Domain Structure of Synaptotagmin (p65)*

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Synaptotagmin (p65) is an abundant and evolutionarily conserved protein of synaptic vesicles that contains two copies of an internal repeat homologous to the regulatory region of protein kinase C. In the current study, we have investigated the biochemical properties of synaptotagmin, demonstrating that it contains five protein domains: an intravesicular amino-terminal domain that is glycosylated but lacks a cleavable signal sequence; a single transmembrane region; a sequence separating the transmembrane region from the two protein kinase C-homologous repeats; and a conserved carboxyl-terminal sequence following the two repeats homologous to protein kinase C. The two protein kinase C-homologous repeats and a conserved carboxyl-terminal sequence following the two repeats homologous to protein kinase C. Sucrose density gradient centrifugations and gel electrophoresis indicate that synaptotagmin monomers associate into dimers and are part of a larger molecular weight complex. A sequence predicted to form an amphipathic \( \alpha \)-helix that may cause the stable dimerization of synaptotagmin is found in its third domain between the transmembrane region and the protein kinase C-homologous repeats. Synaptotagmin contains a single hypersensitive proteolytic site that is located immediately amino-terminal to the amphipathic \( \alpha \)-helix, suggesting that synaptotagmin contains a particularly exposed region as the peptide backbone emerges from the dimer. Finally, subcellular fractionation and antibody bead purification demonstrate that synaptotagmin co-purifies with synaptophysin and other synaptic vesicle markers in brain. However, in the adrenal medulla, synaptotagmin was found in both synaptophysin-containing microvesicles and in chromaffin granules that are devoid of synaptophysin, suggesting a shared role for synaptotagmin in the exocytosis of small synaptic vesicles and large dense core catecholaminergic vesicles.

Synaptotagmin (p65) is an abundant integral membrane protein of synaptic vesicles whose primary structure contains two copies of a repeat homologous to the regulatory region of protein kinase C (1, 2). In the preceding study, synaptotagmin was shown to be structurally and functionally conserved from Drosophila to humans (3). These experiments demonstrated that the protein kinase C-homologous repeats of synaptotagmin are conserved between vertebrates and invertebrates and that these repeats most likely mediate the ability of recombinant synaptotagmin from humans, rats, and Drosophila to bind to phosphatidylserine (2).

The unique structural and functional properties of synaptotagmin suggest a central role of the protein in the exocytosis of synaptic vesicles. Therefore, the elucidation of its subcellular distribution with respect to small synaptic vesicles and large dense core vesicles and the determination of the transmembrane orientation, glycosylation, and other structural features of synaptotagmin is of great importance.

In order to study the biochemical properties of synaptotagmin, we have now raised antibodies against five different epitopes of the rat protein. In addition, the 5' end of the synaptotagmin message was mapped to ensure the validity of the proposed amino terminus of synaptotagmin. Sucrose density gradient centrifugations demonstrate that synaptotagmin is part of a high molecular weight complex in the synaptic vesicle membrane that contains synaptotagmin dimers as its basic unit. Our results demonstrate that rat synaptotagmin is a glycoprotein that contains an amino-terminal intravesicular sequence which is translocated without a cleaved signal sequence. We suggest that synaptotagmin dimerizes via an amphipathic \( \alpha \)-helix that is located between the transmembrane region and the internal repeats homologous to protein kinase C. A single hypersensitive proteolytic site is found in synaptotagmin that maps to the amino-terminal end of the amphipathic \( \alpha \)-helix, compatible with the notion that the synaptotagmin peptide backbone is particularly exposed at this point because it emerges from a rigidly interacting domain. Together, these results lead to a five-domain model of the structure of synaptotagmin in which the last two domains, the carboxyl terminus and the protein kinase C-homologous repeats, are the conserved and presumably functionally active parts of the protein.

EXPERIMENTAL PROCEDURES

Materials—Reverse transcriptase was obtained from Life Sciences, and proteases and endoglycosidase F from Boehringer Mannheim. Restriction enzymes, DNA modifying enzymes, and DNA molecular weight markers were purchased from New England Biolabs. CHAPS1 and Zwittergent 3-10 were obtained from Calbiochem. RNA molecular weight markers were from Bethesda Research Laboratories, and reagents for SDS-PAGE including protein molecular weight markers and protein assay components were from Bio-Rad. Antibodies against synaptophysin, synaptobrevin, rab\( \beta \), and cytochrome b\( \alpha \) were described previously (4-7). The antibody against the 116,000 subunit of the proton pump was raised against a synthetic peptide corresponding to the carboxy-terminal 18 amino acids of the subunit. Peroxidase-labeled secondary antibodies were obtained from Cappel. Eupergit C12M methacrylate Microbeads were purchased from Röhm Pharma.

Production of Synaptotagmin Antibodies—Three antibodies were made to

1The abbreviations used are: CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2T. C. Sudhof and M. S. Perin, unpublished observation.
raised against synthetic peptides coupled to keyhole limpet hemocyanin as described (4). The synthetic peptides had the following sequences (residue numbers as in (2), the topography of synaptotagmin, the synthetic peptides for identifying the protein domains: CMVSASH (residues 1 to 11, amino terminus), CAINMKDVKDLGKTMKDQALKD (residues 100 to 120, between transmembrane region and A repeat), and CMDVGGLSDPVVKHL (residues 302 to 316, B repeat). The amino-terminal cysteine in each peptide is not present in the protein sequence and was introduced to allow efficient coupling. In addition to these three antibodies, the antibodies were raised against two bacterial recombinant proteins incorporating sequences comprised of residues 78 to 421 (complete sequence carboxy-terminal to the transmembrane region) or residues 265 to 381 (B repeat). The production of the recombinant proteins was as described previously (2, 3, 8), and they were used for immunization after partial purification of inclusion bodies containing recombinant protein.

**Subcellular Fractionation and Immunoblot Purification—** Purification of rat brain cortex synaptic vesicles with controlled pore-glass chromatography as the final step was performed as described previously (9, 10). For most biochemical experiments, vesicles of lesser purity obtained before the controlled pore-glass chromatography were used. Subfractionation of bovine adrenal medulla via centrifugation through a 1.6 M sucrose step gradient for the purification of chro- maffin granules was performed as described (11). Immunobead purification of synaptophysin-containing vesicles was performed as described (8). Bovine synaptotagmin-containing microsomes were coated with a purified monoclonal antibody against synaptophysin (12).

**Protease and Endoglycosidase F Digestion of Synaptotagmin—** Synaptic vesicles (75 µg of protein) were digested with different concentrations of pronase or trypsin in 0.15 M NaCl, 20 mM HEPES-NaOH, pH 7.4, for 30 min at 37°C. Reactions were stopped by boiling in electrophoresis sample buffer (62 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS). Endoglycosidase F digestions were performed in 0.13 M NaCl, 20 mM HEPES-NaOH, pH 7.4, 1% CHAPS, 10 mM 2-mercaptoethanol, and 0.2 unit of endoglycosidase F (13). In the experiments in which synaptic vesicles were subjected both to endoglycosidase F and pronase digestion, synaptic vesicles were first digested with trypsin as described above. The reaction was stopped with 1 mg/ml N-tert-l-lysyl chloromethyl ketone, and CHAPS and 2-mercaptoethanol were added to 1% and 10 mM, respectively. 0.2 unit of endoglycosidase F was added, and the mixture was incubated overnight at 27°C.

**SDS-PAGE and immunoblotting** were performed as described (4, 7, 14, 15). Proteins reactive with antibodies were visualized with peroxidase-labeled secondary antibodies. Protein assays were performed according to Bradford (16).

**Sucrose Gradient Centrifugations—** Synaptic vesicles (1.5 mg of protein) were solubilized in 0.1 M NaCl, 50 mM HEPES-NaOH, pH 7.4, 1.8% CHAPS, or Zwittergent 3-10 by stirring for 1 h on ice, and insoluble residue was removed by centrifugation (150,000×g for 30 min). The supernatant was loaded on 5-20% linear sucrose gradients containing 0.9% CHAPS or Zwittergent 3-10 and 50 mM HEPES-NaOH, pH 7.4, and centrifuged for 16 h at 210,000×g. Gradient fractions were analyzed by immunoblotting for synaptophysin, synaptobrevin, synaptotagmin, and the M₆, 116,000 kDa protein pump subunit. In separate, identically performed sucrose gradients, bovine serum albumin (M₆, 65,000), alcohol dehydrogenase (M₆, 150,000), catalase (M₆, 250,000), and β-galactosidase (M₆, 520,000) were fractionated as molecular weight standards.

**Purification of synaptotagmin in the bovine adrenal medulla using Drosophila synaptotagmin (3).**

**Subcellular Localization of Synaptotagmin in Rat Brain and Bovine Adrenal Medulla—** Using these antibodies, we examined whether synaptotagmin is highly enriched in synaptic vesicles or if it is shared with other organelles. For this purpose, rat brain homogenates were depleted of mitochondria and nuclei by low speed centrifugation (25,000×g, for 20 min). Synaptic vesicles were immunoprecipitated from the resulting supernatant using beads coated with a monoclonal antibody against synaptophysin, a well characterized synaptic vesicle-specific protein in brain (4, 20, 21). Synaptotagmin was quantitatively co-immunoprecipitated with synaptophysin from the low speed supernatant by these antibody beads (Fig. 2). No synaptotagmin remained in the supernatant after synaptophysin-containing membranes had been removed, suggesting that in brain little synaptotagmin is localized in membranes other than synaptic vesicles.

In addition to antibody bead precipitation, the distribution of synaptotagmin on synaptic vesicles during the classical synaptic vesicle purification scheme with controlled pore-glass chromatography as the last step (9, 10) was studied and compared with that of synaptophysin, synaptobrevin (5), and rab3 (6). Again we observed complete co-purification of all of these synaptic vesicle markers with synaptotagmin (data not shown). These results suggest that, like synaptophysin, synaptobrevin, and rab3, synaptotagmin is highly enriched on synaptic vesicles in brain.

Several proteins such as synaptophysin and rab3 are exclusively localized to synaptic vesicles in brain but are also expressed in the adrenal medulla (6, 21, 22). Here, synaptophysin, synaptobrevin, and rab3 are found on synaptic-like microvesicles that are distinct from the secretory granules in these cells (6, 22). We therefore studied the subcellular localization of synaptotagmin in the bovine adrenal medulla using centrifugation and antibody bead precipitation. Total med-
The structure of synaptotagmin is shown on top with the transmembrane region (TMR) shown in black and the two repeats labeled A and B in white. The structure of protein kinase C, is shown below with the two regulatory domains (C₁ and C₂), and the two catalytic domains (C₃ and C₄) are identified separately. The degree of identity between the A and B repeats (44%) and between the A or B repeat and the C₂-domain of protein kinase C (39% each) is indicated.

**FIG. 1.** Diagram of the structure of rat synaptotagmin and its relation to protein kinase C. **Co-purification of synaptotagmin (p65) and synaptophysin during antibody bead purification of synaptic vesicles from rat brain.** Mitochondria and nuclei were removed from homogenates of rat brain cortex by centrifugation, and the supernatant (left lane, 10 μg of protein) was extracted with antibody beads (immunobeads) coated with a monoclonal antibody against synaptophysin. Note that the antibody beads (right lane, <2 μg of protein) quantitatively remove synaptotagmin together with synaptophysin, a known synaptic vesicle-specific protein in brain cortex (20, 21) but precipitate less than 5% of the total protein, indicating a complete co-localization of these two proteins in rat brain cortex. The middle lane shows the supernatant from the antibody bead precipitation that has been depleted of synaptic vesicles by the antibody bead precipitation (10 μg of protein).

**FIG. 2.** Co-purification of synaptotagmin (p65) and synaptophysin during antibody bead purification of synaptic vesicles from rat brain. Mitochondria and nuclei were removed from homogenates of rat brain cortex by centrifugation, and the supernatant (left lane, 10 μg of protein) was extracted with antibody beads (immunobeads) coated with a monoclonal antibody against synaptophysin. Note that the antibody beads (right lane, <2 μg of protein) quantitatively remove synaptotagmin together with synaptophysin, a known synaptic vesicle-specific protein in brain cortex (20, 21) but precipitate less than 5% of the total protein, indicating a complete co-localization of these two proteins in rat brain cortex. The middle lane shows the supernatant from the antibody bead precipitation that has been depleted of synaptic vesicles by the antibody bead precipitation (10 μg of protein).

**FIG. 3.** Differential distribution of synaptotagmin (p65) and synaptophysin in bovine adrenal medulla. Adrenal medullary homogenate (left lane, 10 μg of protein) was subfractionated by low speed centrifugation into a supernatant retaining the synaptophysin-containing microvesicles (second lane, 10 μg of protein) and a crude chromaffin granule pellet from which purified chromaffin granules were obtained by sucrose gradient centrifugation (right lane, 10 μg of protein). As shown, chromaffin granules contain virtually no synaptophysin, but the majority of the adrenal medullary synaptotagmin and cytochrome b₅₆₁. Synaptophysin is retained in the low speed supernatant together with some of the synaptotagmin and cytochrome b₅₆₁. Purification of the microvesicles containing synaptophysin from this supernatant by antibody bead (immunobeads) oligonucleotide (fourth lane, <2 μg of protein) results in a co-purification of synaptotagmin and cytochrome b₅₆₁ (none of which remains in the supernatant, third lane, 20 μg of protein), suggesting that the synaptotagmin and cytochrome b₅₆₁ that are not on chromaffin granules are on the synaptophysin-containing microvesicles.

**Glycosylation of Synaptotagmin and Transmembrane Ori-
when synaptic vesicles were subjected to endoglycosidase F digestion after proteolysis, only the smaller amino-terminal then only the cytoplasmic sequences were affected (4). Numbers on the right indicate positions of molecular weight markers. Both the lower and upper immunoreactive band of $M_r = 65,000$ and 130,000 are sensitive to endoglycosidase F and both are recognized by five different antibodies to synaptotagmin (data not shown), suggesting that they represent synaptotagmin monomers and dimers.

equally cleaved by these two proteases despite their different substrate specificities (Fig. 5). Using the antibody directed against the carboxyl-terminal domain of synaptotagmin, the same proteolytic product of approximately $M_r = 39,000$ was detected over a 30-fold concentration range of both proteases.

The proteolytic fragment produced by mild trypsin or pronase treatment of synaptotagmin was soluble, whereas intact synaptotagmin was quantitatively pelleted with synaptic vesicles (data not shown). Higher protease concentrations led to a complete digestion of the carboxyl-terminal synaptotagmin epitope in intact synaptic vesicles. When proteolysis of synaptophysin was investigated under similar conditions, it was left intact except at the highest protease concentrations, and then only the cytoplasmic sequences were affected (4).

The release of a soluble carboxyl-terminal synaptotagmin fragment from intact synaptic vesicles after mild proteolysis suggests that the carboxyl-terminal sequences of synaptotagmin containing the homologies to protein kinase C are indeed cytoplasmic, and that a membrane-bound amino-terminal fragment should remain. To test this hypothesis, untreated and mildly proteolyzed synaptic vesicles were analyzed by immunoblotting using both the antibody against the carboxyl-terminal $C_2$-domains and an antibody raised against the 11 amino-terminal residues of synaptotagmin. As shown in Fig. 6, both antibodies recognized the same protein in intact synaptic vesicles. However, after partial proteolysis, the amino-terminal antipeptide antibody now labeled an $M_r = 28,000$ band as opposed to the $M_r = 39,000$ fragment detected by the antibody against the cytoplasmic epitope. Furthermore, when synaptic vesicles were subjected to endoglycosidase F digestion after proteolysis, only the smaller amino-terminal fragment changed in electrophoretic mobility (Fig. 6). These results suggest that synaptotagmin contains a small intravesicular amino-terminal and large cytoplasmic carboxyl-terminal sequence. The amino-terminal fragment is N-glycosylated; inspection of the amino terminus of the synaptotagmin sequence reveals the presence of a single N-linked glycosylation site at residue 24 (2).

To localize the hypersensitive proteolytic site in the synaptotagmin sequence, we studied which of the four antibodies that recognize epitopes carboxyl-terminal to the transmembrane region reacted with the cytoplasmic proteolytic fragment of synaptotagmin. In addition, its size was compared to that of recombinant synaptotagmin containing all sequences carboxyl-terminal to the transmembrane region (Fig. 7). The proteolytic fragment was found to be slightly smaller than the recombinant fragment whose amino terminus corresponds to residue 78 and to react with all antibodies against cytoplasmic sequences of synaptotagmin. The weakest response was obtained with the antibody against a synthetic peptide corresponding to residues 100 to 120, suggesting that the proteolysis affects this epitope. These results indicate that the hypo-

![Fig. 4. Endoglycosidase F digestion of synaptotagmin.](image)

![Fig. 5. Presence of a hypersensitive proteolytic site in synaptotagmin.](image)

![Fig. 6. Analysis of the endoglycosidase F sensitivity of the amino- and carboxyl-terminal proteolytic fragments of synaptotagmin.](image)

![Fig. 7. Mapping of the hypersensitive proteolytic site in synaptotagmin by an analysis of the size and immunoreactivity of its carboxyl-terminal proteolytic fragment.](image)
Mapping of the 5’ End of the Synaptotagmin Message—The discrepancy between the apparent molecular weight of synaptotagmin as determined by SDS-PAGE and its predicted size cannot be entirely accounted for by N-linked glycosylation since endoglycosidase F treatment of synaptotagmin causes only a small molecular weight shift (Figs. 4 and 6). There are several plausible explanations for the apparent high molecular weight of synaptotagmin. It may migrate at a higher molecular weight on SDS-PAGE because it contains additional modifications such as O-linked sugars, or it may have unusual secondary structures, or the predicted amino terminus of synaptotagmin maybe incorrect due to cloning artifacts. We therefore performed a series of experiments to rule out the possibility of cloning artifacts.

The finding that an antibody against the 11 amino-terminal residues of synaptotagmin reacts with the protein in synaptic vesicles demonstrates that these 11 amino acids are present in the mature protein. This suggests that a possible cloning artifact must be localized to the 5’ untranslated region. To test whether the 5’ untranslated region found by cDNA cloning is correct, blots of poly(A+)-enriched RNA from rat brain were hybridized with oligonucleotides from the 5’ untranslated region and 100 of synaptotagmin. (data not shown), suggesting that the $M_0 = 130,000$ band represents a synaptotagmin dimer that is partially resistant to SDS denaturation. To further investigate the possible association of synaptotagmin into dimers and high molecular weight complexes, synaptic vesicle proteins solubilized in CHAPS or in Zwittergent 3-10 were fractionated by sucrose density gradient centrifugation. The sucrose gradient fractions were then analyzed for synaptotagmin, synaptophysin, and the 116,000 subunit of the synaptic vesicle proton pump by immunoblotting (Fig. 10).

On sucrose density gradients, synaptotagmin migrates in a single peak as a high molecular weight complex of approximately $M_0 = 220,000$ in the presence of CHAPS, a nondenaturing detergent. Its molecular weight is higher than that of synaptophysin, which migrates as a broad peak comprised of dimers, trimers, and tetramers (23), and smaller than the proton pump complex (approximately $M_0 = 530,000$) (24). In Zwittergent 3-10, the proton pump is dissociated into its subunits and synaptotagmin co-migrates with the 116,000 subunit of the proton pump on the sucrose gradients. This suggests that Zwittergent 3-10 also partially dissociates the synaptotagmin complex but leaves synaptotagmin dimers intact (Fig. 10).

What is the mechanism of synaptotagmin dimerization? Analysis of the primary structure of synaptotagmin indicates the presence of a sequence that has a high potential of forming an amphipathic α-helix (shown in an α-helical wheel presentation in Fig. 11). Parallel strands of such an α-helix would not only be held together by their hydrophobic surface but would also form oppositely charged amino acid pairs along the helix, suggesting a mechanism by which stable dimers could be formed.

DISCUSSION

Synaptotagmin is an abundant synaptic vesicle membrane protein that contains two copies of an internal repeat homologous to protein kinase C (2). Synaptotagmin is highly conserved evolutionarily from Drosophila to humans, and it ap-

FIG. 8. RNA blot analysis of the synaptotagmin message in rat brain using an oligonucleotide complementary to the 5’ untranslated region (lane A) and to the coding region of the message predicted from the cDNA sequence (lane B). Oligonucleotides are the same as in Fig. 9. The numbers on the right indicate positions of molecular weight standards.

FIG. 9. Primer extension analysis of the 5’ end of the rat brain synaptotagmin message using three different oligonucleotides. The positions of the three oligonucleotides complementary to different parts of the cDNA are diagrammed at the bottom. On top, the primer extension products obtained with the three different oligonucleotides were resolved on three gels containing 4.5% (A), 10% (B), and 10% (C) acrylamide. Positions of co-electrophoresed radiolabeled molecular weight markers (MspI-cut-pBR322) are shown on the right of each extension product, and the size of each product is given on the left. The size of the longest 920-base pair (bp) product obtained with oligonucleotide A was determined by larger molecular weight markers (HaeIII-cut φX174 RF DNA) in addition to those shown here.
The primary structure of rat synaptotagmin contains a sequence with a strong potential of forming an amphipathic \( \alpha \)-helix. This sequence could dimerize synaptotagmin via its hydrophobic face and form salt bridges between the two parallel helices (Fig. 11). Interestingly, the hypersensitive proteolytic site of synaptotagmin was mapped to the amino-terminal end of this amphipathic \( \alpha \)-helix, compatible with the notion that the protein is particularly exposed to proteolytic attack at the point where its peptide backbone emerges from a leucine zipper-like dimer. 

These biochemical data together with a sequence compari-
This page contains a segment of text discussing synaptotagmin. It mentions the structure and function of synaptotagmin, including its domains, amino acids, and its presence in various types of vesicles. The text also references several studies and researchers to support the findings. The references at the end of the page list various scientific works that provide additional information on synaptotagmin's structure and function.

**Structure of Synaptotagmin**

- Son between different species lead to a five-domain model of synaptotagmin. The first domain consists of the intravesicular glycosylated 52 amino acids, the second domain of the transmembrane region, and the third domain is formed by the dimerizing amphipathic α-helix. The internal repeats homologous to protein kinase C constitute the fourth domain, and is found both on chromaffin granules and on synaptic-like microvesicles, whereas synaptophysin is exclusively localized to the latter (Figs. 2 and 3).

Several studies on the distribution of synaptophysin in the adrenal medulla have been reported with originally conflicting results. However, more recently, a consensus seems to have evolved that most if not all synaptophysin in chromaffin cells is not on the chromaffin granules but on unidentified “synaptic-like microvesicles” in agreement with our results (e.g. see Refs. 6 and 31). Two studies have also previously investigated the distribution of both synaptophysin and synaptotagmin (p65) in the adrenal medulla. In both studies, synaptotagmin was found to be on chromaffin granules in agreement with our findings, although synaptophysin in both studies was found to have a more general distribution different from what is now recognized to be its restricted presence in synaptic-like microvesicles. The presence of synaptotagmin on chromaffin granules, which are similar to large dense core vesicles in brain, raises the possibility that this particular synaptic vesicle protein may have a general function as a docking or fusion protein in regulated secretion by neurons.

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