaptic Vesicles*

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Prenylated Rab GTPases cycle between membrane-bound and soluble forms. Membrane-bound GDP-Rabs interact with GDP dissociation inhibitor (GDI), resulting in the dissociation of a Rab·GDI complex, which in turn serves as a precursor for the membrane re-association of Rabs. We have now characterized the binding of Rab3A to synaptic vesicles *in vitro* using either purified complexes or rat brain cytosol as source for GDI·Rab3A. Binding of Rab3A results in the immediate release of GDI from the membrane. Furthermore, binding does not require the presence of additional guanine nucleotides (GDP or GTP) or of cytosolic factors. Although nucleotide exchange follows binding, binding is initially reversible, suggesting that binding of GDP-Rab3A and nucleotide exchange are separate and independent events. Comparison with the binding of Rab1B revealed that both Rab proteins bind preferentially to their respective resident membranes although some promiscuity was observable. Binding is saturable and involves a protease-sensitive binding site that is tightly associated with the vesicle membrane.

Rab proteins represent a Ras-related family of small GTPases that are essential for intracellular membrane traffic. Individual Rab proteins are specifically associated with membraneous compartments and contribute to the selectivity of vesicle targeting (for review see Refs. 1–4). Recent evidence from both yeast and mammalian cells suggests that Rab proteins are required for the initial contact formation of vesicles destined to fuse (5, 6), probably via GTP-dependent interaction with other proteins (7). Although Rab proteins appear not to be part of the fusion reaction itself, they may be involved in the control of soluble NSF attachment protein receptor proteins, which mediate fusion. (8, 9).

Rab proteins cycle between the GTP- and GDP-bound forms. Sets of specific proteins interact with GTP- and GDP-Rab proteins, either shepherding them to the next step of the cycle or executing a particular task in membrane traffic. The GTP forms of Rabs are considered to represent the active conformations. GTP-Rab proteins bind to effector proteins. A number of such effector proteins has been identified that structurally belong to diverse protein families, and individual Rab proteins may interact with multiple effectors (for review see Refs. 1 and 10). In addition, proteins are known that activate the GTPase of specific Rabs (GTPase-activating proteins), that inhibit the dissociation of GDP (GDP dissociation inhibitor, GDI),[1] and that mediate nucleotide exchange (guanine nucleotide exchange factors).

Most Rab proteins contain two hydrophobic geranylgeranyl moieties at their C terminus that are responsible for membrane anchoring. Despite these highly hydrophobic side chains, Rab proteins undergo reversible membrane dissociation-association cycles during membrane traffic that are correlated to their GTP/GDP cycles. After GTP hydrolysis, membrane-bound GDP-Rabs are recognized by GDP dissociation inhibitor (Rab·GDI) (11). Rab·GDI forms a complex with GDP-Rabs that shields their geranylgeranyl moieties and leads to their dissociation from the membrane. GDI·Rab complexes serve then as the precursor form, which mediates the re-binding of Rab proteins to their appropriate membrane (1, 2, 4). In addition to Rab·GDI, calmodulin has recently been suggested to remove Rab3A from membranes in a calcium-dependent manner, but the significance of this finding remains to be established (12).

In contrast to proteins mediating other parts of the Rab cycle, the protein factors associated with the GDI-mediated binding of Rab proteins to membranes are not well understood. Since the various GDI isoforms appear to interact indiscriminately with all Rab proteins, membrane binding must be the discerning step that is responsible for the specificity of membrane binding. Consequently, the binding mechanism must be able to recognize the specific type of Rab protein. In yeast, a novel Golgi-associated membrane protein, termed Yip1p, has recently been identified that appears to mediate the binding of the Rab protein Ypt31p (13). Less is known about the binding sites of mammalian Rabs. Membrane specificity has been demonstrated for *in vitro* binding of several Rab proteins including Rab4 and Rab5 (14, 15), which are localized to early endosomes, and Rab 7 and Rab9 (16, 17), which are localized to late endosomes. These studies revealed that binding of Rab proteins involves three distinct steps. First, the GDI·Rab complex binds to the membrane. Second, GDI dissociates after a delay of several minutes. Recently, evidence for a protein factor has been obtained that may be responsible for GDI-dissociation and that appears to be specific for endosomal GDI·Rab complexes (18). Third, GTP is exchanged for GDP with the aid of guanine nucleotide exchange factor proteins.

The present study was undertaken in order to investigate the membrane-association of Rab3A. Rab3A is the predominant Rab protein in the brain, which is exclusively localized to the membrane of synaptic vesicles (19, 20) and which functions in the regulation of neurotransmitter release (21, 22). Two addi-

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[1] The abbreviations used are: GDI, GDP dissociation inhibitor; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; CPG, controlled pore glass bead; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

tional isoforms of Rab3A, Rab3B and Rab3C, respectively, are also localized to synaptic vesicles (23, 24), whereas a fourth isoform, Rab3D, is expressed in non-neuronal tissues (Ref. 25; for review, see Refs. 10 and 26–28).

We have previously shown that the cycle of Rab3A is closely linked to the recycling of synaptic vesicles. Before exocytosis, vesicle-bound Rab3A is in the GTP form (29). Stimulation of neurotransmitter release leads to GTP hydrolysis (29) and causes a dissociation of Rab3A from the vesicle membrane that is reversed during recovery from the stimulus (30). Here, we show that the re-binding of Rab3A to synaptic vesicles can be reconstituted *in vitro*. Binding involves the presence of a specific protein receptor that discriminates between Rab3A and Rab1B, which was used for control. Furthermore, we found that both Rab3A binding and dissociation of GDI occur independently of guanine nucleotide exchange and that newly bound Rab3A can be re-extracted with GDI as long as no nucleotide exchange occurs.

EXPERIMENTAL PROCEDURES

Antibodies—GDI purified from bovine brain was used as antigen for generating monoclonal antibodies in mice using standard procedures (31, 32). A rabbit serum specific for Rab1B was raised using purified Rab1B (see below) as antigen, employing standard procedures of the Yale Animal Care Facility. The serum reacted with only a single band in immunoblots of rat brain cytosol and did not cross-react with either Rab3A or Rab5A (data not shown). The following monoclonal antibodies were described previously and are now commercially available (Synaptic Systems): Rab3A (Cl 42.2; Ref. 33), Rab5 (Cl 621.1; Ref. 34), synaptophysin (C 7.2; Ref. 32), synaptotagmin (luminal domain, Cl 604.4), and synaptobrevin (VAMP) 2 (Cl 69.1; Ref. 35). Rabbit sera against GDI and sec 61 were generously provided by Dr. T. C. Südhof (University of Texas, Southwestern Medical Center, Dallas, TX) and Dr. T. Rapoport (Harvard University, Boston, MA), respectively. The hybridoma line producing antibodies for the myc epitope was obtained from American Type Culture Collection. Rabbit anti-mouse Fc-specific antibodies were obtained from Jackson ImmunoResearch. A monoclonal antibody specific for $G\alpha O_2$ was raised against recombinant protein and will be described elsewhere.[2]

Generation of Rab·GDI Complexes—GDI was purified from bovine brain using ammonium sulfate precipitation and chromatography on DEAE-Sephacel and Mono-Q, respectively. All steps were carried out as described in Ref. 11, except that GDI was eluted from the Mono-Q column by prolonged wash instead of a salt gradient.

Rab3A and Rab1B were expressed using the baculovirus system, largely following the procedure described by others for Rab1 and Rab5 (36, 37). cDNAs for Rab3A and Rab1B were provided by Dr. P. De Camilli (Yale University School of Medicine, New Haven, CT), and for Rab5A by Dr. T. C. Südhof. Full-length cDNAs encoding Rab3A, Rab5A, and Rab1B were amplified by polymerase chain reaction from the PET11d-Rab3A, PGEX-2T-Rab5A, and PALTER-Ex1-Rab1B constructs, respectively, using appropriate 5′- and 3′-oligonucleotide primers according to standard procedures. Unless indicated otherwise, the 5′ primers were designed to add a myc epitope upstream of the respective 5′ ends. The products were cloned into the *Bam*HI site of the baculovirus transfer vector pBlueBac His2 A, which contains an N-terminal His_6 tag. Constructs containing a single insert in the appropriate orientation were selected by restriction analysis and confirmed by DNA sequencing. Construction and purification of recombinant viruses was performed using the MaxBac baculovirus system (Invitrogen) according the manufacturer's instructions. Sf9 cells were grown at 27 °C to a density of $\sim 2 \times 10^6$ cells/ml in spinner flasks and infected with recombinant viruses at a multiplicity of infection (virions/cell) of 5, and incubation was continued for 72 h. The cells were harvested, washed with phosphate-buffered saline, and resuspended in lysis buffer (50 mM HEPES-KOH, pH 7.2, 1 mM $MgCl_2$). Usually, the cells were frozen in liquid N_2 and stored at −80 °C at this stage.

To purify prenylated Rab proteins from Sf9 cells, a crude membrane fraction was prepared. The cell suspension was supplemented with 0.3 M NaCl, 1 mM PMSF, 0.5 μg/ml leupeptin, and 1 μM pepstatin, sonicated on ice four times each for 30 s with 30-s intervals at 50% intensity, and centrifuged for 5 min at 900 × *g* to remove cell debris and nuclei. Membranes were then isolated from the supernatant by centrifugation at 100,000 × *g* for 1 h and washed once in the same buffer and once in extraction buffer (20 mM Tris, pH 7.8, 150 mM NaCl) supplemented with protease inhibitors as above. The pellet was then extracted in extraction buffer containing 1% (v/v) CHAPS and centrifuged at 100,000 × *g* for 30 min to remove non-soluble components.

The following steps involve formation of proteoliposomes, collection of these liposomes by centrifugation, and subsequent re-extraction of these liposomes by detergent. This procedure was shown previously to greatly enrich membrane proteins with almost complete recovery, while efficiently removing proteins without a membrane anchor such as non-prenylated Rab proteins (38). To the resulting supernatant, 100 mg/ml Biobeads SM2 were added in order to lower the detergent concentration. Following incubation on ice for a few minutes, the turbid extract was removed from the beads and applied to 10 volumes of a Sephadex G50 (fine) column for complete detergent removal. The turbid fractions containing proteoliposomes were collected and centrifuged at 100,000 × *g* for 30 min. The pellet was re-extracted in lysis buffer containing 1% CHAPS. The extract was then used for purification on Ni^{2+}-nitrilotriacetic acid-agarose according to standard procedures. The final products contained no significant contaminations (as judged by SDS-PAGE); they were stored at −30 °C in 50% glycerol until further use.

For the formation of Rab·GDI complexes, equimolar amounts of Rab protein and GDI (2 μM each) were mixed in the presence of 100 μM GDP and 8 mM $MgCl_2$, followed by extensive dialysis against a buffer containing 64 mM HEPES-NaOH, pH 8.0, 100 mM NaCl, 8 mM $MgCl_2$, 2 mM EDTA, 0.2 mM dithiothreitol, 0.01 mM GDP, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μM pepstatin. The mixture was clarified by ultracentrifugation in a TLA 100.2 rotor at 95,000 rpm for 10 min at 4 °C. The supernatant was collected as Rab·GDI complex. The complexes were kept on ice and normally used within 72 h.

Binding of Rab Proteins to Membranes—As acceptor membranes in standard binding assays, a fraction enriched in synaptic vesicles (LP2 fraction) was prepared by first isolating synaptosomes from rat brain, followed by hypotonic shock and differential centrifugation (39). When indicated, synaptic vesicles were further purified from LP2 using, consecutively, sucrose density gradient centrifugation and chromatography on controlled pore glass beads (CPG) (39, 40). The eluate from the controlled pore glass bead column was divided in three fractions, one enriched in large membranes (CPG I), one intermediate fraction (CPG II), and one highly enriched in synaptic vesicles (CPG III; see Ref. 41 for further details). Membranes enriched in endoplasmic reticulum (ER) were obtained by consecutive differential and density gradient centrifugation using Percoll and Nycodenz gradients (42). LP2 from Rab3A-deficient mice was prepared as above. The mice (21) were kindly provided by Dr. T. C. Südhof.

Binding or Rab proteins to membranes was measured as follows unless indicated otherwise. The reaction mixture contained 10 μg of membrane protein, 100 ng (4.5 nM) of Rab·GDI complex, binding assay buffer (25 mM HEPES-KOH, pH 7.2, 115 mM KCl, 1.5 mM $Mg(OAc)_2$, 0.2 mM dithiothreitol, 100 mM $(NH_4)_2SO_4$, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μM pepstatin), 100 μM guanine nucleotide as indicated, and an ATP regeneration system (1.4 mM $MgCl_2$, 0.4 mM ATP, 6 mM phosphocreatine, 8 units of creatine phosphokinase), which, however, was omitted in later experiments. The final assay volume was 250 μl. After incubation for 30 min at 37 °C the reaction was stopped by addition of 500 μl of ice-cold assay buffer. Membranes were sedimented by centrifugation in a TLA 100.2 rotor at 95,000 rpm for 10 min at 4 °C. The pellets were washed once with 500 μl of assay buffer and then analyzed by SDS-PAGE, followed by immunoblotting. In all experiments, the supernatants were also analyzed in order to check whether unbound Rab·GDI complex and/or released GDI were recovered in the supernatants at the end of the binding reaction. This was the case in all experiments described here. Under standard conditions, about 40–50% of Rab3A remained in the supernatant with an overall recovery (supernatant + pellet) of about 80%. Furthermore, parallel incubations were carried out for each data point in which the membranes were omitted, in order to rule out that Rab recovery in the pellet fraction is due to aggregation. Each binding experiment shown here was repeated independently at least three times, yielding very similar results.

Guanine Nucleotide Exchange—Guanine nucleotide exchange was monitored by the binding of radiolabeled GTPγS. In order to reduce background binding of GTPγS to endogenous Rab proteins, the membranes (200 μg of protein) were pre-incubated with 1 μM GDI in the presence of 100 μM GDP using conditions identical to those in Rab binding experiments. Incubations for nucleotide exchange were carried out exactly as for Rab binding, except that 5 nM [^{35}S]GTPγS (about 0.7

[2] J. H. Chou and R. Jahn, manuscript in preparation.

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μCi/reaction) were present. The reactions were stopped by the addition of 900 μl of ice-cold assay buffer. Protein-bound radioactivity was recovered by filtration through a Millipore HA type filter (0.45-μm pore size) and quantitated by liquid scintillation counting.

Other Methods—SDS-PAGE was carried out according to Laemmli (43). Immunoblotting on nitrocellulose membranes was performed according to Ref. 44 using alkaline phosphatase enzymatic reaction (Roche Molecular Biochemicals), ^{125}I Protein A radiography (Amersham Pharmacia Biotech), or enhanced chemiluminescence (Amersham Pharmacia Biotech) detection. Protein concentrations were determined according to Bradford (45) or with the BCA method (Pierce).

Protease treatment of membranes was carried out at a concentration of 8 mg/ml membrane protein and 1 mg/ml trypsin (Roche Molecular Biochemicals), bromelain (Sigma), and elastase (Roche Molecular Biochemicals), respectively, for 1 h at 37 °C. The incubations were stopped by 10-fold dilution with ice-cold incubation buffer (320 mM sucrose, 10 mM HEPES, pH 7.4) and the addition of 2 mg/ml appropriate protease inhibitors (trypsin inhibitor (Roche Molecular Biochemicals), bromelain inhibitor (Sigma), and elastatinal (Sigma)), respectively. Protease-treated membranes were then isolated by ultracentrifugation (100,000 × *g*, 15 min, 4 °C) and tested for Rab binding activity using standard assay conditions.

RESULTS

Generation and Characterization of GDI·Rab Complexes—GDI was purified from bovine brain (11). For the characterization of GDI and Rab·GDI complexes, we generated a panel of mouse monoclonal antibodies specific for GDI. Clone Cl 81.2 showed the strongest reaction and was therefore used in all subsequent experiments. When rat brain cytosol was analyzed by SDS-PAGE and immunoblotting, a single band of an approximate M_r = 55,000 was detected by this antibody which comigrated with purified GDI (Fig. 1*A*). We then examined whether the antibody was able to immunoprecipitate Rab·GDI complexes from cytosol. As shown in Fig. 1*B*, both GDI and Rab3A were detectable in the immunoprecipitate. In contrast, the α-subunit of the trimeric GTPase GO_2 that is abundantly expressed on synaptic vesicles (46) did not co-precipitate, showing the specificity of the immunoprecipitation procedure.

Posttranslationally modified Rab3A and Rab1B were produced in Sf9 cells using the baculovirus expression system. All proteins contained an N-terminally attached His_6 tag for affinity purification. Furthermore, a myc epitope was added downstream of the His_6 tag in order to differentiate the recombinant Rab proteins from their endogenous counterparts. For Rab3A, a version lacking the myc epitope was also prepared. When the proteins were extracted and purified according to Ref. 36, the resulting protein fractions were of low yield and purity, particularly for Rab3A and myc-Rab3A. Therefore, an additional purification step was included. It involves the reconstitution of all membrane proteins into proteoliposomes, separation of these proteoliposomes from non-incorporated proteins, and resolubilization of the liposomes by detergent prior to affinity chromatography. Yield and purity were significantly improved (see "Experimental Procedures" for details). Rab3A purified by this procedure bound stoichiometric amounts of GTP (data not shown), demonstrating that its nucleotide binding pocket is correctly folded.

Rab·GDI complexes were formed by dialysis (see "Experimental Procedures"). To examine whether GDI and Rab proteins were indeed bound to each other after dialysis, GDI was immunoprecipitated with monoclonal antibody Cl 81.2 and then probed for the presence of the respective Rab protein. As shown in Fig. 1*C*, virtually all of Rab3A coprecipitated with GDI, indicating that no free Rab3A was present in the complex fraction. Similar results were obtained for the other Rab·GDI complexes.

Basic Parameters of Rab3A Binding to Synaptic Vesicles—In the first series of experiments, an assay for binding of Rab3A to synaptic vesicles was established. A membrane fraction enriched about 5–8-fold in synaptic vesicles (LP2, see Refs. 32 and 39) was prepared from rat brain and used as acceptor in all experiments unless indicated otherwise. After incubation of the membranes with Rab3A·GDI complexes, bound Rab3A was separated from unbound Rab3A by ultracentrifugation and binding was measured by immunoblotting. LP2 contains endogenously bound Rab3A that needed to be differentiated from the exogenously added variant. Therefore, the myc-tagged form of Rab3A was used in most experiments, which is well separated from endogenous Rab3A on SDS-polyacrylamide gels. Unless indicated otherwise, both endogenous and exogenous Rab3A were detected with a Rab3A-specific monoclonal antibody to allow for direct comparison. In order to control for membrane recovery, synaptobrevin, an integral membrane protein of synaptic vesicles, was monitored in parallel.

First, we defined the time dependence of the binding reaction. The myc-Rab3A·GDI complex was incubated with synaptic vesicles under standard conditions (see "Experimental Pro-

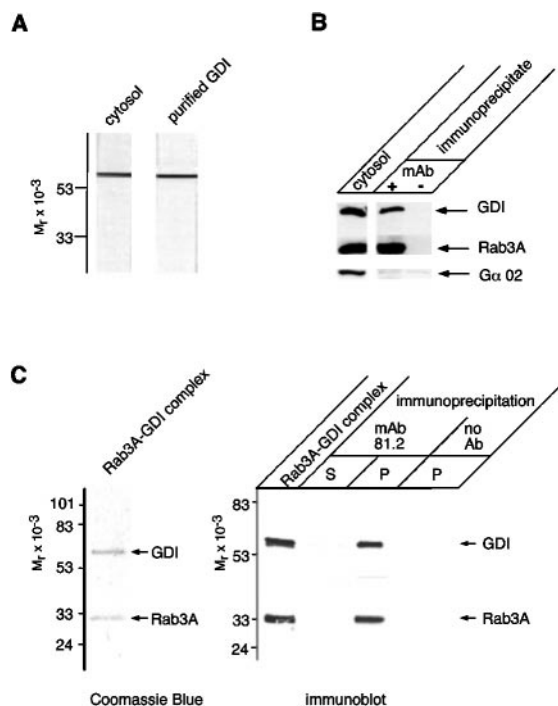

FIG. 1. **Monoclonal antibody Cl 81.2 specifically reacts with GDI and immunoprecipitates Rab·GDI complexes.** *A*, immunoblot of rat brain cytosol (approximately 6 μg) and purified GDI using monoclonal antibody Cl 81.2, detected with the alkaline phosphatase method. *B*, coprecipitation of GDI and Rab3A from rat brain cytosol. For precipitation, 20 μl of ascites were mixed with 200 μg of cytosol protein in 320 mM sucrose, 100 mM NaCl, and 10 mM HEPES-KOH, pH 7.4, at a final volume of 200 μl, followed by incubation at 4 °C for 1 h. Hundred μl of protein G-Sepharose slurry was added, and the incubation was extended for 1 h. The beads were collected by centrifugation and washed three times. Three percent of the precipitate was analyzed by SDS-PAGE/immunoblotting using the enhanced luminescence method. For detection, a polyclonal antiserum for GDI and monoclonal antibody Cl 42.2 for Rab3A were used. As control for nonspecific binding, the blot was probed with a monoclonal antibody specific for the α-subunit of the trimeric GTPase GO2, no cosedimentation was observed. *C*, coprecipitation of GDI and Rab3A using purified Rab3A·GDI complexes in order to determine the efficiency of complex formation. 1.5 μg of Rab3A·GDI complex in phosphate-buffered saline (Coomassie Blue staining of the electrophoretically separated complex is shown in the *left panel*) was incubated with 20 μl of Cl 81.2 ascites in a final volume of 200 μl and precipitated as above. After sedimentation of the Sepharose beads, both the bead pellet and the first supernatant (containing unbound material) were analyzed for GDI and Rab3A by SDS-PAGE and immunoblotting using the alkaline phosphatase method for detection. Virtually all of Rab3A coprecipitated with GDI, indicating that the purified complex contains no unbound Rab3A.

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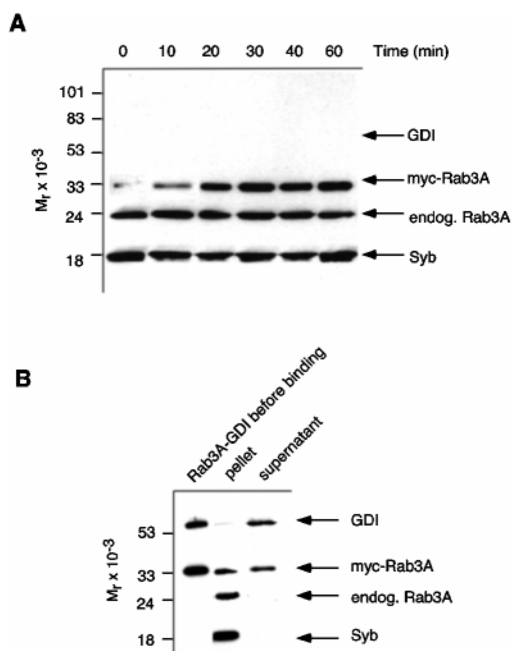

FIG. 2. **Binding of myc-Rab3A to synaptic vesicles under standard assay conditions.** *A*, time dependence of Rab3A binding to synaptic vesicles. The myc-Rab3A·GDI complex was incubated with synaptic vesicles (LP2) for different time periods using standard assay conditions. The figure shows an immunoblot analysis of the bound (pellet) fraction. Both myc-Rab3A and endogenous Rab3A were detected with the Rab3A-specific monoclonal antibody Cl 42.2. In this and all following experiments, the immunoblots were developed with the enhanced chemiluminescence method unless indicated otherwise. Binding of myc-Rab3A increased from 0 to 30 min but did not increase further upon extension of the incubation time. The amount of endogenous Rab3A present in the vesicle fraction did not change during the course of the incubation. *B*, recovery of GDI and Rab3A in the pellet (bound) and supernatant (unbound) fractions. For comparison, all fractions are normalized to the same relative volume. Note that both synaptobrevin (a membrane marker) and endogenous Rab3A are recovered in the pellet, whereas myc-Rab3A, derived from exogenously added myc-Rab3A·GDI complex, was distributed between bound (approximately 35% of starting material) and unbound (approximately 40% of the starting material). See "Experimental Procedures" for details.

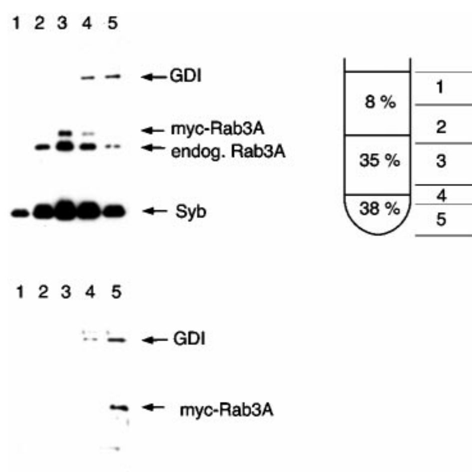

FIG. 3. **Bound myc-Rab3A comigrates with vesicle membranes on a sucrose density flotation gradient.** The binding reaction mixture was brought to 38% sucrose in a final volume of 0.74 ml at the end of a standard assay incubation and overlaid with 0.75 ml each of 35% and 8% sucrose, respectively. After centrifugation at 50,000 rpm for 2.5 h in a Beckman SW 41 rotor, fractions were collected as indicated and analyzed by immunoblotting. *Top*, standard assay; *bottom*, myc-Rab3·GDI complex only.

cedures") for different time periods. Rab3 binding increased from 0 to 30 min but did not increase further during prolonged incubation (Fig. 2*A*). The amount of endogenous Rab3A on synaptic vesicles did not change during the incubation. In addition, no accumulation of GDI on the membrane was observed at any time. Binding was dependent on the amount of vesicles in an approximately linear manner between 5 and 50 μg of vesicle protein (data not shown), and 10 μg were used in all subsequent experiments. Under standard conditions (10 μg of vesicle protein, 30 min of incubation), about 35% of the Rab3A added to the assay (as Rab·GDI complex) was recovered in the membrane pellet, with approximately 40% remaining unbound (Fig. 2*B*). The loss of about 20–25% is probably due to adsorption.

Next, we investigated whether the Rab3 pool found in the membrane pellet after the binding reaction is indeed attached to vesicles instead of representing aggregated proteins cosedimenting with the membranes. For this purpose, we used a flotation gradient in order to separate the membranes from unbound protein. The binding assay mix was adjusted to 38% sucrose at the end of the reaction and overlaid with layers consisting of 35% and 8% sucrose, respectively. After ultracentrifugation, the distribution of vesicles, GDI, and Rab3A on the gradient was determined. As shown in Fig. 3, both GDI and myc-Rab3A remained in the dense fraction when membranes were omitted. In the presence of vesicles, however, the majority of myc-Rab3A was found in fractions of lower density together with the vesicles, as seen by the parallel distribution of synaptobrevin. GDI largely remained in the bottom of the gradient, confirming that GDI does not bind to the vesicles.

Nucleotide Dependence of Rab3A Binding—Previous work on the binding of Rab4, Rab5, and Rab9 to membranes demonstrated that membrane binding is closely linked to nucleotide exchange. We therefore tested whether binding of myc-Rab3A to synaptic vesicles is influenced by the presence of GDP or the non-hydrolyzable GTP analog GTPγS. As shown in Fig. 4*A*, no change in the amount of bound myc-Rab3A was observed when either of these nucleotides was present. We then investigated whether nucleotide exchange does occur during the binding reaction. This was indeed the case (Fig. 4*B*), but exchange appeared to be slower than binding under our assay conditions with no saturation reached after 60 min of incubation. Note that, in this experiment, endogenous GDP-Rab proteins were removed from the membrane by preincubation with free GDI. This step was needed in order to reduce background binding of GTPγS, which is known to be high (19).

These observations suggested that binding and guanine nucleotide exchange are separate and independent events. Apparently, GDI delivers Rab3A to the membrane and dissociates during the binding reaction without the need for nucleotide exchange, although, as shown above, nucleotide exchange does occur if GTP (or GTP analogs) are present. Since free GDI is capable of removing GDP-Rab proteins but not GTP-Rab proteins from the membrane, these findings prompted us to investigate whether binding is reversible as long as no nucleotide exchange occurs.

First, we tested whether addition of free GDI (in addition to Rab·GDI complexes) to the binding reaction would shift the equilibrium toward unbound Rab·GDI complexes. As shown in Fig. 5, binding of myc-Rab3A was reduced in the presence of excess free GDI. This effect was consistently seen in several experiments.

The effects of free GDI are best explained by an equilibrium between binding and dissociation in which GDP-Rab3 delivered by GDI·Rab complexes is subsequently being removed by free GDI. In order to examine this issue further, we performed binding and GDI-mediated dissociation sequentially. We also tested whether nucleotide exchange after binding of GDP-

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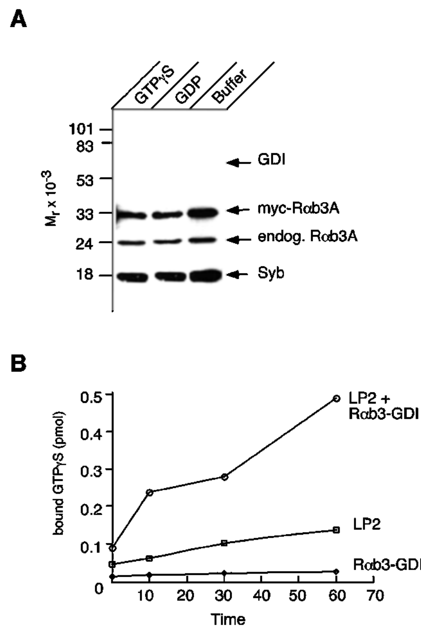

FIG. 4. **Binding of Rab3A is accompanied by, but does not depend on, guanine nucleotide exchange.** *A*, Rab3A binding is independent of the presence of guanine nucleotides. The myc-Rab3A·GDI complex was incubated with synaptic vesicle membranes without nucleotides (*Buffer*) or in the presence of 500 μM GTPγS or 500 μM GDP using standard assay conditions. *B*, Rab3A binding is accompanied by nucleotide exchange. The synaptic vesicle fraction used in the assay was pre-treated with GDI in order to reduce background binding (see text). Binding was performed in the presence of [^{35}S]GTPγS. Bound radioactivity was quantitated using a filtration assay (see "Experimental Procedures" for details).

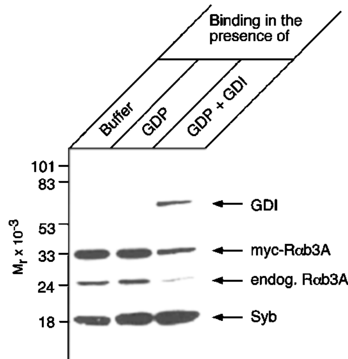

FIG. 5. **Addition of free GDI reduces binding of Rab3A.** Binding of myc-Rab3A·GDI to synaptic vesicles was performed under standard assay conditions in the presence of 1 μM GDI, leading to a reduction of both bound exogenous and bound endogenous Rab3A.

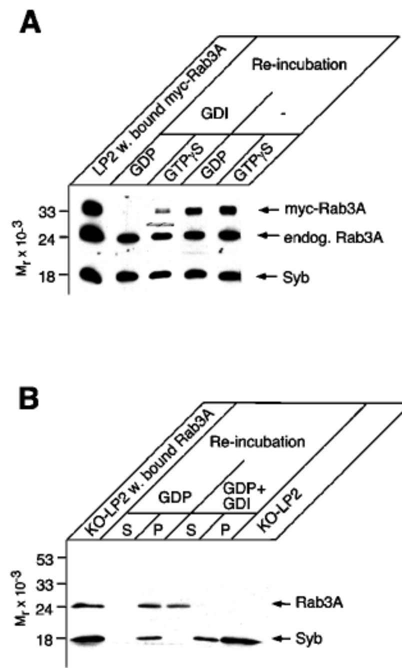

FIG. 6. **Newly bound Rab3A is dissociated by GDI in the presence of GDP but becomes refractory after nucleotide exchange.** *A*, myc-Rab3A·GDI complex was first bound to synaptic vesicles (LP2) using standard assay conditions. The membranes were then collected by centrifugation, resuspended and re-incubated in the presence or absence of 1 μM GDI and either 0.1 mM GTPγS or 0.1 mM GDP using standard assay conditions. Note that GTPγS partially protects Rab3A from re-dissociation by GDI. *B*, binding of Rab3A to synaptic vesicles is reversed by GDI when rat brain cytosol is used as a source for Rab3A·GDI complex. One hundred μg of rat brain cytosol was first incubated with 10 μg of a synaptic vesicle fraction derived from Rab3A-deficient mice (21) (KO-LP2, analyzed in the *right lane* to show that endogenous Rab3A is absent) under standard conditions. The membranes were then isolated and re-incubated as in *A*, except that dissociation was only analyzed in the presence of GDP using rat brain cytosol as source for Rab3A·GDI complex. Immunoblot analysis was performed on half of the recovered membranes.

Rab3A would protect bound Rab3A from subsequent dissociation by GDI. In this experiment, binding was first carried out in the presence of GDP under standard conditions and the membranes were separated from unbound material by centrifugation. In a second step, these membranes were resuspended and re-incubated with GDI in the presence of either GDP or GTPγS.

The result of this experiment is shown in Fig. 6*A*. When vesicles containing bound myc-Rab3A were re-incubated with GDI in the presence of GDP, myc-Rab3A was completely removed from the membrane. Interestingly, the same result was obtained when the first binding reaction was carried out in the presence of GTP. When GTPγS was present instead of GDP, part of the newly bound myc-Rab3A remained on the membrane. Control incubations carried out in parallel showed that this effect is due to GDI and not to the nucleotides alone, as no removal was observed in the absence of GDI. For further confirmation, part of the experiment was repeated using rat brain cytosol containing endogenous Rab3A·GDI complex instead of purified Rab3A·GDI complex as Rab donor. To avoid interference by vesicle-bound Rab3A, synaptic vesicles (LP2) were prepared from transgenic mice, which lack a functional Rab3A gene (KO-LP2) (21). The experiment depicted in Fig. 6*B* shows that cytosol can be indeed used as Rab3 donor in the binding experiment and confirms that binding is reversible as long as no nucleotide exchange occurs. Furthermore, it shows that the presence of cytosol (including soluble exchange factors such as MSS4; Ref. 47) does not influence binding and its subsequent reversal by GDI.

Characterization of the Rab3A Binding Site—In the following experiments, we investigated whether exogenous Rab3A only binds to synaptic vesicles and whether binding is dependent on proteins in the target membrane. This issue is of importance as the re-binding reaction is supposed to be the discerning step that is responsible for the highly selective association of individual Rab proteins with their respective organelle.

In the first series of experiments, we compared the binding of Rab3A with that of Rab1B. In contrast to the vesicle-specific Rab3A, Rab1B functions in the transport of vesicles from the ER to the Golgi and, at least under steady-state conditions, is mostly associated with Golgi membranes (for review, see Ref. 4). GDI complexes of either myc-Rab3A or myc-Rab1B were incubated in parallel with either brain-derived synaptic vesicles (LP2) or with an ER fraction prepared from rat liver, *i.e.* a tissue in which Rab3A is not expressed. Surprisingly, myc-Rab3A bound to both ER and LP2 membranes, with only a

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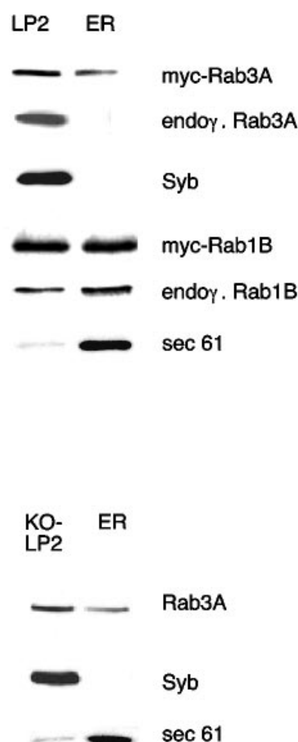

FIG. 7. **Comparison of Rab3A- and Rab1B-binding to membranes enriched either in synaptic vesicles (LP2) or in ER.** *Top*, myc-Rab3A·GDI and myc-Rab1B·GDI complexes were incubated with either LP2 or ER membranes under standard assay conditions. As reference, the distribution of the respective endogenous proteins was analyzed in parallel. *Bottom*, rat brain cytosol was used as a source for Rab3A·GDI complexes. Cytosol was incubated either with LP2 derived from Rab3A-KO mice (devoid of endogenous Rab3A, see Fig. 6) or with ER membranes. All experiments were performed and analyzed using standard conditions.

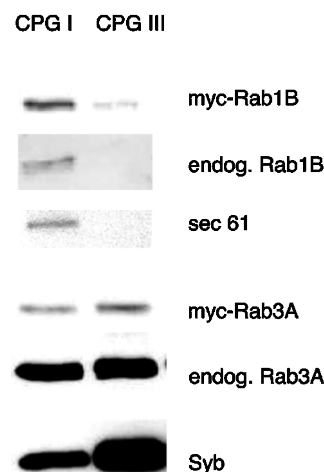

FIG. 8. **Comparison of Rab3A and Rab1B binding with the distribution of endogenous proteins using fractions obtained during purification of synaptic vesicles by CPG chromatography.** CPG I contains mostly large membranes in addition to some synaptic vesicles, whereas CPG III contains highly purified synaptic vesicles with virtually no contamination by larger membranes (see "Results" for details). All experiments were carried out under standard conditions. Note that myc-Rab3A binds preferably to CPG III membranes, in accordance with the distribution of the endogenous proteins. The distributions of both endogenous Rab1B and myc-Rab1B-binding are parallel to each other and are inversely related to that of Rab3B.

slight preference for LP2 (Fig. 7, *upper panel*). Conversely, myc-Rab1B bound equally well to both membranes despite the fact that endogenous Rab1B, like the ER-resident protein sec 61, was enriched in the ER fraction in comparison to LP2. Changing the binding conditions, for instance by substituting GTPγS for GDP or by using Rab3A·GDI complex instead of myc-Rab3A·GDI complex, did not change the result (data not shown). For confirmation, we again used unfractionated rat brain cytosol as source for the Rab3A·GDI complex and compared binding to ER and an LP2 fraction derived from Rab3A-KO mice. Again, there was preferential binding to LP2 but significant amounts of Rab3A became also associated with the liver-derived ER fraction (Fig. 7, *lower panel*). Increasing concentrations of cytosol increased the amount of bound Rab3A but did not change the membrane preference (data not shown).

Although LP2 is enriched in synaptic vesicles, it still contains significant membrane contaminants from other sources including the ER (39). Vice versa, it is possible that the liver-derived ER fraction is contaminated with small trafficking vesicles that may contain a binding site interacting with Rab3A, even though Rab3A is not expressed in liver. For these reasons, we subfractionated LP2 further by continuous sucrose density gradient centrifugation followed by chromatography on CPG. CPG chromatography separates synaptic vesicles from larger membranes, resulting in membrane fractions either highly enriched (CPG III) or relatively de-enriched (CPG I), respectively, in synaptic vesicles (39, 41) eluting from the same column. When these fractions were analyzed for endogenous Rab1B and Rab3A, an inverse distribution was found that corresponded to that of the respective marker proteins synaptobrevin and sec 61 (Fig. 8). Binding of the corresponding myc-Rab3A and myc-Rab1B, respectively, largely paralleled the distribution of the endogenous proteins (Fig. 8). Together, these results support the view that it is the membrane-rebinding reaction which defines the specificity of the subcellular localization of Rab proteins.

In order to confirm the independence of the binding sites for Rab3A and Rab1B, we performed competition experiments. Binding of myc-Rab3A was measured in the presence of increasing concentrations of myc-Rab1B. Binding of myc-Rab3A and myc-Rab1B were assayed using anti-myc tag monoclonal antibodies, allowing for a direct comparison of the protein quantities. As shown in Fig. 9*A*, increasing amounts of myc-Rab1B failed to interfere with the binding of myc-Rab3A, with only a slight reduction in the presence of a 10-fold excess of Rab1B·GDI complex.

We next investigated whether Rab3-binding is saturable, a feature suggested by the result of the competition experiments. When increasing amounts of myc-Rab3A·GDI complex were used in the binding reaction, saturation was observed at a concentration of about 100 nM myc-Rab3A·GDI complex (Fig. 9*B*). The amount of endogenous Rab3A remained unchanged over the entire concentration range, suggesting that saturation is not caused by an increased dissociation rate under these conditions.

Together, these results strongly suggest that Rab3 binding is mediated by a protein receptor present in the vesicle membrane. To address the nature of the receptor on synaptic vesicle membranes, synaptic vesicles were treated with 1 M KCl or 0.1 M Na_2CO_3, pH 11, in order to strip off peripheral membrane proteins. Binding of myc-Rab3A was not influenced by these treatments (data not shown). We therefore used limited proteolysis of synaptic vesicles with three different proteases in order to prove the proteinaceous nature of the receptor. Under our experimental conditions, the cytoplasmic domains of synaptic vesicle proteins were largely proteolyzed, shown here for synaptotagmin (Fig. 10), where only the luminal domain was detectable after digestion. Treatment with all three proteases greatly reduced the binding of Rab3A to vesicle membranes,

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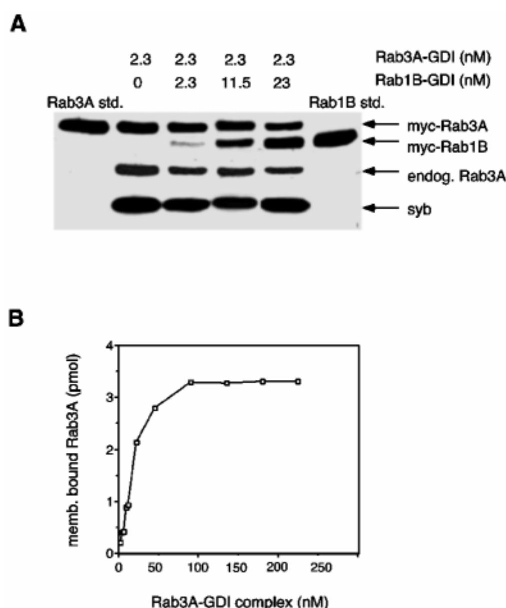

FIG. 9. **Binding of Rab3A is saturable and is not competed for by Rab1B.** *A*, competition of myc-Rab3A binding to LP2 with increasing amounts of Rab1B under standard assay conditions. In the presence of a 10-fold excess of Rab1B·GDI complex, binding of Rab3A was only slightly reduced. *B*, saturation analysis of myc-Rab3A binding using increasing amounts of myc-Rab3A·GDI complex under standard assay conditions. Immunoblots were quantitated by densitometry and calibrated with a standard curve of purified Rab3A separated in parallel.

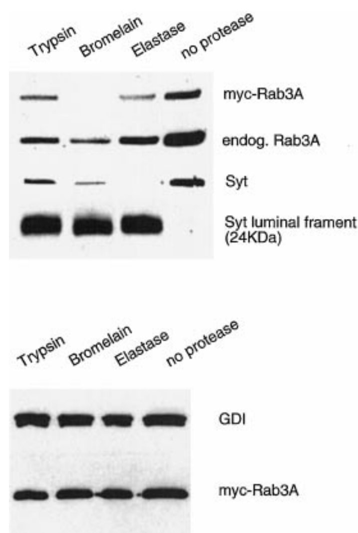

FIG. 10. **The Rab3A binding site on synaptic vesicles is sensitive to proteases.** Synaptic vesicles (LP2) were pretreated with trypsin, bromelain, and elastase (see "Experimental Procedures" for details) and then analyzed for the binding of myc-Rab3A using standard assay conditions. *Top*, membrane fractions containing bound myc-Rab3A. As a control for proteolysis, the degradation of synaptotagmin was monitored. Cleavage resulted in the generation of a 24-kDa fragment corresponding to the intravesicular domain of the protein, which is protected from protease attack (see, *e.g.*, Ref. 50 for details). Furthermore, the fate of endogenous Rab3A was monitored in parallel. Treatment with all three proteases greatly reduced binding of Rab3A to vesicle membranes with bromelain being most effective. *Bottom panel*, analysis of the supernatant fractions recovered after centrifugation at the end of the binding assays. No change was observed, demonstrating that no residual protease activity was present during the incubation, which may have resulted in a breakdown of the complex.

with bromelain being most effective (Fig. 10, *upper panel*). Reduction was not due to a diminished membrane recovery since equal amounts of the luminal fragment of synaptotagmin were recovered. Furthermore, no breakdown of unbound myc-Rab3A·GDI complex was observed in the unbound fraction (Fig. 10, *bottom*), showing that the loss of binding is due to a loss of a Rab3 receptor protein and not due to a digestion of Rab3A·GDI complex by residual proteases.

DISCUSSION

In the present study, we have used several approaches to study the binding of Rab3A to synaptic vesicles *in vitro*. Binding is a saturable, protein-mediated event that displays selectivity for the appropriate target organelle. Furthermore, binding is independent of nucleotide exchange and is reversible as long as no nucleotide exchange occurs. Our findings complement previous studies on the binding of endosomal Rab proteins (14–17) and support the idea that the membrane recruitment of Rab proteins proceeds in distinct steps involving binding of the GDI·Rab complex, dissociation of GDI, and nucleotide exchange.

Several approaches were used to define whether the binding reaction is specific for synaptic vesicles and thus reflects the localization of endogenous proteins. For comparison, we tested binding of Rab1B, which functions in the trafficking of vesicles derived from the endoplasmic reticulum. Rab3A associates preferentially with synaptic vesicles, whereas Rab1B preferentially associates with larger membranes derived from the endoplasmic reticulum. However, specificity was not absolute since some cross-binding was observed (particularly with respect to ER binding of Rab3). Similar cross-binding was previously reported with respect to binding of Rab7, Rab9, and Rab1B to endosomes where binding of all three Rab proteins was comparable although the levels of the endogenous proteins were not determined in that study (17).We assume that binding as observed by our *in vitro* assay includes a nonspecific component of Rab binding to any membrane. Lack of specificity was much more pronounced when proteoliposomes reconstituted from synaptic vesicle detergent extracts or phospholipid vesicles were used as acceptor membranes.[3] The enhanced nonspecific component prevented the use of a detergent-based reconstitution approach for the purification of the receptor protein(s).

Our data shed some new light on the initial phase of Rab binding to membranes. The rate and the extent of Rab3A binding is not influenced by the presence of either GDP or GTPγS. As found for other Rab proteins, nucleotide exchange does occur after binding but it has no effect on the binding reaction. Furthermore, addition of cytosol had no influence on the binding reaction, suggesting that all components required for binding are present on the surface of synaptic vesicles.

Interestingly, we were unable to detect membrane-bound GDI even at short incubation times. Clearly, we cannot exclude that GDI binds but then dissociates during our centrifugation/washing procedure. Furthermore, a recent study has suggested that GDI may be associated with membranes *in vivo*, even when not bound to a Rab protein, resulting in the proposal that an additional, hitherto unknown Rab recycling factor is responsible for directing GDI to newly formed Rab-GDP species (48). Furthermore, a specific GDI dissociation factor has been described that specifically displaces GDI from Rab9 and that may be responsible for the initial step in the binding reaction (18).

The finding that GDP-Rab3A persists on the membrane after GDI dissociation prompted us to investigate whether binding is reversible before nucleotide exchange. Excess GDI inhibits binding, in good agreement with similar observations on the binding of endosomal Rab proteins (17). Interestingly, however, bound Rab3A can be subsequently dissociated by GDI as long as no nucleotide exchange occurs. Thus, at least in our *in vitro*

[3] J. H. Chou and R. Jahn, unpublished observations.

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system, the initial phase of membrane recruitment involves an equilibrium between binding and dissociation. The efficiency of recruitment would be determined by the concentration of free GDI as much as that of GDI·Rab complexes and by the rate of nucleotide exchange. Indeed, excess GDI was shown to inhibit *in vitro* transport reactions although it is noteworthy that overexpression of GDI in intact cells appears to be less effective (see Ref. 48 for a more detailed discussion and for references of the older literature).

The fact that membrane-bound, GDI-sensitive GDP-Rab is an intermediate of the re-binding reaction raises the question how (and if) newly bound GDP-Rab proteins are distinguished from GDP-Rab proteins that have just done their job in hydrolyzing GTP as a result of an interaction with GTPase-activating protein. Clearly, it is possible that nucleotide exchange is more tightly coupled to binding in an intact systems than in our *in vitro* experiments. However, since GDI appears to be able to operate on both GDP-Rab3A pools, differentiation between them may be due to other factors such as the above mentioned GDI dissociation factors or Rab recycling factors. Interestingly, several lines of evidence suggest that, although GDI is an essential gene product, GDI-mediated dissociation may not be a mandatory under all circumstances (see, *e.g.*, Ref. 49). Thus it is conceivable that membrane-bound GDP-Rab, generated by hydrolysis from GTP-Rab, is directly re-converted into the GTP form by guanine nucleotide exchange factor without intermediate involvement of GDI.

Binding of Rab3A is saturable, supporting that binding is dependent on a Rab3A receptor on the vesicle surface. The nature of the binding site remains to be established. Protease pretreatment of vesicles greatly reduced binding, in agreement with earlier reports on the binding of Rab4 (15). For Rab3A, bromelain was most effective, but in contrast to similar experiments with Rab4 (15) we were unable to reconstitute binding by re-addition of the protease supernatant.[3]

In summary, our data demonstrate that synaptic vesicles possess a specific binding mechanism for Rab3A whose properties resemble those previously characterized for endosomal Rab proteins. They lend further support to a rebinding pathway that proceeds in distinct steps (1). In the first step, GDI·Rab complexes bind to the target membrane. Second, GDI is released, probably involving a specific GDI dissociation factor (18), although this step could not be resolved from the first step in the present study. After GDI dissociation, GDP-Rab remains bound to the membrane and subsequently undergoes nucleotide exchange, resulting in active GTP-Rab, which does not interact with GDI. In turn, GTP-Rab recruits appropriate downstream effectors, which are required for its biological activity.

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MEMBRANE TRANSPORT STRUCTURE FUNCTION AND BIOGENESIS:
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