The COOH Terminus of Synaptotagmin Mediates Interaction with the Neurexins*

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The interaction of the synaptic vesicle protein, synaptotagmin, and the presynaptic α-latrotoxin receptor, a neurexin, has been proposed to be involved in docking of synaptic vesicles at active sites or modulation of neurotransmitter release. Here I report the investigation of the domain of synaptotagmin responsible for this interaction. Pieces of synaptotagmin containing the carboxyl terminus are capable of purifying neurexins from solubilized brain homogenates. Pieces as small as a synthetic peptide corresponding to the COOH-terminal 34 amino acids are capable of enriching neurexins 100-fold. The binding of neurexins to synaptotagmin is calcium-independent and of moderate affinity. This COOH-terminal segment of synaptotagmin is conserved in all species characterized to date. Reflective of this, a synthetic peptide corresponding to the carboxyl terminus of Droso phila synaptotagmin is capable of purification of rat neurexins, suggesting the possibility that this interaction may also exist in Drosophila. I propose that the carboxyl terminus of synaptotagmin binds to the carboxyl terminus of the neurexins and that this interaction may mediate docking of synaptic vesicles or modulation of neurotransmitter release.

Synaptotagmin has been hypothesized to be involved in calcium-dependent exocytosis of synaptic vesicles at the synapse (1). This was first based on molecular characterization of synaptotagmin showing that the cytoplasmically exposed part of the protein contained two repeats with homology to a domain involved with calcium-dependent membrane interaction. This domain was first identified in protein kinase C (2), but has subsequently been found in an arachidonic acid-specific phospholipase A2, a phospholipase C, and GTPase-activating protein (GAP) (3). Synaptotagmin has been shown to bind calcium in the presence of acidic phospholipids with an affinity in the range of 10−50 μM (4), a concentration range hypothesized to be involved in calcium-dependent exocytosis of synaptic vesicles at synapses (5). Recently, genetic analysis of synaptotagmin in Drosophila and Caenorhabditis elegans has shown that neurotransmission is dramatically altered in the absence of synaptotagmin (6–9). Electrophysiology of partial lack of function mutants point towards a direct role of synaptotagmin in calcium activation of exocytosis of synaptic vesicles (7).

Additional evidence on the involvement of synaptotagmin in neurotransmitter release has centered on the co-purification of synaptotagmin and a presynaptic receptor for the black widow spider venom toxin α-latrotoxin. Purification of this receptor on a column of immobilized latrotoxin yields two proteins of 200 and 160 kDa. Partial amino acid sequences from these proteins led to the molecular cloning of a family of proteins that have been named the neurexins (10). Also eluting off this column is synaptotagmin (11). This co-purification suggests that synaptotagmin, a synaptic vesicle protein, and the α-latrotoxin receptor/neurexins, a presynaptic membrane protein, interact. This potentially has additional significance because α-latrotoxin causes massive exocytosis of synaptic vesicles even in the absence of external calcium. This raises the possibility that the interaction of synaptotagmin and the α-latrotoxin receptor may mediate synaptic vesicle docking or mediate modulation of neurotransmitter release.

The neurexin family is complex due to multiple genes with extensive alternative splicing (10, 12). To date it has not been determined which forms of the neurexins represent receptors for α-latrotoxin. All transmembrane forms of the neurexins have extensive homology in their short carboxyl-terminal cytoplasmic domain. This domain of the neurexins has been shown to be capable of mediating binding to synaptotagmin (13).

We have investigated the domain of synaptotagmin responsible for binding neurexins based on evolutionary conservation of synaptotagmin between greatly separated species. Synaptotagmin has now been cloned from species as evolutionarily distant as fruit flies and man (14–16). A comparison between these species is revealing. Overall these proteins are 55% identical but this conservation is not evenly distributed. Sequence identity is restricted to the C2 repeats (79% identity) and the COOH-terminal 43 amino acids (65% identity). This pattern of conservation suggests that these domains are functional modules. The C2 domains, based on their homology to a similar domain in other proteins, have been suggested to be involved in calcium-dependent membrane interactions, potentially fusion (1). In this paper, we show that the COOH terminus is sufficient for substantial purification of neurexins from solubilized brain membranes. This implicates this domain in binding neurexins, again suggesting a potential role for synaptotagmin-neurexin binding in docking or modulation of neurotransmitter release.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA modifying enzymes were from New England Biolabs. Peptides were chemically synthesized. All other chemicals were of reagent-grade and used without further purification.

Antibodies—Three neurexin antibodies were raised against synthetic peptides coupled to keyhole limpet hemocyanin as described (17) or synthesized directly as a polymeric resin (18). The synthetic peptides had the following sequences: LR1, CSANKKKNNKKEYV residues 1493–1507; LR2 YRNDEGSYHVDES residues 1455–1468; LR3, CGLFPGAEQWTRFPKW residues 30–46 (residue numbers as in neurexin 1a (16)); LR4 was kindly provided by Dr. Martin Geppert and Dr. Thomas Sudhof, and was raised to a recombinant bacterial portion of the cytoplasmic domain of neurexin 1a. The amino-terminal cysteine in each peptide is not present in the protein sequence and was introduced to allow efficient coupling. Synaptotagmin 1 antibodies were as previously described (19). Monoclonal antibody that recognizes synaptotagmin A and B was kindly provided by Dr. Colin Barnstable.
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**Fig. 1. Pieces of synaptotagmin containing the COOH-terminal domain enrich neuraxins.** Chromatography was as described under "Experimental Procedures." 100 μg of solubilized brain membranes (SM), 100 μg of flow through (PT), and 50 μg of eluate 1 (E1) and 60 μg (E2) from chromatographies using affinity columns of recombinant pieces of synaptotagmin or synthesized peptides (b) were run on 7% SDS-PAGE gels, transferred to nitrocellulose, and blotted with LR1 antibody (a) or run on 10% gels and blotted with synaptotagmin antibody (c). Latrotoxin receptor/neurexin (LR) control (2 μg) was purified on a microcolumn of α-latrotoxin as described by Petrenko et al. (23). STD, molecular weight standards.

**Bacterial Expression—**Bacterial expression of cytoplasmic sequences of synaptotagmin in the pET vector has been described previously (1, 14). Briefly sequences corresponding to the appropriate protein region were amplified using the polymerase chain reaction with synthetic oligonucleotides containing restriction sites to insert each fragment into an expression vector. Polymerase chain reactions were performed as described (14, 23). Briefly sequences corresponding to the appropriate protein region were amplified using the polymerase chain reaction with synthetic oligonucleotides containing restriction sites to insert each fragment into an expression vector. Peptides were coupled to activated CH-Sepharose (Pharmacia LKB Biotechnology Inc.). After coupling, SDS was removed by extensively washing the column with 0.2% Triton X-100. Soluble proteins (PET 5 and PET 6) were directly coupled to activated CH-Sepharose. Peptides were coupled to thiopropyl-Sepharose using their introduced amino-terminal cysteine. Triton X-100-solubilized rat brain membranes were used for enrichment of neuraxins via chromatography on small recombinant pieces of synaptotagmin. Synaptotagmin has at least 6 identifiable domains based on structural considerations or sequence conservation between evolutionarily distant species. These include a short amino-terminal intravesicular sequence, a single membrane spanning sequence, a linker domain, two C2 repeats, and a short carboxy-terminal sequence. Recombinant pieces of synaptotagmin were constructed to include most combinations of these domains, excluding the luminal amino terminus and the membrane spanning sequence (Fig. 1b). Our ideas directing this search were influenced by the conservation of synaptotagmin between evolutionarily distant species. When comparing synaptotagmin sequences between species as different as man and...
fruit fly (14), or different synaptotagmin genes within a species (15, 24), three domains of the protein have high percentages of sequence identity. These are the two C2 domains and the carboxyl-terminal sequence that follows the second C2 domain. DNA coding for these pieces was generated by subcloning or by polymerase chain reaction using oligos containing convenient restriction sites. This DNA was ligated in-frame into PET vectors (21) transformed in bacteria, and recombinant protein was produced after induction with IPTG as has been previously described (1, 14). Recombinant proteins were purified using standard chromatographies (PET 5 and PET 6) or purified by preparative PAGE on a Bio-Rad Prep Cell (PET 1, PET 4, and PET 8). In addition, two peptides, corresponding to part of the linking domain (residues 100-120, rat sequence) and to the last 34 carboxyl-terminal residues of synaptotagmin (residues 387-421, rat sequence), were chemically synthesized.

The recombinant pieces of synaptotagmin and the two peptides were coupled to separate columns and tested for the ability to purify neurexins from solubilized rat brain membranes (Fig. 1a). I followed a similar chromatography as was used for the purification of the latrotoxin receptor on immobilized latrotoxin columns (23). As has been reported (23), neurexins are greatly enriched via chromatography on columns of recombinant protein corresponding to the cytoplasmic sequence of synaptotagmin (PET 1, Fig. 1a). I specifically followed the purification of the neurexins using antibodies raised to the carboxy-terminal 15 amino acids of neurexin Ia. This domain is highly conserved between neurexins I, II, and III (three to four changes in 15 amino acids; Refs. 10 and 12). Immunoreactivity to neurexins is barely detectable in brain membranes when assayed by Western blotting (Fig. 1a). After chromatography, neurexin immunoreactivity is easily detectable. This enrichment represents at least a 50-fold increase in neurexin immunoreactivity as measured by dilution of the elute necessary to get a comparable staining to starting membranes (data not shown). Using this approach, I investigated whether smaller pieces of synaptotagmin could yield similar purification of the neurexins. The bacterially produced recombinant proteins that were tested are diagrammed in Fig. 1b. Peptides tested included a 34-amino acid COOH-terminal peptide (p65–9) and a 21-amino acid peptide in the linking domain (p65–6). As shown in Fig. 1a, PET 1, PET 4, PET 8, and the COOH-terminal peptide support enrichment of neurexins. PET 6, PET 5, and the linking peptide do not enrich neurexins. In addition, uncoupled activated CH-Sepharose, thiophyll-Sepharose, or

these matrices coupled to bovine serum albumin or 2-mercaptoethanol yield no enrichment of neurexins (data not shown). All synaptotagmin pieces containing the COOH-terminal sequence were capable of enriching neurexins; all synaptotagmin pieces that did not contain the COOH-terminal sequence were not capable of neurexin enrichment. The result that the COOH-terminal 34-amino acid peptide was sufficient for substantial enrichment of neurexins is particularly dramatic. The p65–9 column consistently gave the highest enrichment of the neurexins, usually to a 4-fold higher level that the next best column, PET 1. A concern of this chromatography approach is that synaptotagmin exists in the synaptic vesicle as a tetramer (19). Because of this, we wanted to be able to exclude the possibility that this enrichment of neurexins is mediated by prior enrichment of native synaptotagmin on these columns. To test this, we assayed for native synaptotagmin in the starting and elution fractions from chromatographies over each column. This is shown in Fig. 1c for chromatographies from the COOH-terminal synaptotagmin peptide (p65–9) and PET 6 (linking domain and first C2 domain) columns. For both columns (and the other tested columns including controls), native synaptotagmin is carried through the chromatography. Importantly, enrichment of synaptotagmin in eluates is not greater in a column (p65–9) that enrichs neurexins compared to one (PET 6) that does not.

Additional evidence that the COOH terminus of synaptotagmin is functionally important is its conservation between evolutionarily distant species. This is shown in Fig. 2 where the COOH-terminal sequences (approximately last 40 amino acids) of cloned synaptotagmins (1, 14–16) and a synaptotagmin-like protein rabphilin (25) are aligned. Residues for the first and last amino acid of each COOH-terminal sequence are listed on the left and right of each sequence. Sequences are as in Refs. 1, 8, 14–16, and 25.
neurexin immunoreactivity, we tested whether the reactivity of this band was blocked by preincubation with the COOH-terminal neurexin peptide. As shown in Fig. 3, peptide preincubation completely blocks the reaction of this antibody with this 200-kDa band. In addition, we tested three other antibodies raised against either peptides (LR2 residues 1456–1469, LR4 residues 30–46, of neurexin 1α) or recombinant protein (LR4) of the neurexins. All recognized the same 200-kDa band. A 180-kDa latrotoxin receptor/neurexin purified from α-latrotoxin columns was not apparent in our elutions when brain membranes were chromatographed over these columns in 100 mM KCl. In contrast, when solubilized brain membranes were chromatographed over the same columns in 200 mM KCl, both 160- and 200-kDa bands were present in the elutions, although at less than one-fifth the amount of the 200-kDa band with chromatography at 100 mM KCl (data not shown).

Neurexins are not eluted from the COOH-terminal synaptotagmin peptide column by removal of calcium. Similarly, enrichment of neurexins on synaptotagmin columns occurs in the absence of calcium in loading and washing buffers (data not shown), suggesting that the interaction of synaptotagmin and neurexins does not require calcium. In addition, we investigated the conditions for elution of neurexins from the COOH-terminal peptide column. Solubilized rat brain membranes were chromatographed over the COOH-terminal peptide column in 100 mM KCl, 20 mM HEPES, 1 mM calcium chloride. The column was extensively washed with the same buffer. The column was then eluted with increasing concentrations of KCl in 50 mM steps in the presence of 10 mM EDTA. As shown in Fig. 4, Neurexins elute from the COOH-terminal peptide column from 150 to 400 mM KCl. During this elution, I also assayed for enrichment of synaptotagmin and the presynaptic protein, HPC1/syntaxin. I investigated the potential purification of HPC1/syntaxin because it has been reported to co-immunoprecipitate with synaptotagmin (26–29). HPC1/syntaxin is present in eluate fractions but it is not enriched by chromatography on the COOH-terminal peptide column. This is shown in Fig. 4b where eluate fractions have been loaded at twice the protein concentration of the starting solubilized membranes. Under these conditions, HPC1/syntaxin immunoreactivity is barely visible. This suggests that synaptotagmin does not bind to HPC1/syntaxin via synaptotagmin's COOH-terminal sequence.

The conservation of the COOH-terminal sequence of synaptotagmin between evolutionarily distant species suggests that the interaction between synaptotagmin and neurexins may also be conserved between species. Because neurexins have as yet only been identified in mammals, we investigated whether the COOH-terminal peptide from divergent species could be sufficient to enrich mammalian neurexins. As an initial test of this, we assayed whether mouse neurexins could be purified on rat COOH-terminal synaptotagmin peptide. As shown in Fig. 5, a 200-kDa mouse protein, immunoreactive to rat neurexin antibodies, is enriched by chromatography on a column of the rat COOH-terminal synaptotagmin peptide. For a further test of the evolutionary conservation of synaptotagmin/neurexin binding, we synthesized a peptide to the COOH terminus of Drosophila synaptotagmin (residues 451–474, Ref. 14). When coupled to a gel matrix, it also is sufficient for enriching rat neurexin. Chromatography of solubilized Drosophila head membranes over this column resulted in a prominent enrichment of a 200-kDa Drosophila protein (data not shown). We are currently investigating whether this Drosophila protein may be a neurexin.

**DISCUSSION**

The potential for an interaction between synaptotagmin and the latrotoxin receptor/neurexins was first noted because of the co-purification of synaptotagmin, a synaptic vesicle protein, during chromatography for the latrotoxin receptor, a presynaptic membrane protein. This suggested that the latrotoxin receptor and potentially many of the neurexins may be presynaptic membrane receptors for synaptotagmin. We have investigated the localization of sequence elements of synaptotagmin 1 that mediate its interaction with the latrotoxin receptor/neurexins by assaying enrichment of neurexins from solubilized membranes by chromatography over columns of smaller pieces of synaptotagmin. This approach has identified the extensive (50–100-fold) enrichment of neurexins on a 34-amino acid peptide corresponding to the COOH-terminal sequence of synaptotagmin 1 and on all recombinant pieces that contain this element. The position of an interaction element at the end of the protein is consistent with the structural and functional organization of synaptotagmin. Synaptotagmin has four domains in the cytoplasmically exposed part of the protein. These are the linking domain, the first and second C2 repeat,
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and the carboxyl-terminal sequence. Proteolysis of synaptotagmin reveals that trypsin or Pronase only cuts in the linking domain, even though synaptotagmin has many lysine residues (19, 30). This suggests that the two C2 domains and carboxyl-terminal sequence form a tight structure. Given that the two C2 repeats have been shown to bind acidic phospholipids (1, 14) and be involved in calcium-dependent membrane association (4), the carboxyl-terminal sequence would be ideally situated to orient or modulate the COOH-terminal sequence. Potentially reflecting this, and be involved in calcium-dependent membrane association C2 repeats have been shown to bind acidic phospholipids (1,14) (19,30). This suggests that the two C2 domains and carboxyl-domain, even though synaptotagmin has many lysine residues (13), in complementary work, have shown that a small carboxyl-terminal peptide can enrich mammalian neurexins to a synaptotagmin column and the enrichment of synaptotagmin COOH terminus. Solubilized rat or mouse brain membranes were chromatographed on p65-9 columns or rat brain membranes were chromatographed on D65-3 column. 50 μg of eluate (E2) was run on 7% gels, transferred to nitrocellulose, and blotted with LR1 antibody.

There is emerging evidence from study of other secretory pathways and analysis of the targets of proteolysis by clostridial neurotoxins that docking and fusion of synaptic vesicles may involve complexes of synaptic vesicle, cytosolic and presynaptic cell membrane proteins (31). In addition to binding latrotoxin receptor/neurexins, synaptotagmin has been suggested to be part of a large synaptic vesicle-protein complex (32), to bind to the presynaptic membrane proteins, HPC1/syntaxin (26, 28), and voltage-gated calcium channels (26-28), and perhaps indirectly to such cytoplasmic proteins as N-ethyl-maleimide-sensitive fusion protein, α-soluble NSF attachment protein and synaptoosome-associated protein 25 to form a large fusion complex (31). Because an interaction of synaptotagmin with HPC1/syntaxin has also been suggested to possibly mediate docking, we tested whether HPC1/syntaxin might also bind to the carboxyl terminus of synaptotagmin. We did not get enrichment of HPC1/syntaxin on p65-9 columns. This suggests that the carboxyl-terminal domain of synaptotagmin is not mediating an interaction with HPC1/syntaxin and that HPC1/syntaxin is not required for the interaction of synaptotagmin with the neurexins. This is also supported by the work of Hata et al. (13). In their investigation, the binding of purified recombinant pieces of neurexins to a synaptotagmin column occurred in the absence of other proteins such as syntaxin. This does not rule out that HPC1/syntaxin interacts with other domains of synaptotagmin. It seems likely that HPC/syntaxin or other nerve terminal proteins form a large complex, perhaps one that may be centered on the binding of synaptotagmin and neurexins.

The specificity of binding of neurexins to synaptotagmin, the orientation of both proteins, and the potential association of both proteins with neurotransmitter release suggests that their interaction may also be directly involved with neurotransmitter release. I would suggest two hypotheses on how this interaction would function in release. One hypothesis is that this interaction is involved in docking synaptic vesicles at the active site. Such a function would be attractive given synaptotagmin's role in calcium activation of neurotransmitter release (7). This would fit with the orientation of the two proteins. It also could fit with the calcium independence of this interaction because docking presumably occurs before calcium influx and fusion. A caveat to this hypothesis is that multiple proteins may mediate docking (31). A second possibility is that binding by neurexins modulates synaptotagmin function. If synaptotagmin mediates calcium-dependent neurotransmitter release, then neurexins may be a focal point for modulation of neurotransmission. That at least some neurexins are receptors for the black widow spider venom toxin, latrotoxin (23, 10), and that latrotoxin causes massive exocytosis of synaptic vesicles even in the absence of external calcium (33) suggests the possibility for the second hypothesis. Unfortunately, it is presently difficult to directly test these hypotheses. Docking or modulation must be intimately entwined with release. The recent demonstration of genomest and minigene rescue of synaptotagmin null mutants in C. elegans and Drosophila (7-9) raises the possibility of in vivo tests of rescue with synaptotagmin constructs missing the carboxyl-terminal sequence.

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