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The Subcellular Localizations of Atypical Synaptotagmins III and VI

SYNAPTOTAGMIN III IS ENRICHED IN SYNAPSES AND SYNAPTIC PLASMA MEMBRANES BUT NOT IN SYNAPTIC VESICLES*

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Multiple synaptotagmins are expressed in brain, but only synaptotagmins I and II have known functions in fast, synchronous Ca^{2+}-triggered neurotransmitter release. Synaptotagmin III was proposed to regulate other aspects of synaptic vesicle exocytosis, particularly its slow component. Such a function predicts that synaptotagmin III should be an obligatory synaptic vesicle protein, as would also be anticipated from its high homology to synaptotagmins I and II. To test this hypothesis, we studied the distribution, developmental expression, and localization of synaptotagmin III and its closest homolog, synaptotagmin VI. We find that synaptotagmins III and VI are present in all brain regions in heterogeneous distributions and that their levels increase during development in parallel with synaptogenesis. Furthermore, we show by immunocytochemistry that synaptotagmin III is concentrated in synapses, as expected. Surprisingly, however, we observed that synaptotagmin III is highly enriched in synaptic plasma membranes but not in synaptic vesicles. Synaptotagmin VI was also found to be relatively excluded from synaptic vesicles. Our data suggest that synaptotagmins III and VI perform roles in neurons that are not linked to synaptic vesicle exocytosis but to other Ca^{2+}-related nerve terminal events, indicating that the functions of synaptotagmins are more diverse than originally thought.

Synaptotagmins represent a family of at least 12 proteins that are thought to function in membrane traffic (reviewed in Ref. 1). All synaptotagmins are composed of a short N-terminal sequence that is intraluminal and/or extracellular, a single transmembrane region, and a large cytoplasmic sequence that contains two C_2-domains (1). In all synaptotagmins, the N-terminal sequence is translocated across the membrane during translation but lacks a cleaved signal peptide (2, 3), and the transmembrane regions contain multiple cysteine residues that may be palmitoylated (4, 5). The C_2-domains, the most conserved feature of synaptotagmins (1), are separated from the transmembrane region by a variable connecting sequence. C_2-domains are general Ca^{2+}-binding motifs that function as Ca^{2+}-binding modules in most synaptotagmins (reviewed in Ref. 6). In some synaptotagmins, however, the C_2-domains contain sequence changes that probably abolish Ca^{2+} binding (*e.g.* synaptotagmins IV and XI; Refs. 3 and 7), suggesting that C_2-domains can also perform Ca^{2+}-independent functions.

Synaptotagmins I and II, the first molecularly characterized synaptotagmins (8, 9), are abundant synaptic vesicle proteins that are differentially expressed; synaptotagmin I is primarily present in rostral regions, and synaptotagmin II is primarily present in caudal brain regions (9, 10). Knockout experiments in mice revealed that synaptotagmin I is essential for fast Ca^{2+}-triggered neurotransmitter release but is not required for exocytosis as such; slow Ca^{2+}-triggered neurotransmitter release and Ca^{2+}-independent release were still functional in the knockout animals (11). These results indicated that synaptotagmin I functions as a Ca^{2+} sensor for fast synaptic vesicle exocytosis in upper brain regions. Synaptotagmin II probably performs an equivalent role in lower brain regions because it is very homologous to synaptotagmin I and is also enriched in synaptic vesicles (9, 11).

Similar to synaptotagmins I and II, synaptotagmins III-XII are expressed primarily in brain (3, 12–17). However, the mRNAs of most of these other synaptotagmins are detectable at low levels outside of the brain (3, 12–17). Although the enrichment of synaptotagmins III-XII in brain and their homology to synaptotagmins I and II led to the notion that synaptotagmins III-XII may also reside on the synaptic vesicle, their localizations and potential functions have not been investigated in detail. Synaptotagmin III is particularly interesting because it is the most abundant synaptotagmin after synaptotagmins I and II and because it exhibits Ca^{2+}-binding characteristics that resemble the Ca^{2+} requirements of the slow component of Ca^{2+}-dependent neurotransmitter release (18–20). These findings have led to the hypothesis that synaptotagmin III may mediate the slow component of neurotransmitter release (18). In support of this hypothesis, synaptotagmin III is enriched in synapses, detectable in purified synaptic vesicles, and heteromultimerizes with synaptotagmin I *in vitro* (10, 21). Furthermore, in pancreatic β-cells, synaptotagmin III is present on secretory granules and may mediate Ca^{2+}-triggered exocytosis of these granules (22). If synaptotagmin III indeed functions as an exocytotic Ca^{2+} sensor similar to synaptotagmins I and II, it should also be on the secretory vesicles that undergo exocytosis. However, the localization of synaptotagmin III (or other synaptotagmins except for synaptotagmins I and II) was not investigated systematically and quantitatively, and its precise distribution is unknown.

In the current study, we have examined the subcellular localization of synaptotagmin III and its closest relative, synaptotagmin VI. Surprisingly, we find that synaptotagmins III and VI are not enriched in synaptic vesicles. Instead, synapto-

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tagmin III is highly concentrated on synaptic plasma membranes, and synaptotagmin VI is widely present in membranes. Our data suggest that synaptotagmins may be functionally more diverse than previously envisioned.

EXPERIMENTAL PROCEDURES

Production and Affinity Purification of Antibodies—Specificity tests revealed that our previously raised antibodies against the C_2-domains of synaptotagmins III and VI (L181 and I471, respectively) weakly cross-react with other synaptotagmins, presumably because their C_2-domains are so similar. Therefore we generated a new set of antibodies to the sequence between the transmembrane region and the C_2A-domain of synaptotagmins III and VI (residues 88–296 and 86–229 of synaptotagmins III and VI, respectively (3, 12)). Rabbits were immunized with glutathione *S*-transferase-fusion proteins (produced in the pGEX-KG vector (23)), and the resulting sera (S754 and S756 for synaptotagmins III and VI, respectively) were affinity-purified on immobilized maltose-binding protein fusion proteins containing the same sequences of synaptotagmins (in pMal-C2; New England Biolabs). The specificity of all antibodies was tested by probing extracts from COS cells transfected with individual synaptotagmins and by comparing their reactivities with brains from wild type and knockout mice that lack either synaptotagmin III or VI.[1]

Immunocytochemistry—Cryostat sections (5–15 μm) from rat and mouse brains or bovine retina were probed with affinity-purified synaptotagmin antibodies followed by horseradish peroxidase-labeled or biotin-labeled secondary antibodies and development of the signal with the StreptABComplex/HRP kit (DAKO) or by metal enhancement essentially as described previously (24–26). The specificity of the signals was ascertained by two controls: 1) blocking of the signal with the recombinant protein used for antibody production, and 2) analysis of brains from knockout mice lacking synaptotagmin III.[1]

Subcellular Fractionations—Rat brain homogenates were subfractionated into crude synaptic vesicles, mitochondria, myelin, and synaptic plasma membranes by the method of Jones and Matus (27). For this purpose, whole brains including cerebella, olfactory bulbs, and brain stem from 4–6-week-old rats were homogenized in ice-cold 0.32 M sucrose using a motorized glass-Teflon homogenizer. The homogenate was centrifuged at 800 × *g* for 10 min to remove nuclei and debris, and the resulting supernatant was re-centrifuged at 9,000 × *g* for 20 min to obtain synaptosomes and the synaptosomal supernatant. The synaptosomes in the pellet were washed once in 0.32 M sucrose, lysed hypotonically, and centrifuged at 25,000 × *g* for 20 min to isolate large synaptosomal membranes in the pellet, whereas the free synaptic vesicles remained in the supernatant under these conditions. The synaptosomal membranes were resuspended in dH_2O, sucrose was added to 1.1 M, and the solution was placed at the bottom of a Beckman SW28 rotor tube. The sample was overlaid with 0.855 and 0.32 M sucrose solutions and centrifuged for 2.5 h at 19,000 rpm, resulting in the isolation of myelin (in the 0.32/0.855 M sucrose interface), synaptic plasma membranes (in the 0.855/1.1 M sucrose interface), and mitochondria (in the pellet). For the purification of synaptic vesicles, hypotonically lysed synaptosomes were used as a starting point for the isolation of crude synaptic vesicles that were further purified by velocity-density gradient centrifugation and controlled pore glass chromatography essentially as described previously (28, 29). For organelle immunoisolation, beads with immobilized monoclonal antibodies to synaptotagmin I (Cl41.1) or synaptobrevin II (Cl69.1) and control beads containing only immobilized glycine were prepared as described previously (30, 31). Rat brains were homogenized in 5 mM HEPES-NaOH, pH 7.4, 0.32 M sucrose, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/liter leupeptin, 1 mg/liter pepstatin A, and 10 mg/liter aprotinin. The homogenates were centrifuged for 10 min at 7,800 × *g* to generate a postnuclear supernatant. Beads were incubated for 30 min at 4 °C under rotation with the postnuclear supernatant and washed four times, and bound proteins were analyzed by quantitative immunoblotting using ^{125}I-labeled secondary antibodies.

Quantitative Immunoblotting—To quantitate the relative levels of proteins in various fractions for the purification of synaptic vesicles by subcellular fractionation or by immunobead precipitation, we reacted immunoblots of the various fractions with ^{125}I-labeled secondary antibodies and measured the respective signals in a phosphorimager. All blots were probed simultaneously with antibodies to the protein under study and antibodies to synaptogyrin as an internal vesicle protein standard (32). The synaptotagmin signals were then normalized for the synaptogyrin signal by designating the ratio of the two signals observed in starting brain homogenates or postnuclear supernatants as 1.0. For the immunobead experiments, the signals obtained in pulldowns with glycine control beads were subtracted from the values from the immunobead pulldowns before the ratios were calculated.

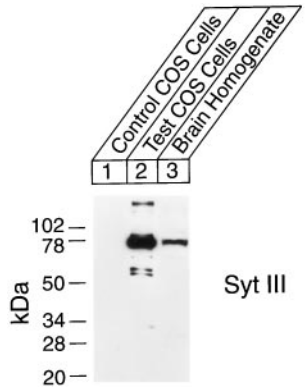

FIG. 1. **Characterization of synaptotagmin III antibodies.** Proteins from COS cells transfected with control DNA (*lane 1*) or with a rat synaptotagmin III expression vector (*lane 2*) and rat brain homogenates (*lane 3*) were analyzed by immunoblotting with affinity-purified antibodies to synaptotagmin III. Signals were visualized by ECL. Note that in brain only a single band is apparent; the low abundance, smaller proteins in *lane 2* probably represent proteolytic breakdown products. *Numbers* on the *left* indicate positions of molecular mass markers.

Miscellaneous Procedures—SDS-polyacrylamide gel electrophoresis and immunoblotting were performed using standard procedures (33, 34). Immunoblots were developed by enhanced chemiluminescence except for the quantitative experiments described above.

RESULTS AND DISCUSSION

We raised multiple antibodies to synaptotagmins III and VI, affinity-purified the antibodies, and tested their specificity using COS cells transfected with various synaptotagmins (data not shown). Our initial antibodies were directed against the C_2-domains of synaptotagmins III and VI. These antibodies, however, exhibited cross-reactivity with the more abundant synaptotagmins I and II, thereby limiting their usefulness. Therefore, we subsequently raised more specific antibodies using the sequence that connects the transmembrane region with the C_2-domains and varies considerably between different synaptotagmins (7–10, 12–17) as an antigen. At least one of these antibodies for each synaptotagmin was specific for that particular isoform, based on the following evidence: 1) as shown for synaptotagmin III in Fig. 1, the antibodies specifically reacted with the cognate protein expressed in COS cells and recognized a single band (synaptotagmin III) or two closely co-migrating bands (synaptotagmin VI; see below) in total brain extract; and 2) immunoblots of total brain homogenates from wild type mice and from knockout mice lacking either synaptotagmin III or synaptotagmin VI were analyzed (Fig. 2). The generation and analysis of these knockout mice will be reported in a later, more detailed report.[1] The mouse immunoblots confirmed that the synaptotagmin III antibodies reacted with a single band and that the synaptotagmin VI antibodies reacted with a set of two closely spaced bands in brain. In the knockouts that contain all other synaptotagmins, the reactivity was abolished (Fig. 2).

Using these antibodies, we examined the regional distribution of synaptotagmins I, III, and VI in rat brain (Fig. 3). As described previously (9), synaptotagmin I was expressed in much higher levels in upper brain regions than in hindbrain and spinal cord (which in turn synthesize higher levels of synaptotagmin II). CASK as a general synaptic marker was uniformly present in all brain regions (35, 36). The expression of synaptotagmins III and VI, however, exhibited a different

[1] R. Fernandez Chacon, R. E. Hammer, and T. C. Südhof, unpublished observation.

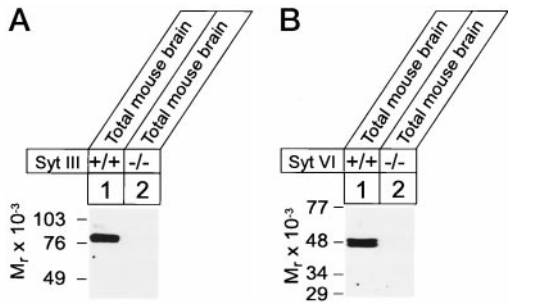

FIG. 2. **Definition of antibody specificity to synaptotagmins III and VI by immunoblot analysis of brains from wild type mice and from knockout mice lacking synaptotagmins III (*A*) or VI (*B*).** Brain homogenates from the indicated mice were immunoblotted with affinity-purified antibodies to synaptotagmins III (*A*) and VI (*B*). Note that the immunoreactive bands are absent in the knockout mice, indicating that the antibodies we raised are highly specific for those particular isoforms.

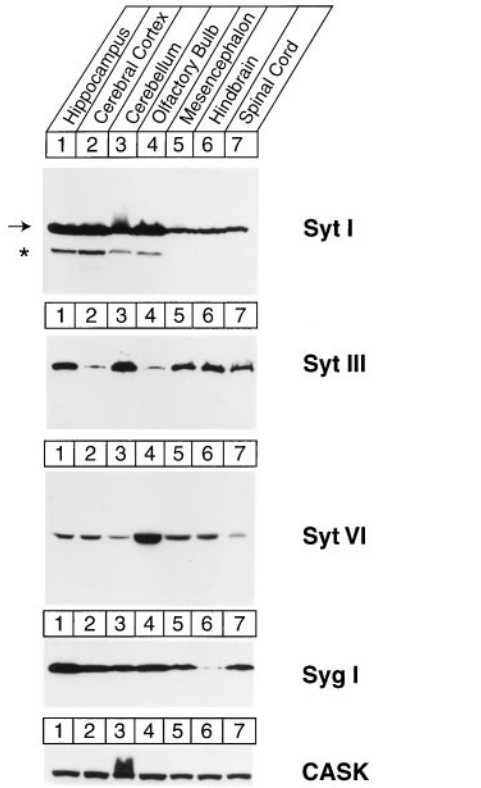

FIG. 3. **Heterogeneous regional expression of synaptotagmins I, III, and VI in rat brain.** Equivalent amounts of protein from the indicated brain regions were analyzed by immunoblotting with antibodies to synaptotagmins I (*Syt I*), III (*Syt III*), and VI (*Syt VI*) and to synaptogyrin I (*Syg I*) and CASK. Note the contrast between the relatively uniform expression pattern of CASK, the striking rostral-caudal gradient of synaptotagmin I, and the heterogeneous distributions of synaptotagmins III and VI. The *arrow* in the synaptotagmin I blot identifies the full-length protein, and the *asterisk* identifies the major proteolytic breakdown product of this protein (2). Signals were visualized by ECL. The synaptotagmin VI doublet is not resolved in the percentage of acrylamide used for this gel (see Fig. 2).

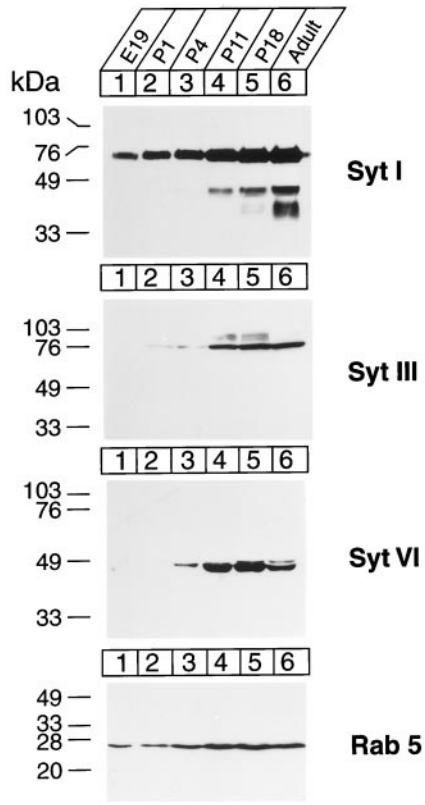

FIG. 4. **Developmental time course of the expression of synaptotagmins I, III and VI.** Brain homogenates from rats of embryonic day 19 (*E19*) and postnatal days 1–18 (*P1–P18*) and from adult rats were analyzed by immunoblotting with ECL detection using antibodies to synaptotagmins I (*Syt I*), III (*Syt III*), and VI (*Syt VI*) and rab5. Positions of molecular mass markers are shown on the *left*.

pattern: both synaptotagmins were present in all brain regions but displayed large regional variations. Synaptotagmin III was almost undetectable in the cerebral cortex and olfactory bulb but was highly expressed in the hippocampus and cerebellum. In addition, it was present at moderate levels in spinal cord and hindbrain (Fig. 3). Synaptotagmin VI was synthesized at rather low levels in all brain areas except for the olfactory bulb, which contained exceptionally high levels of this isoform.

Previous data suggested that synaptotagmin III is localized to synapses (10). This result was supported in the current study by two independent observations. First, we examined the developmental expression profile of synaptotagmins. The level of synaptic vesicle proteins goes up dramatically postnatally in parallel with synaptogenesis (37). When we analyzed brain proteins from rat forebrain as a function of age, we found that synaptotagmin I levels increased postnatally, as expected (10) (Fig. 4). By contrast, the general trafficking protein rab5 exhibited only a moderate developmental change. Synaptotagmins III and VI, similar to synaptotagmin I, experienced a striking postnatal increase in levels that paralleled the time course of synaptotagenesis (Fig. 4). In adults, the levels of synaptotagmin VI, but not synaptotagmins I and III, decreased slightly. Two closely migrating bands were observed for synaptotagmin VI that may be due to alternative splicing; the molecular basis for this heterogeneity is unclear.

The second observation confirming a synaptic localization of synaptotagmin III was obtained by immunocytochemistry. Unfortunately, the quality of our synaptotagmin VI antibodies precluded a morphological localization; even the signal for synaptotagmin III in immunocytochemical stains was insufficient to allow immunoelectronmicroscopy. At the light level, however, synaptotagmin III was present in a pattern strongly suggestive of a synaptic localization (Fig. 5; data not shown). This was most clear in the retina, which contains two sharply delineated synaptic layers, the thin outer plexiform layer composed of photoreceptor synapses and the broader inner plexiform layer comprising multiple sublayers of synapses (38). Synaptotagmin III was highly concentrated in the two synaptic layers in the retina, similar to synaptotagmin I (Fig. 5). The

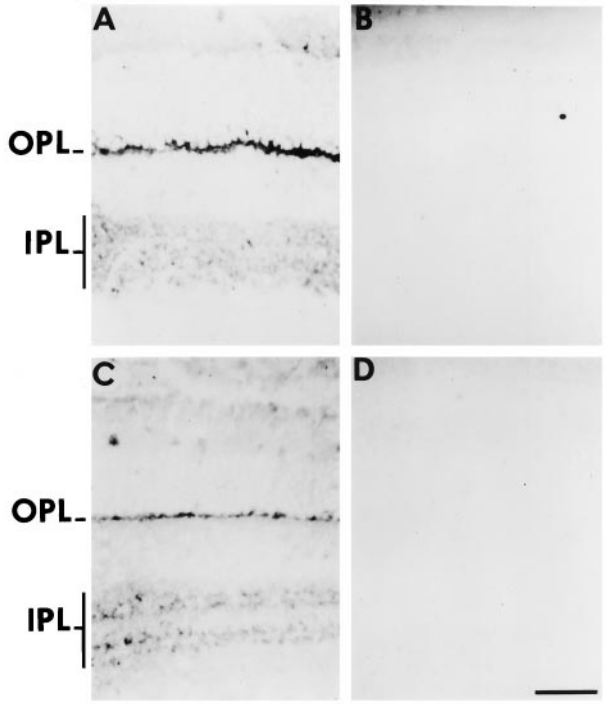

FIG. 5. **Localizations of synaptotagmins I and III in retinal synapses.** Cryostat sections of bovine retina were probed with antibodies to synaptotagmin I (*A* and *B*, control without primary antibody) or synaptotagmin III (*C* and *D*, control with synaptotagmin III antibody and recombinant synaptotagmin III as a blocking agent). Signals were developed with biotin-labeled secondary antibodies and streptavidin ABC complexes. Synaptic layers are identified on the *left*. *OPL*, outer plexiform layer; *IPL*, inner plexiform layer. *Scale bar*, ≈50 μM.

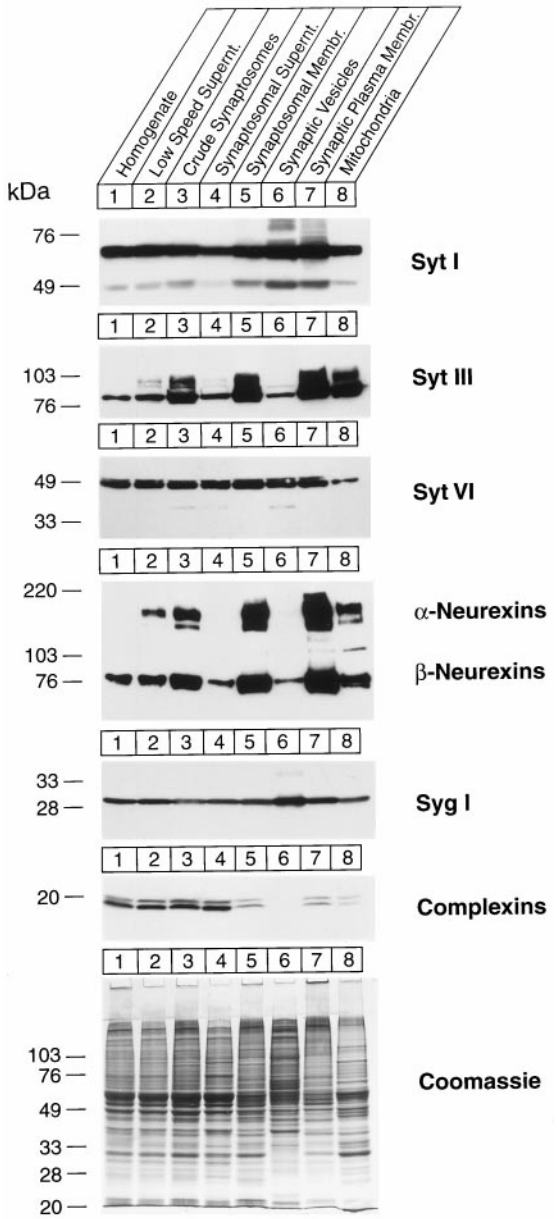

FIG. 6. **Analysis of the distributions of synaptotagmins in rat brain by subcellular fractionation.** Fractions were immunoblotted with antibodies to the proteins indicated on the *right*; the *bottom panel* exhibits a Coomassie Blue-stained SDS-gel of the same factions. Molecular masses are indicated on the *left*.

only difference between synaptotagmins I and III was that in the inner plexiform layer, synaptotagmin I was present throughout the synaptic zone, whereas synaptotagmin III was enriched in a few sharply delineated sublayers (Fig. 5). These data confirm that synaptotagmin III is highly enriched in synapses.

Because synaptotagmins I and II are known stoichiometric components of synaptic vesicles (1) and are highly homologous to synaptotagmins III and VI (3, 12), a logical presumption is that synaptotagmins III and VI are also vesicle proteins. This is supported by the finding that all synaptotagmins are co-enriched in brain, where they appear to be concentrated in the synapses, and that at least some synaptotagmin III protein can be detected in the vesicles (10, 12). However, the relative distributions of synaptotagmins between various brain fractions was never quantitatively examined. Therefore, we studied this question systematically by subcellular fractionation (Fig. 6). As expected, synaptotagmin I was enriched in two fractions: 1) free synaptic vesicles (Fig. 6, *lane 6*), and 2) synaptic plasma membranes that contain active zones with firmly docked vesicles (*lane 7*). Synaptotagmin III was also detectable in free synaptic vesicles but, surprisingly, was not enriched in the vesicles (Fig. 6, *lane 6*). Instead, it was highly concentrated in synaptic plasma membranes (*lane 7*). The pattern of synaptotagmin III closely resembled that of neurexins, which are known components of the presynaptic plasma membrane (39). Synaptotagmin VI was evenly present in all fractions and was also not particularly enriched in synaptic vesicles. The distributions of synaptogyrin I as an additional synaptic vesicle marker (32) and of complexins as a marker for the cytosol (40) were analyzed as controls in the same blots and found to be localized in the appropriate fractions (Fig. 6).

The subcellular fractionation data raised the possibility that synaptotagmin III, although present in synapses, may not actually be a synaptic vesicle protein. To test this hypothesis, we performed systematic studies on the enrichment of synaptotagmins with synaptic vesicles. We used two complementary techniques in these studies: 1) purification of synaptic vesicles by CPG[2] chromatography (Fig. 7), which is widely accepted as the method that yields the most highly purified vesicles (28, 29), and 2) immunoprecipitation of synaptic vesicles by antibodies to synaptotagmin I or synaptobrevin II (Fig. 8), which allows separation of the synaptic vesicles from similarly sized organelles (30, 31).

First, we examined the levels of various synaptotagmins by quantitative immunoblotting in four fractions obtained during the isolation of highly purified vesicles. In this purification procedure, starting homogenate (Fig. 7, *lane 1*) is used to purify

[2] The abbreviation used is: CPG, controlled pore glass.

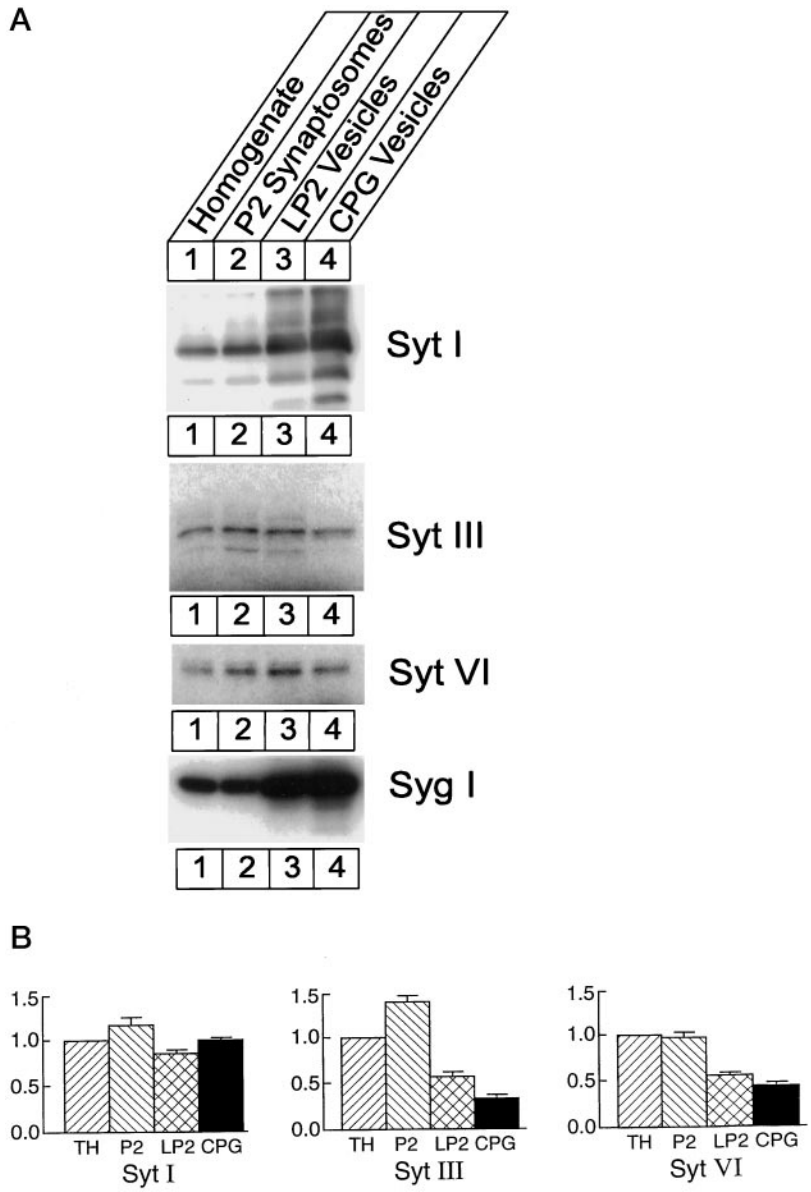

FIG. 7. **Relative levels of synaptotagmins I, III, and VI in synaptic vesicles purified by CPG chromatography.** *A*, immunoblotting analysis of the four major fractions obtained during the isolation of highly purified synaptic vesicles (*CPG Vesicles*). Signals for synaptotagmins III and VI (*Syt III* and *Syt VI*) are relatively weak because an autoradiogram of a blot reacted with ^{125}I-labeled secondary antibodies is shown that is less sensitive than ECL detection. Equivalent exposures for all synaptotagmins are depicted, leading to an overexposure of the synaptotagmin I (*Syt I*) and synaptogyrin I (*Syg I*) blots. *B*, quantitation of the relative enrichment of synaptotagmins I, III, and VI in synaptic vesicles isolated by CPG column chromatography. Blots were reacted with antibodies to either synaptotagmin I, III, or VI and to synaptogyrin I followed by ^{125}I-labeled secondary antibodies. The ratio of the synaptotagmin to synaptogyrin signal was quantified on a phosphorimager and set at 1.0 for the starting homogenate. Data shown are from three independent experiments (*TH*, total brain homogenate; *P2*, synaptosomes; *LP2*, LP2 vesicles obtained from synaptosomes; *CPG*, synaptic vesicles purified by CPG chromatography).

synaptosomes (*lane 2*) that yield free synaptic vesicles (*lane 3*; see also Fig. 6) that are further purified by CPG chromatography (*lane 4*). Coomassie Blue-stained SDS-gels revealed that the protein composition of synaptic vesicles changed dramatically upon purification of the vesicles by CPG chromatography (data not shown). We then measured the relative enrichment of various synaptotagmins by immunoblotting with ^{125}I-labeled secondary antibodies and phosphorimager detection; all blots were probed for synaptogyrin as an internal standard. Data were calculated as the signal ratios of the protein of interest to synaptogyrin I. For the purpose of normalization, the ratio observed in the starting fraction was set as 1.0. As expected, the abundance of synaptotagmin I and synaptogyrin I, two known vesicle proteins, increased in parallel during purification; thus, their ratio remained constantly close to 1.0 (Fig. 7*B*). Synaptotagmins III and VI, however, did not increase with synaptotagmin I during synaptic vesicle preparation; their ratio to synaptogyrin decreased significantly in pure synaptic vesicles (Fig. 7*B*). These results suggest that synaptotagmins III and VI are not obligatory vesicle components but de-enrich from the vesicles.

To confirm this unexpected result by an independent method, we performed experiments in which organelles were immunoisolated from rat brain homogenates with beads coated with antibodies to either synaptotagmin I or synaptobrevin II, which are known synaptic vesicle proteins. As a control, incubations were performed with beads that were coated only with glycine (30, 31) (Fig. 8*A*). The resulting fractions were again analyzed by quantitative immunoblotting, and the signals obtained for glycine beads were subtracted from the synaptotagmin I or synaptobrevin II bead signals. As in the biochemical preparations, we normalized all results for synaptogyrin analyzed on the same gels. As before, immunoisolation dramatically increased the levels of synaptotagmin I and synaptogyrin I in the samples, and their ratio remained constant. In contrast, a quantitative loss of synaptotagmins III and VI from the immunoisolated vesicles was observed; their ratio to synaptogyrin decreased significantly in the immunisolated samples. (Fig. 8*B*). The two kinds of beads coated with the different antibodies gave identical results, thereby confirming each other.

Conclusion—Synaptotagmins are a large family of homologous proteins that are enriched in brain and thought to function in Ca^{2+}-regulated membrane traffic. Although synaptotagmins I and II have known functions in synaptic vesicle exocytosis, little is known about the localizations, properties,

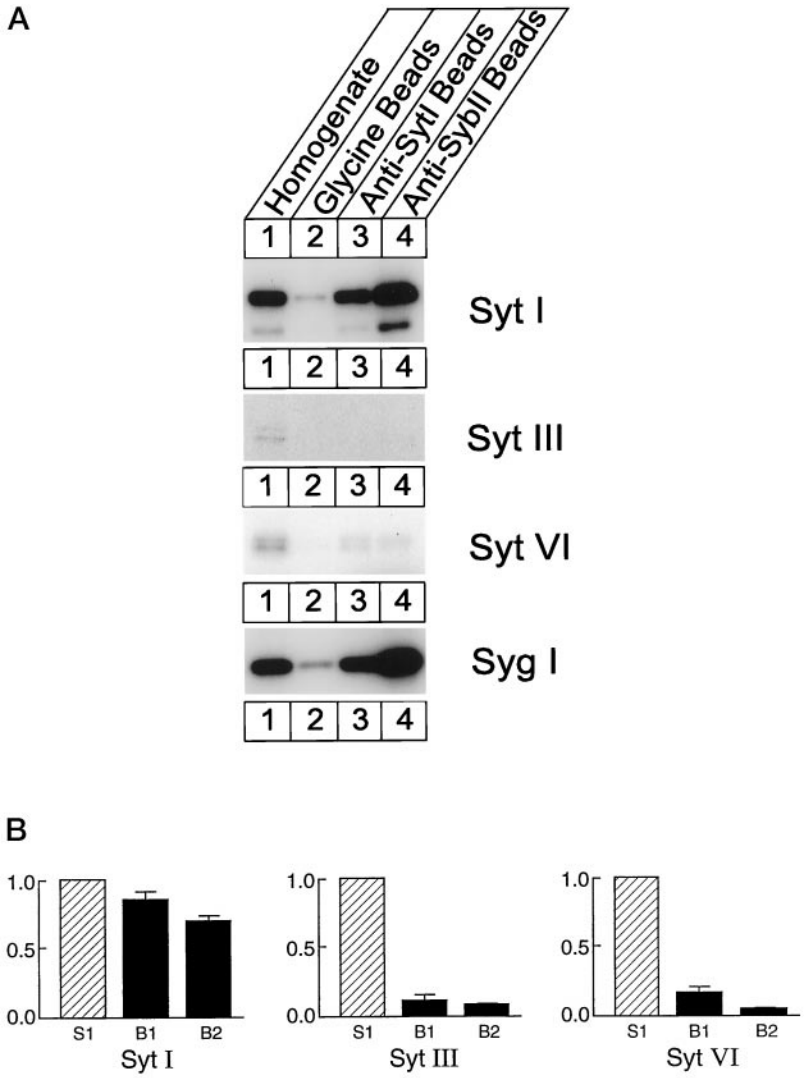

FIG. 8. **Analysis of synaptotagmins III and VI in vesicles precipitated from brain homogenates with antibodies to synaptotagmin I or synaptobrevin II.** *A*, immunoblots of the starting homogenate (postnuclear supernatant) and the pellets obtained with beads coated with glycine or with antibodies to synaptotagmin I or synaptobrevin II. Pictures shown represent equivalent exposures of blots reacted with ^{125}I-labeled secondary antibodies. *B*, ratios of synaptotagmins I, III, and VI to synaptogyrin I in immunobead pellets from multiple independent experiments. Analysis was performed as described for Fig. 7, except that the signal obtained with glycine beads was subtracted as background. Note that the enrichment value of ≈1 found for synaptotagmin I in these experiments shows that synaptotagmin I is present in synaptic vesicles in the same ratio to synaptogyrin as in brain homogenates, whereas the ratio decreases dramatically for the other synaptotagmins.

and functions of the other synaptotagmins. In the current study, we examined the localizations of synaptotagmins III and VI. Unexpectedly, we have found that synaptotagmin III, although synaptic, is not on synaptic vesicles, and that synaptotagmin VI also fails to co-purify with synaptic vesicles. Our data suggest that the functions of nerve terminals must involve other, as yet unidentified Ca^{2+}-regulated membrane trafficking pathways that involve these synaptotagmins. The high concentration of synaptotagmin III in synaptic plasma membranes raises the possibility that this isoform may, in fact, not be on an intracellular vesicle but may be localized to the plasma membrane. One possibility is that synaptotagmin III is a plasma membrane protein that could regulate the expansion of presynaptic plasma membranes as a function of activity; another possibility is that an as yet unidentified organelle co-purifies with synaptic plasma membranes and represents the true localization of this isoform. Future experiments will have to test this and other possible hypotheses about what the functions of these proteins might be in synapses that do not directly involve synaptic vesicles.

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