Synapsin Is a Novel Rab3 Effector Protein on Small Synaptic Vesicles

I. IDENTIFICATION AND CHARACTERIZATION OF THE SYNAPSIN I-Rab3 INTERACTIONS IN VITRO AND IN INTACT NERVE TERMINALS*

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Synapsins, a family of neuron-specific phosphoproteins, have been demonstrated to regulate the availability of synaptic vesicles for exocytosis by binding to both synaptic vesicles and the actin cytoskeleton in a phosphorylation-dependent manner. Although the above-mentioned observations strongly support a pre-docking role of the synapsins in the assembly and maintenance of a reserve pool of synaptic vesicles, recent results suggest that the synapsins may also be involved in some later step of exocytosis. In order to investigate additional interactions of the synapsins with nerve terminal proteins, we have employed phage display library technology to select peptide sequences binding with high affinity to synapsin I. Antibodies raised against the peptide YQYIESTMQ (syn21) specifically recognized Rab3A, a synaptic vesicle-specific small G protein implicated in multiple steps of exocytosis. The interaction between synapsin I and Rab3A was confirmed by photoaffinity labeling experiments on purified synaptic vesicles and by the formation of a chemically cross-linked complex between synapsin I and Rab3A in intact nerve terminals. Synapsin I could be effectively co-precipitated from synaptosomal extracts by immobilized recombinant Rab3A in a GTP-dependent fashion. In vitro binding assays using purified proteins confirmed the binding preference of synapsin I for Rab3A-GTP and revealed that the COOH-terminal regions of synapsin I and the Rab3A effector domain are required for the interaction with Rab3A to occur. The data indicate that synapsin I is a novel Rab3 interactor on synaptic vesicles and suggest that the synapsin-Rab3 interaction may participate in the regulation of synaptic vesicle trafficking within the nerve terminals.

Information transfer among neurons is controlled by neurotransmitters stored in synaptic vesicles (SV) and released to the extracellular space by an efficient process of regulated exocytosis. Synaptic vesicles are organized in two distinct functional pools, a large reserve pool in which SV are restrained by the actin-based cytoskeleton, and a quantitatively smaller releasable pool in which SV contact the presynaptic membrane and eventually fuse with it upon stimulation (for review see Refs. 1 and 2). Both SV trafficking and neurotransmitter release depend on a precise sequence of events that includes release from the cytoskeletal constraint, targeting to the active zone, docking, priming, fusion, and endocytotic retrieval of SV. These steps are believed to be mediated by a series of specific interactions among cytoskeletal, SV, presynaptic membrane, and cytosolic proteins that, by acting in concert, promote the spatial and temporal regulation of the exocytotic machinery.

The synapsins are members of a multigene family of SV-specific phosphoproteins that are implicated in the regulation of neurotransmitter release and synapse formation (see Refs. 6 and 7 for review). A large body of experimental data has demonstrated that the synapsins are both necessary and sufficient for SV to bind actin filaments and are responsible for the formation and maintenance of SV clusters within the nerve terminal (8–12). Modulation of SV and actin binding after synapsin phosphorylation by Ca2+/calmodulin-dependent protein kinase (CaMK) II (10, 13–17), protein kinase A, and mitogen-associated protein kinase Erk 1/2 (18–20) may control the transition of SV from the reserve pool to the releasable pool, thus regulating the efficiency of neurotransmitter release. Such a model has been confirmed by an array of in vivo observations.

In living hippocampal neurons, synapsin I dissociates from SV and disperses into the axon during firing, whereas it reclusters upon return to the resting state, and the dispersion of the proteins depends on phosphorylation by CaMK and extracellular signal-regulated kinase (21, 22). Injection of synapsin I into...
developing neurons stimulated the maturation of quantal neurotransmitter release and increased SV clustering (12, 23, 24). Conversely, neutralization of endogenous synapsins by the intracellular injection of anti-synapsin antibodies disrupted the clusters of SV, decreased neurotransmitter release evoked by high frequency stimulation in the lamprey giant reticulospinal neurons (11), and induced the disappearance of post-tetanic potentiation, appearance of post-tetanic depression, and increased synaptic depression in *Aplysia* ganglion neurons (25). Mutant mice lacking synapsin I, synapsin II, or both exhibited a decrease in the number of SV and in the maximal release of neurotransmitters, depression during high frequency stimulation, and increased recovery times after synaptic depression (26–30).

Although the above-mentioned observations strongly support a pre-docking role of the synapsins in the assembly and maintenance of a large reserve pool of SV and in the regulation of short term synaptic plasticity, recent results indicate that the synapsins are also involved in some later step of exocytosis. The kinetics of release was slowed in the squid giant terminal ganglion neuron terminals after injection of a conserved synapsin COOH-terminal peptide (31), as well as in *Aplysia* ganglion neuron terminals after neutralization of endogenous synapsin by antibody injection (25). However, there is no clear evidence, as yet, of an interaction of synapsins with presynaptic or SV proteins involved in the post-docking events of exocytosis. Synaptic vesicles docked or fused with the presynaptic membrane following electrical stimulation are only partially depleted of synapsins (11, 32, 33), and synapsin I staining is found on the membranes of vesicles fused with the plasma membrane (34). This raises the possibility that the putative post-docking effects of the synapsins are obtained via specific interactions with nerve terminal proteins playing a direct role in exocytosis.

With the aim of uncovering potential synapsin partners, we have identified high affinity synapsin-binding peptides by phage display library analysis and have generated anti-peptide antibodies to identify nerve terminal proteins bearing the selected peptide motifs. By using this approach, we have observed that the small G protein Rab3A is one of the synapsin I interactors and that the binding of synapsin I to Rab3A occurs in purified SV and intact nerve terminals.

**EXPERIMENTAL PROCEDURES**

**Materials**

125I- and 35S-labeled secondary antibodies were from Amersham Biosciences; the Renaissance enhanced chemiluminescence detection system was from PerkinElmer Life Sciences. Isoform-specific anti-synapsin polyclonal antibodies (G143, G211, G278, G281, G304, G306, G455, and RU316), anti-synapsin I (mAb 10.22 and 19.11), anti-synapsin II (mAb 19.21) monoclonal antibodies, and anti-Rab3A polyclonal antibodies raised against a 12-mer peptide corresponding to the NH2 terminus of rat Rab3A were made in our laboratories. Other antibodies were obtained from the following sources: rabbit anti-Rabphilin-3, Transduction Laboratories (Lexington, KY); goat anti-His6 and anti-glutathione S-transferase (GST), Amersham Biosciences; peroxidase-conjugated anti-mouse and anti-rabbit antibodies, Bio-Rad; and alkaline phosphatase-conjugated secondary antibodies, Santa Cruz Biotechnology (Santa Cruz, CA). The anti-RabGDI (DPD dissociation inhibitor) polyclonal antibody was a gift of Dr. M. Zerial (EMBL, Heidelberg, Germany). The pET3b His/SG vector was a gift of Dr. W. E. Balch (Scripps Research Institute, La Jolla, CA). Glutathione-Sepharose, protein G-Sepharose, CH-Sepharose 4B, and pGEX-2T were from Amersham Biosciences; human recombinant Rab3A and bovine serum albumin (BSA) were from Calbiochem; Ni-NTA-agarose was from Qiagen (Valencia, CA); nitrocellulose membranes were from Schleicher & Schuell; sulfo- succinimidyl 2-(p-azidosalicylamido)-ethyl-1,3-dithiopropionate (SASD) and disuccinimidyl suberate (DSS) were from Pierce. The pET-30a/pET-30c vectors and the S protein horseradish peroxidase conjugate were from Novagen Inc. (Madison, WI).

**Purification of Synapsin I and Preparation of Brain Subcellular Fractions**

Synapsin I was purified from bovine brain and subjected to cysteine-specific cleavage as described previously (13, 35). Cellular fractionation of rat forebrain from homogenate to purified SV was carried out through the step of controlled pore glass chromatography as described (36). Purified SV containing endogenous synapsins (untreated SV (USV)) were quantitatively depleted of synapsin I by exposure to mild salt treatment (synapsin-depleted SV (SSV)) and reassociated in vitro with purified synapsin I (synapsin-rebound SV) as described previously (8). Synaptosomes were purified from forebrain P2 fractions by centrifugation on discontinuous Percoll gradients as described previously (37).

**Expression and Purification of Recombinant Proteins**

Bacterial cells (*Escherichia coli* BL21 strain) were transformed to ampicillin resistance by electroporation using either pGEX-2T alone or pGEX-2T-in-frame with sequences encoding for wild-type rat Rab3A or rat Rabphilin-3 (1–206). Mutated forms of Rab3A carrying the mutation Q81L that abolishes the intrinsic GTPase activity or the mutations Q61L, V55E in the Rab effector domain were also expressed. Large scale cultures of Luria broth containing ampicillin (50 μg/ml) supplemented with small and overnight cultures, grown at 37 °C to log phase, and induced with isopropyl β-D-thiogalactopyranoside (100 μM) for 3–5 h. GST and GST-Rab3A fusion proteins were extracted from bacterial lysates, purified to homogeneity by affinity chromatography on glutathione-Sepharose, and dialyzed against 25 mm Tris-Cl, 50 mM NaCl, pH 7.4 (38). Purified GST-Rabphilin-3 was cleaved with thrombin and renatured. Rat Rab3A was expressed as a His6-tagged protein in BL21(DE3) pLyS strain and purified to homogeneity on NTA-agarose affinity columns. The DNA's corresponding to COOH-terminal truncations of rat synapsin Ia were amplified by PCR and subcloned into the pET-30c vector (38). The resulting expression plasmids were then verified by sequencing and transformed into *E. coli* BL21(DE3) Lys E. The following mutants, containing His6 and S tags at the NH2 terminus, were made: DE peptide (syn-(413–704)), D peptide (syn-(413–652)), D1 peptide (syn-(413–617)), D2 peptide (syn-(413–585)), D3 peptide (syn-(413–556)), and D4 peptide (syn-(413–538)). Recombinant proteins were extracted from bacterial lysates and purified to homogeneity on NTA-agarose affinity columns. The His6 tag was removed using thrombin cleavage and renatured.

**Phage Display Library Analysis**

We have used a previously described nonapexonin peptide display library (40) containing ~107 independent clones. Random oligonucleotides were inserted into the pCS9 phagemid at the 5′ end of the gene encoding for the major coat protein pVIII. The resulting chimeric pVIII protein was incorporated into phage particles by introduction of the phagemid into *E. coli* cells subsequently superinfected with M13-K07 helper phage. Selection of the phages displaying peptides that bound to purified synapsin I immobilized on polystyrene beads (6.4 μm diameter; Precision Plastic Balls, Chicago, IL) was performed by the standard panning method as described previously (41). Generally, two or three cycles of panning were needed to achieve a reliable enrichment of phage clones. Synapsin-positive clones, identified by immunoscreening analysis, were isolated, and single-stranded genome DNAs were purified and sequenced (39, 42). The deduced amino acid sequences were aligned, and consensus sequences were derived by using Wisconsin Sequence Analysis Software package (Genetics Computer Group, Madison, WI).

**ELISA and Phage For Western Overlays**

The relative affinity of interaction of the selected phage clones with native synapsin I was tested by ELISAs as described previously (42). Briefly, polystyrene beads coated with synapsin 1 (1 μg/ml) were incubated with the most reactive phage clones (50 μl of supernatant) for 1 h at 37 °C on a rocking platform. After washing the wells were incubated for 1 h at room temperature with an anti-pIII monoclonal antibody and revealed using alkaline phosphatase-conjugated secondary rat antibodies or the alkaline phosphatase detection system, and then reading the absorbance at 405–620 nm using a Labsystem reader. The site-specific binding of the selected phage clones was assayed by far Western overlay. Holosynapsin I and its cysteine-specific cleavage fragments (7.5 μg of total protein) (35) were separated by SDS-PAGE and trans-
ferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in TBS (150 mM NaCl, 25 mM Tris-Cl, pH 7.4) containing 5% (w/v) nonfat dry milk (blocking buffer), rinsed with TBS, and incubated overnight at 4°C in blocking buffer supplemented with 0.1% (v/v) Triton X-100 containing 1010 phage/ml. The membranes were then washed and incubated for 2 h at room temperature with a rat anti-pIII monoclonal antibody and revealed using alkaline phosphatase-conjugated secondary antibodies and the alkaline phosphatase detection system.

**Generation of Antipeptide Antibodies**

Peptides corresponding to the sequences of the most reactive clones were synthesized at the Rockefeller University Oligomer Facility. Peptides were coupled to thyroglobulin in 10 mM KPO4, 150 mM NaCl, pH 7.0, by dropwise addition of glutaraldehyde to a final concentration of 0.1% (v/v), incubated for 2 h at 4°C, and used to immunize rabbits in complete Freund’s adjuvant. Sera were collected at various time intervals after immunization, and the antipeptide antibodies were affinity-
purified using columns made by coupling synthetic peptides to CH-Sepharose 4B beads following the manufacturer's instructions (5-ml bed volume; 20–40 ml of serum). After serum loading, the columns were washed sequentially with 50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.4; 25 mM sodium borate, 0.5 mM NaCl, 0.1% Tween 20, pH 8.3; 50 mM sodium acetate, 0.5 mM NaCl, 0.1% Tween 20, pH 5.5; 50 mM Tris-Cl, 150 mM NaCl, pH 7.4. The antibodies were eluted with 100 mM glycine, pH 2.5, immediately neutralized with 1 ml Tris-Cl, pH 9.0, dialyzed against 10 mM MOPS, 150 mM NaCl, pH 7.5, and concentrated to about 1 mg/ml.

**Photoaffinity Labeling and Cross-linking Experiments**

The COOH-terminal fragment of synapsin I, generated by cysteine-specific cleavage, was conjugated in the dark with SADSE (1.4 mM) for 40 min at room temperature and subsequently iodinated on the reagent modified as described previously (18). The modified, 125I-labeled COOH-terminal fragment (100–400 ml final concentration) was incubated for 30 min on ice in the dark with synapsin-depleted SV (10–30 ml of total protein). After high speed sedimentation, SV pellets were photolyzed for 2 min at 254 nm using an ultraviolet lamp (UVP, San Gabriel, CA) and subjected to SDS-PAGE under reducing conditions. Cross-linking of synaptosomal proteins was performed by incubating intact synaptosomes at 37 °C for 45 min with the cell-permeable cross-linker DSS dissolved in dimethyl sulfoxide and used at a final concentration of 5 mM (43). After quenching the reaction with 100 mM glycine for 30 min, synaptosomes were osmotically lysed and fractionated by differential centrifugation, and the resulting crude SV fraction was subjected to SDS-PAGE and immunoblot assay.

**Analysis of the Synapsin I-Rab3A Interaction**

**Affinity Purification of Rab3A-binding Proteins from Brain Synaptosomes**—Wild-type or mutated GST-Rab3A fusion proteins (30 mg) were loaded with either GDP or GTPγS (500 ml) in buffer containing 20 mM Tris-Cl, 25 mM NaCl, 4 mM EDTA, pH 7.4 (loading buffer), for 10 min at 30 °C. After the incubation, the samples were chilled at 4 °C, and MgCl2 was added to a final concentration of 5 mM. When the loading procedure was carried out with a trace amount of either [35S]GTPγS or [35S]HMGDP, the average nucleotide binding stoichiometry was 0.22 ± 0.05 mol of nucleotide/mol of GST-Rab3A. Preloaded GST-Rab3A or GST alone was coupled to glutathione-Sepharose (30 ml of settled beads) by an overnight incubation at 4 °C under gentle rotation in binding buffer (10 mM Hepes, 150 mM NaCl, 1% (v/v) Triton X-100, 2 mg/ml BSA, pH 7.4) and 5 mM MgCl2. Samples were then extensively washed with 20 volumes of binding buffer and mixed with 1 ml of a 1% (v/v) Triton X-100 extract of Percoll-purified synaptosomes (1 mg of protein/ml) (37). After a 3–5-h incubation at 4 °C under gentle rotation, samples were extensively washed and eluted with Laemmli sample buffer (44). The eluted proteins were separated by SDS-PAGE and analyzed by immunoblotting.

**Rab3A-Synapsin I Binding Assays**—The binding of wild-type and mutated GST-Rab3A to purified synapsin I or to truncation mutants of the COOH-terminal region of synapsin I was assessed by co-precipitation experiments as follows. GST-Rab3A was loaded with either GDP or GTPγS as described above. Preloaded GST-Rab3A fusion proteins or GST alone was coupled to glutathione-Sepharose (0.04 nmol of fusion protein per ml of settled beads) in binding buffer containing 5 mM MgCl2. After extensive washing, protein-coupled beads (15 ml) were incubated for 3–5 h at 4 °C with increasing concentrations of either purified synapsin I (62.5–2000 ml) or synapsin truncation mutants (400 ml in 500 ml of binding buffer). After the incubation, the beads were pelleted by centrifugation, extensively washed with binding buffer and detergent-free binding buffer, resuspended in Laemmli sample buffer, and boiled for 2 min. Binding was analyzed by SDS-PAGE followed by quantitative immunoblotting. The recovery of recombinant bait proteins in the pellets was routinely assessed by protein stain of the gels or of the corresponding blots.

**Miscellaneous Procedures**

Protein concentrations were determined using the Bradford (Bio-Rad) assay with BSA as standard. One- and two-dimension gel electrophoresis (SDS-PAGE and nonequilibrium pH gradient electrophoresis) was performed according to Laemmli (44) and O’Farrell et al. (45). Proteins in the gels were electrophoretically transferred to nitrocellulose membranes and analyzed by immunoblotting by using either chemiluminescence or 125I-labeled secondary antibodies as detection system. Quantitative immunoblotting was performed either by laser scanning densitometry (Ultrascan XL, Amersham Biosciences) of the films obtained in the linear range of the emulsion response or by direct radioactivity counting, followed by interpolation of the values into a suitable standard curve.

**RESULTS**

**Identification of High Affinity Synapsin-binding Peptides Using Phage Display Libraries**—In order to identify new potential ligands of synapsin I, we have panned a random nonapeptide library displayed on the surface of a filamentous bacteriophage. Affinity selection of specific synapsin ligands was performed by screening the phage library on polystyrene beads coated with synapsin I. The amino acid sequences deduced from the nucleotide sequences obtained from the selected phages were aligned, and four families of reactive clones exhibiting common motifs (group 1, qnY; group 2, +Y--; group 3, WY; group 4, WXY) were identified (Fig. 1A). The relative affinity of interaction of the selected phage clones with native synapsin I was tested by ELISA (Fig. 1B). The most reactive phage clones were YPRYDHYDW (syn18), WSHYSSEPL (syn4), PSMSSYTTW (syn20), YQYIETSMQ (syn21), and CDWELCAYE (syn13); lower but comparable affinity levels were observed with YYYSTPSVA (syn6), NYDYRAIE (syn2), VADLITYYW (syn24), and AHNYYFREW (syn1). To identify which region of synapsin I was involved in binding the most reactive phage clones, synapsin I was cleaved at cysteine resi-
FIG. 3. Synapsin I interacts with Rab3A in synaptic vesicles. A and B, the COOH-terminal fragment of synapsin I covalently bound to the iodinated cleavable photoaffinity reagent SAD (43764-ASD Syn) was incubated with synapsin-depleted SV. After photolysis, the samples were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and subjected to autoradiography (AX) and immunoblotting (IB). In addition to the prominent 36–38-kDa doublet present in both photolyzed (UV+) and nonphotolyzed (UV−) samples corresponding to the labeled ligand, two bands of 48 kDa (previously identified as the α subunit of CaMKII) (15) and 25 kDa were specifically labeled in a UV-dependent fashion (A). Retrospective immunoblotting with anti-Rab3A antibodies (αRab3A) performed after one- (A) and two-dimensional nonequilibrium pH gradient electrophoresis (B) separations revealed that the labeled 25-kDa protein corresponds to Rab3A.

dues, and the resulting fragments were analyzed by far Western overlay for their ability to interact with the various phage clones. Although a few phage clones positive in the ELISA (syn1, syn4, syn6, syn13, and syn18) did not exhibit significant binding to synapsin I and synapsin fragments in the latter assay (not shown), clones syn2, syn20, syn21, and syn24 bound to both undigested synapsins la/1b and to their COOH-terminal fragments (peptides βγ) (Fig. 1C).

Generation of Anti-peptide Antibodies—As the amino acid sequences of the selected peptides were too short and variable to unambiguously identify proteins in the data base, an immunological approach was used to uncover potential nerve terminal proteins bearing the above-mentioned or homologous amino acid sequences. Peptides corresponding to the inserts displayed by the most reactive phage clones were synthesized, conjugated with thyroglobulin, and used to immunize rabbits. The resulting anti-peptide antibodies were affinity-purified and analyzed for their ability to recognize specific protein bands in immunoblots of subcellular fractions from rat forebrain. Most interestingly, the antibody (Ab 279) raised against the peptide YQYI-ETSMQ (syn21) specifically recognized a protein of 25-kDa apparent molecular mass that was enriched in crude SV fractions (LP2) and untreated highly purified SV (USV) (Fig. 2A). This protein was not dissociated from the SV membrane after exposure to high salt (synapsin-depleted SV) and was virtually absent from the synaptosolic fraction (LS2).

Identification of the Synapsin Interactor as Rab3A—The apparent molecular mass of the protein recognized by Ab 279 and its SV enrichment suggested that it may correspond to Rab3A/C, a family of SV-associated small G proteins implicated in multiple steps of exocytosis, of which Rab3A is the most abundant and widespread isoform in the brain (46). Double immunostaining performed on blots obtained after both monochromatic and two-dimensional gel separations of either purified recombinant human Rab3A or the LP2 fraction from rat forebrain (Fig. 2, B and C) revealed that the 25-kDa protein indeed corresponds to Rab3A and that purified recombinant Rab3A is recognized to a similar extent by both the anti-peptide antibody and an anti-Rab3A-specific antibody. Retrospective alignment of the syn21 sequence revealed that it shares 78% similarity with residues 159–167 of Rab3A.

Analysis of the Interactions between Synapsin I and Rab3A in Synaptic Vesicles and Intact Nerve Terminals—To verify whether an interaction between synapsin I and Rab3A indeed occurs in nerve terminals, we employed both cross-linking and affinity chromatography approaches. The purified COOH-terminal region of synapsin I was coupled to the photoactivatable, 125I-labeled and cleavable reagent SAD, bound to synapsin-depleted SV, and subjected to photolysis and cleavage. In addition to the previously reported 50-kDa SV band corresponding to the α-subunit of CaMKII (15), an additional protein of 25 kDa was specifically labeled upon photolysis (Fig. 3A). Immunolabeling of blots from one- and two-dimensional gel separations using anti-Rab3A-specific antibodies revealed that the protein specifically labeled upon the interaction with the photoactivatable synapsin I derivative was Rab3A (Fig. 3, A and B).

In resting nerve terminals, Rab3 is present primarily on SV
Synapsin I Is a Novel Rab3A Interactor

**Fig. 5.** Rab3A affinity purifies synapsin I from synaptosomal extracts in a GTP-dependent fashion. A, affinity resins were prepared by coupling glutathione-Sepharose to either wild type or mutated Rab3A (Rab3AQ81L, Rab3AQ81LV55E) GST-Rab3A which had been loaded with either GDP or GTP·S (500 μM), as described under "Experimental Procedures." Rab3A-coated beads were incubated with a Triton X-100 extract of Percoll-purified forebrain synaptosomes. Bound proteins were eluted with SDS, separated by SDS-PAGE, and analyzed by immunoblotting using antibodies (αSyn I, anti-Rabphilin-3 (αRph3), and αRabGDI (αGDI) antibodies. B, Rabphilin-3 inhibits the binding of synapsin I to Rab3A. GST-Rab3A, which had been coupled to glutathione-Sepharose, was loaded with either GDP or GTP·S (500 μM) and incubated with a Triton X-100 extract of rat forebrain synaptosomes in the absence or presence of either EDTA (10 mM) or an excess of purified Rabphilin-3 (1 μM). The amounts of synapsin I bound to Rab3A were analyzed by immunoblotting as described above (LOAD, 3 μg of total synaptosomal proteins).

in the GTP-bound state, complexed with the SV-associated Rab3 effector protein Rabphilin-3 (43, 47, 48). To test if synapsin I is also complexed with Rab3A on SV in intact nerve terminals, we incubated resting and depolarized synaptosomes with the cell-permeant cross-linker DSS and analyzed the resulting protein patterns by immunoblotting with anti-synapsin I (αSyn I), anti-Rabphilin-3 (αRph3), and anti-RabGDI (αGDI) antibodies. As shown in Fig. 4, an adduct of ~110-kDa molecular mass containing both Rab3A and synapsin I was generated in the presence of DSS under both resting and depolarized conditions.

As an alternative approach, we investigated whether synapsin I could be pulled down using GST-Rab3A coupled to glutathione beads from a detergent extract of Percoll-purified rat forebrain synaptosomes. To analyze the specificity of the interaction, native or mutated Rab3A was loaded with either GDP or GTP. Two Rab3A mutants were used, namely GST-Rab3AQ81L, a GST fusion protein carrying a mutation (Q81L) abolishing the intrinsic GTPase activity, and GST-Rab3AQ81LV55E, a fusion protein carrying another mutation (V55E) in the effector domain that affects the interaction with target proteins such as Rabphilin-3. In agreement with previous reports, Rabphilin-3 associated with both Rab3A and Rab3AQ81L in the GTP-bound state, whereas the binding to Rab3AQ81LV55E was completely abolished. In contrast, Rab-GDI specifically bound to the GDP-bound form of both wild-type Rab3A and Rab3A mutants. Most interestingly, synapsin I was also purified with high efficiency by GTP-Rab3A, and its binding was abolished by mutation of the Rab3A effector domain (Fig. 5A) or by removing the guanine nucleotide by exposure to EDTA (Fig. 5B).

Immunoblotting with isoform-specific anti-synapsin antibodies following the Rab3A pull-down assays revealed that synapsins Ia/Ib are the major isoforms co-precipitated by Rab3A, but that also synapsins IIa/IIb (but not synapsin IIIa) are precipitated to a lower extent. Although the possibility of a direct interaction between synapsin II and Rab3A exists, the presence of synapsin I/synapsin II hetero-oligomers (49) may contribute to the co-precipitation of synapsin II (Fig. 6).

**Analysis of the Binding between Purified Rab3A and Synapsin I**—To better characterize the synapsin I-Rab3A interaction, we analyzed the binding of immobilized GST-Rab3A to purified synapsin I. Although the binding to GST or to nucleotide-free Rab3A was negligible, synapsin I bound in a dose-dependent fashion to nucleotide-bound Rab3A, exhibiting a strong preference for the GTP-bound form (Fig. 7A). Quantitative analysis of the binding isotherms revealed that the binding was saturable, with maximal binding (B max) under the assay conditions used of 415 ± 44 fmol of synapsin I/μg of GTP-loaded GST-Rab3A.
and 178 ± 9 fmol of synapsin I/μg of GDP-loaded GST-Rab3A (means ± S.D.; Fig. 7B) and dissociation constant \( (K_d) \) of 1–1.5 μM. The \( K_d \) value suggests that partial dissociation may have occurred during the washing procedure leading to an underestimation of the \( B_{\text{max}} \) values. A further underestimation of the \( B_{\text{max}} \) values is provided by the fact that only ~20% of the recombinant Rab3A molecules is nucleotide-bound after the loading procedure (see “Experimental Procedures”), and synapsin does not bind to nucleotide-free Rab3A.

In order to identify the site of interaction with Rab3A in the synapsin I molecule, the above-described co-sedimentation assay was used to analyze Rab3A binding to truncation mutants of the COOH-terminal region of synapsin I. Recombinant peptides were made corresponding to domain DE, domain D, and four distinct truncation mutants of domain D (D1, D2, D3, and D4) progressively missing residues at the COOH-terminal region (Fig. 8A). Both GTP-Rab3A and, to a lower extent, GDP-Rab3A bound to the DE and D peptide, whereas the binding to the D1, D2, D3, and D4 truncation mutants was virtually absent (Fig. 8B).

**DISCUSSION**

With the goal of uncovering potential synapsin I partners, we have identified synapsin-binding peptides by panning a phage display library and generated anti-peptide antibodies to identify nerve terminal proteins bearing the peptide motifs. The affinity-purified antibody directed against the syn21 peptide specifically recognized an SV-specific protein that was immunochemically identified as Rab3A, the most abundant monomeric GTPase associated with SV (46). We have investigated the occurrence of this interaction in nerve terminals, and we demonstrated the following: (i) Rab3A is photoaffinity-labeled in intact SV by a derivative of the COOH-terminal region of synapsin I; (ii) heterodimers of synapsin I and Rab3A can be detected in intact synaptosomes treated with membrane-permeable cross-linkers; (iii) at low concentrations, synapsin I exhibits a strong preference for GTP-bound Rab3A, although it can also bind to GDP-Rab3A in *in vitro* binding assays; (iv) both the Rab3A effector domain and a COOH-terminal peptide of rat synapsin Ia encompassing residues 618–652 are necessary but not sufficient for binding, and other regions of the two proteins are likely to be involved.

A point mutation in the Rab3A effector domain virtually abolished the interaction with synapsin I. In contrast, the peptide identified by phage display, which shares homology with Rab3A-(159–167), was unable to inhibit the binding of synapsin to Rab3A (data not shown). The lack of inhibition by the peptide may be ascribed to its short length and/or to the presence of nonrelevant residues in its sequence that may make its affinity for synapsin too low to compete with holo-Rab3.

The use of Rab3A mutants and the competition between Rabphilin-3 and synapsin I suggest that the major synapsin-binding site corresponds to the Rab3 switch I region encompassing the so-called effector domain, with a partial overlap with the Rabphilin-3-binding site. Synapsins do not contain Zn2+ finger motifs typical of Rab3 effectors such as Rabphilin-3 and Rim (50, 51). On the one hand, this aspect can explain the lower affinity of synapsin for Rab3A binding as compared with
Rabphilin-3 or Rim; on the other hand it indicates that the Zn$^{2+}$/H$\alpha$01 finger domain is dispensable for Rab3 binding, as reported previously for the Rab effectors EEA1, Rabaptin-5, p40 and Rabkinesin-6 (52–55). Indeed, structural studies indicate that Rab proteins may direct their effector binding specificity by utilizing different binding surfaces (56).

In addition to synapsins Ia and Ib, synapsins IIa and IIb can be pulled down by Rab3A from synaptosomal extracts. As synapsin II does not share primary sequence homology with the proline-rich COOH-terminal region of synapsin I involved in Rab3 binding, co-precipitation of synapsin isoforms may simply reflect recovery of synapsin I/synapsin II hetero-oligomers present in nerve terminals (49), whose formation is not mutually exclusive with the binding of synapsin I to Rab3A. However, the possibility of a direct binding of synapsin II to Rab3A cannot be ruled out, as the COOH-terminal domains of synapsin II (domains G–I) are also proline-rich and may exhibit structural similarities with the corresponding regions of synapsin I.

Whereas the affinity purification of endogenous synapsin I from synaptosomal extracts by immobilized Rab3A was strictly GTP-dependent, reconstitution of the interaction in vitro with Rab3A and synapsin I revealed that synapsin can also bind GDP-bound Rab3A, although a clear preference for GTP-bound Rab3A was maintained. As synapsin I does not bind to nucleotide-free Rab3A, the data indicate that the strict GTP selectivity observed in synaptosomal extracts is attributable to the presence of additional Rab3 interactors, such as RabGDI or guanine nucleotide exchange factor, or additional molecules that may stabilize the interaction of endogenous synapsin I with Rab3A-GTP or prevent synapsin I from binding to GDP-Rab3A. This possibility may also account for the relatively low stoichiometry observed in in vitro pull-down assays with purified synapsin and Rab3A.

The cycling of Rab3 proteins correlates with the exo-endocytotic cycle of SV and stimulation of neurotransmitter release promotes GTP hydrolysis and dissociation of Rab3A from the SV membrane (57, 58). Rab3 has been proposed to vectorially regulate SV trafficking by participating in SV targeting, regulation of SV fusion, and repriming after endocytosis, as well as in the dynamic assembly of the actin cytoskeleton (for review see Refs. 56, 59, and 60). Synapsins have also been implicated in the regulation of SV trafficking and cytoskeleton assembly (9, 10, 21, 22, 33). In addition, both proteins cycle between a cytosolic and an SV-associated form and may represent clocks for the exo-endocytotic cycle of SV by ensuring directionality and reversibility to the process.

Protein-protein interactions in nerve terminals represent key events for correct targeting of proteins in the appropriate compartment and for the precise spatio-temporal timing of exocytosis. We have demonstrated that synapsin I is a novel Rab3A effector and that an interaction between the two proteins occurs in intact nerve terminals. The existence of an interaction between two SV proteins both involved in the regulation of SV trafficking is particularly challenging. It is tempting to speculate that this interaction, by modulating the biochemical properties of Rab3A and/or synapsin I, participates in the regulation of the exo-endocytotic cycle of SV (61).

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FIG. 8. The COOH-terminal region of synapsin I is involved in Rab3A binding. A, schematic representation of the recombinant rat synapsin I domains DE and D and of the four truncation mutants of domain D (D1–D4) progressively missing residues at the COOH-terminal region. B, the binding of truncation mutants of the domain DE of synapsin I to GST or to recombinant GST-Rab3A was evaluated by co-precipitation assays as described under “Experimental Procedures.” The synapsin peptides recovered in the pellet were separated by SDS-PAGE and analyzed by immunoblotting with an anti-synapsin I monoclonal antibody recognizing to the same extent all recombinant synapsin peptides. Aliquots of the synapsin peptides added to the samples are also shown (Total).
Synapsin I Is a Novel Rab3A Interactor