Mirror Image Motifs Mediate the Interaction of the COOH Terminus of Multiple Synaptotagmins with the Neurexins and Calmodulin†

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ABSTRACT: I have previously reported that the COOH-terminal 34 amino acids of synaptotagmin 1 are capable of interacting with the presynaptic proteins, the neurexins. Multiple synaptotagmins and a synaptotagmin-like protein, rabphilin 3A, are conserved in this domain, raising the possibility that many different synaptotagmins may interact with neurexins. Here I report that the COOH termini of synaptotagmins 1, 2, 4, 5, 6, 7, and 9 and rabphilin 3A are capable of interacting with neurexins. The COOH terminus of rabphilin 3A is still capable of substantial enrichment of neurexins from solubilized brain membranes even though only 11 of 33 residues are identical with the COOH terminus of synaptotagmin 1. Like the purification of neurexins on the COOH terminus of synaptotagmin 1, purification by the COOH terminus of rabphilin 3A is calcium-independent. The conservation between carboxyl termini of these proteins suggests symmetrical motifs are necessary for neurexin binding. These include the sequence Leu-X-His-Trp, followed by 13 amino acids, and the sequence Trp-His-X-Leu. Deletion of the first motif or substitution of residues in the second of these motifs greatly reduces neurexin enrichment. Interestingly, these same COOH termini yield substantial calcium-dependent enrichment of calmodulin mediated by the first of these sequence motifs. This correlates with the binding of ^{125}I-labeled calmodulin by recombinant pieces of synaptotagmin 1 containing the carboxyl terminus. These data suggest that multiple synaptotagmins may interact with neurexins to mediate docking or regulation of neurotransmitter release and that synaptotagmins may be calcium-regulated via interaction with calmodulin.

Synaptotagmin 1 (Perin et al., 1990) is critical for the calcium activation of neurotransmitter release in most neurons (Littleton et al., 1993, 1994; Geppert et al., 1994). Synaptotagmin I may mediate this activation by binding acidic phospholipids (Perin et al., 1990) or interacting with other components of a docking/fusion protein complex at the active site [reviewed in Südhof (1995)]. Synaptotagmin 1 may also mediate docking or modulation of neurotransmitter release by interacting with the presynaptic proteins, the neurexins (Petrenko et al., 1991; Perin, 1994; Hata et al., 1993). In addition, synaptotagmin 1 may be involved in endocytosis by binding AP-2 via its second C2 domain (Zhang et al., 1994). Reflecting the important role of this protein in membrane traffic, at least nine different synaptotagmin genes have been identified in rat [reviewed in Li et al. (1995) and Hudson and Birnbaum (1995)]. Synaptotagmins 1−4 are found exclusively in neurons, whereas synaptotagmins 5−9 have been localized to multiple different tissues. In addition, a synaptotagmin-like protein, rabphilin 3A, has been identified by its interaction with the neuronal small G-protein rab3A (Shirataki, 1993).

I have studied the interaction of synaptotagmin 1 with the neurexins and have shown that the carboxyl-terminal 34 amino acids of synaptotagmin 1 can mediate the recognition of neurexins (Perin, 1994). The localization of synaptotagmin 1 to synaptic vesicles and the localization of neurexins to the presynaptic cell membrane raise the possibility that the interaction between these two proteins may mediate part of the recognition of presynaptic active sites by synaptic vesicles or may regulate neurotransmitter release.

Although the exact functions of the neurexins are not known, this protein family is complex, being composed of potentially over 1000 forms derived from multiple splice variants of at least three genes (Ushkaryov et al., 1992; Ullrich et al., 1995). The structures of the neurexins are intriguing for synapse-specific presynaptic membrane proteins. They contain a short cytoplasmic sequence that has been shown to be capable of interacting with synaptotagmin 1 (Hata et al., 1993), a single transmembrane spanning sequence, and an extracellular domain that in many forms contains three epidermal growth factor (EGF) repeats and multiple domains with homology to laminin (Ushkaryov et al., 1992). The structures and diversity of the neurexins raise the possibility that neurexins may be involved with neuronal cell recognition. Neurexins were first characterized as receptors for the black widow spider toxin, latrotoxin (Ushkaryov et al., 1992), and several cloned forms of the neurexins have been shown to directly interact with this toxin (Davletov et al., 1995). Latrotoxin causes massive exocytosis of synaptic vesicles even in the absence of external calcium (Matteoli et al., 1988), although the exact role of neurexin binding in the action of latrotoxin is currently unknown.

In order to understand potential roles of synaptotagmin and neurexins in synaptic vesicle docking or modulation of neurotransmitter release, I have studied whether the carboxyl termini of other synaptotagmins can interact with neurexins. The conservation of carboxyl termini of synaptotagmins varies between 78% and 28% identity with synaptotamin 1. I find that the carboxyl termini of multiple synaptotagmins can enrich neurexins from solubilized brain membranes. This

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interaction appears to be mediated by distinct sequence motifs, and these same motifs may also mediate an interaction with calmodulin. This raises the possibility that multiple functions of synaptotagmins are regulated by intraterminal calcium.

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 were used without further purification.

Antibodies. Neurexin antibodies have been previously described (Perin, 1994). These included three anti-peptide antibodies to residues 1493−1507 (LR1), residues 1455−1468 (LR2), and residues 30−46 (LR3) of neurexin 1a, respectively. LR1 and LR2 recognize the carboxyl termini of neurexins 1a, 2a, and 3a as expressed as bacterial constructs. LR4 was kindly provided by Dr. Martin Geppert and Dr. Thomas Südhof and was raised to a recombinant bacterial portion of the extracellular domain of neurexin 1a. Anti-calmodulin antibody was purchased from East Acres Biologicals. Monoclonal antibody that recognizes syntaxin 1A and B was kindly provided by Dr. Colin Barnstable. Monoclonal antibodies to synaptobrevin, SNAP25,[1] α-SNAP, and a polyclonal antibody to NSF were kindly provided by Dr. Reinhard Jahn.

Purification on Carboxyl-Terminal Synaptotagmin Affinity Columns. Peptides were coupled to thiopropyl-Sepharose using their introduced amino-terminal cysteine. Ten micromoles of peptide was used for each column. Coupling to thiopropyl-Sepharose was assayed to be greater than 90% for all columns. Triton X-100-solubilized rat brain membranes were used for purification of neurexins on synaptotagmin columns as previously described (Perin, 1994). Ten rat brains were homogenized in 0.32 M sucrose and 1 mM EGTA. Brain membranes were pelleted by centrifugation at 100000 g_{av} for 1 h. Brain membranes were solubilized in 20 mM HEPES, pH 7.4, 1 mM EGTA, and 1% Triton X-100. Unsolubilized material was removed by 1.5-h 100000 g_{av} centrifugation and discarded. For chromatography, solubilized brain membranes were adjusted to 100 mM NaCl and 1 mM free $CaCl_2$ and loaded onto columns over a period of several hours. Columns were washed extensively with 100 mM NaCl, 20 mM HEPES pH 7.4, 1 mM $CaCl_2$, and 0.2% Triton X-100. For most chromatographies, protein was eluted in two steps consisting of a first elution (E1) with 100 mM NaCl, 20 mM HEPES, pH 7.4, 10 mM EDTA, and 0.2% Triton X-100 and then a second elution (E2) of 1 M NaCl, 20 mM HEPES, pH 7.4, 10 mM EDTA, and 0.2% Triton X-100. Elution fractions were concentrated with Centriprep apparati (Amicon) and rinsed with 20 mM HEPES, pH 7.4, and 10 mM EDTA to reduce salt and Triton X-100 concentrations.

SDS−PAGE and immunoblotting were performed as described (Perin, 1994). Proteins reactive with antibodies were visualized with peroxidase-labeled secondary antibodies. For western blotting with anti-calmodulin antibodies, gels were transferred to nitrocellulose and fixed with glutaraldehyde (Van Eldik & Wolchok, 1984). Protein assays were performed according to Bradford (1976) using commercial reagents (Bio-Rad) and bovine immunoglobulins as standards.

Bacterial Expression. Bacterial expression of cytoplasmic sequences of synaptotagmin in the pET vector has been described previously (Perin et al., 1990; Perin, 1994). PET 1 codes for amino acids 78−421 of rat synaptotagmin 1, PET 4 for amino acids 265−421, PET 8 for amino acids 135−421, PET 6 for amino acids 78−263, and PET 5 for amino acids 78−134. PET 2 codes for amino acids 79−422 of human synaptotagmin 1. Recombinant proteins were checked for the correct amino acid sequence by western analysis with antibodies to the p65-9 peptide (residues 387−421, rat sequence) for PET 1, PET 4, and PET 8 or antibodies to p65-6 peptide (linking domain, residues 100−120, rat sequence) for PET 5 and PET 6.

^{125}I-Calmodulin Blot Overlays. Bacteria expressing recombinant pieces of synaptotagmin 1 (PET 2, PET 1, PET 8, PET 6, PET 4, and PET 5) were pelleted, resuspended in SDS buffer, and electrophoresed on SDS−polyacrylamide gels and transferred to PDVF membranes. Blots were blocked with 3% bovine serum albumin in 100 mM NaCl and 20 mM HEPES, pH 7.4 (HBS) for 1 h. Blots were incubated with 1 μCi of ^{125}I-labeled calmodulin (Amersham) in HBS with 100 μM $CaCl_2$ (Fournier & Trifaro, 1988). Blots were washed two times in HBS for 10 min, two times with HBS with 0.05% nonionic detergent NP40, and two times with HBS and exposed to film.

RESULTS

Comparison of synaptotagmins and synaptotagmin-like proteins demonstrates three regions of sequence conservation. These include the two repeats with homology to the C2 domain of protein kinase C (PKC) and the carboxyl terminus of each protein (Li et al., 1995; Hudson & Birnbaum, 1995; Shirataki et al. 1993). The conservation of the carboxyl termini of the synaptotagmins and rabphilin 3A is shown in Figure 1. The homology in this domain ranges between 28% and 78% identity to synaptotagmin 1 (expressed as number of identical residues divided by the residues of the shorter C-terminus multiplied by 100). This homology is highest for synaptotagmin 2 (78%) followed by synaptotagmin 9 (58%), synaptotagmin 8 (55%), synaptotagmin 7 (48%), synaptotagmin 5 (43%), synaptotagmin 4 (39%), synaptotagmin 6 (35%), and rabphilin 3A (34%), and the homology is lowest for synaptotagmin 3 (28%). Comparing all synaptotagmins and rabphilin 3A, three motifs are apparent within the carboxyl termini. These include an amino acid sequence Leu-X-His-Trp near the beginning of the carboxyl-terminal sequence. This is followed by a stretch of 13 residues which in turn is followed by a mirror image of the first motif, Trp-His-X-Leu. These motifs are present in whole or in part for all the synaptotagmins and rabphilin 3A. They are least conserved in synaptotagmin 3, which only contains histadine and tryptophan of the initial motif and only the tryptophan of the final motif. In all synaptotagmins, these motifs are separated by 13 amino acids. Searches of the Swiss-Pro database reveal that the Leu-X-His-Trp-13 amino acids-Trp-

[1] Abbreviations: kDa, kilodaltons; PVDF, poly(vinylidene difluoride); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); NSF, *N*-ethylmaleimide sensitive factor; SNAP25, synaptosome-associated protein 25; α-SNAP, α soluble *N*-ethylmaleimide sensitive factor associated protein; AP-2, adaptor protein 2; C2, conserved domain 2.

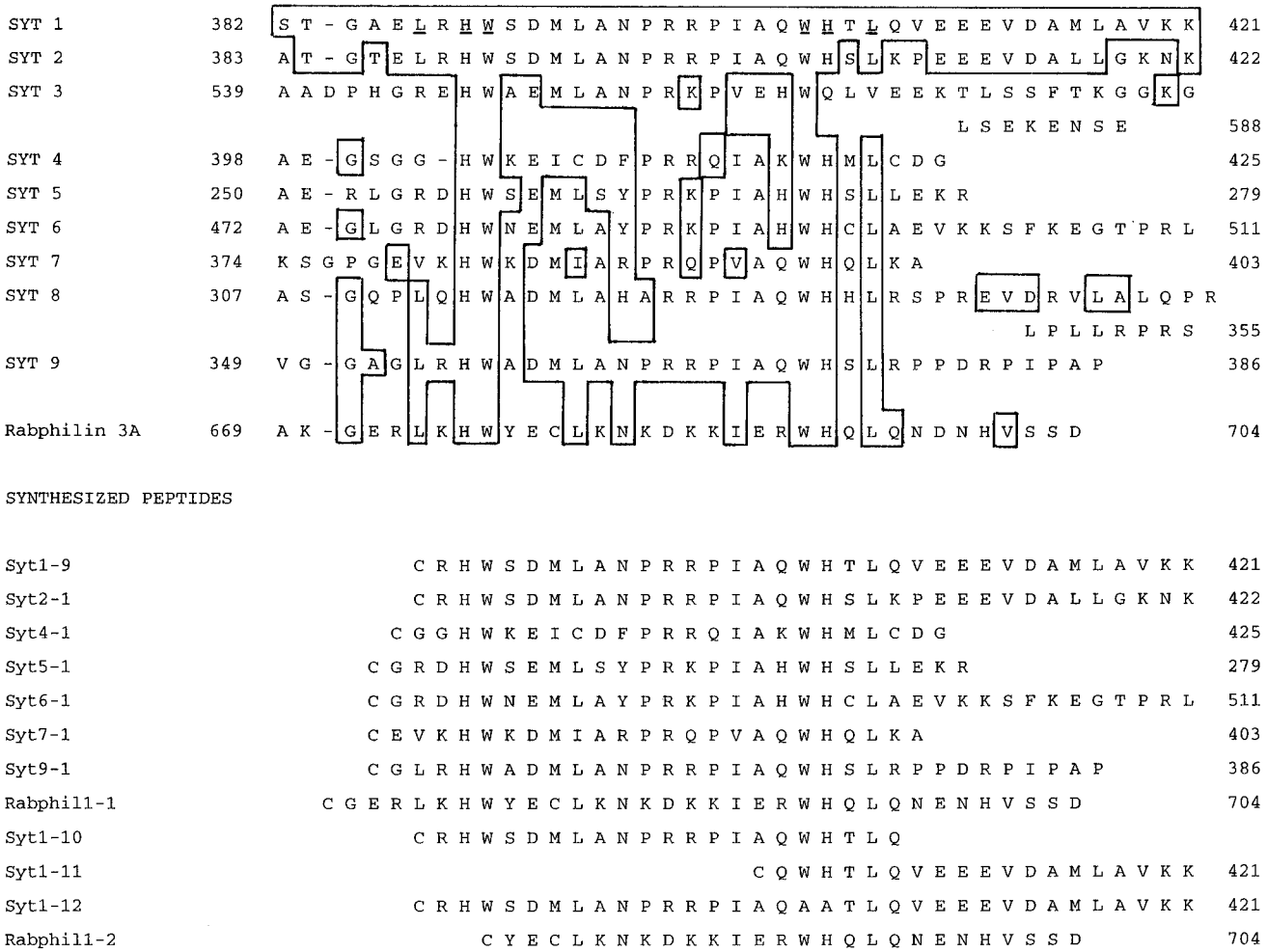

FIGURE 1: Conservation of the carboxyl termini of multiple synaptotagmins within a species. Residues identical to those in rat synaptotagmin 1 are boxed. Residue numbers for the first and last amino acid of each COOH-terminal sequence are listed on the left and right of each sequence. Leu-X-His-Trp and Trp-His-X-Leu motifs are underlined for synaptotagmin 1. Peptides used for construction of affinity columns are listed below, and the residue number for the last amino acid of each peptide is listed on the right of each sequence. Sequences are as reviewed in Li et al. (1995), Perin et al. (1990), Hudson and Birnbaum (1995), and Shirataki et al. (1993).

His-X-Leu or the His-Trp-13 amino acids-Trp structure is unique to the synaptotagmins and synaptotagmin-like proteins. The conservation within this domain raised the possibility that these domains in the synaptotagmins mediate a similar function and, like synaptotagmin 1 (Perin, 1994), may mediate an interaction with the presynaptic proteins, the neurexins (Petrenko et al., 1991; Perin, 1994; Hata et al., 1993).

To determine whether these domains in other synaptotagmins can also mediate an interaction with neurexins or other proteins, peptides corresponding to the carboxyl termini of synaptotagmins 2, 4, 5, 6, 7, and 9 and rabphilin 3A and truncations of these peptides were chemically synthesized (Figure 1). Synthesis of peptides corresponding to the carboxyl termini of synaptotagmins 3 and 8 were attempted but were unsuccessful. Peptides corresponding to the carboxyl termini of synaptotagmins 1 and 2 were synthesized with a cysteine replacing the leucine of the first motif. Previous studies have shown that this synaptotagmin 1 peptide can purify neurexins (Perin, 1994). A longer synaptotagmin 1 peptide including the leucine of the first motif also purifies neurexins (data not shown). As in previous published work (Perin, 1994), I followed the ability of columns of coupled peptide to enrich neurexins from solubilized brain membranes. A 10-μmol sample of each peptide was coupled to thiopropyl-Sepharose through their amino-terminal cysteine residues. To standardize these purifications, a fixed amount of tissue (10 rat brains) was used for each preparation. Membranes were solubilized in Triton X-100 and insoluble material was removed by centrifugation. Solubilized membranes were adjusted to 100 mM NaCl and 1 mM free calcium chloride and run over columns. Columns were washed extensively and sequentially eluted with 100 mM NaCl, 10 mM HEPES, 10 mM EDTA, and 0.2% Triton X-100 (E1) and then with 1 M NaCl, 10 mM HEPES, 10 mM EDTA, and 0.2% Triton X-100 (E2) as described previously (Perin, 1994). Eluates 1 (E1) and 2 (E2) were concentrated with Centripreps to a fixed volume (1 mL), and the protein concentration of each fraction was determined. Gels were loaded with either a fixed amount of protein (25 or 50 μg) of each fraction or a fixed percentage of each fraction ($^1/_{30}$ or $^1/_{375}$). Protein in gels was electrophoretically transferred to nitrocellulose. Purification was followed by Coomassie blue staining and western blotting with antibodies specific for the neurexins as previously described (Perin, 1994).

The enrichments of neurexins on the carboxyl termini of multiple synaptotagmins are shown in Figure 2. Neurexins are difficult to detect in brain membranes due to their low abundance (SM and FT fractions). As previously shown

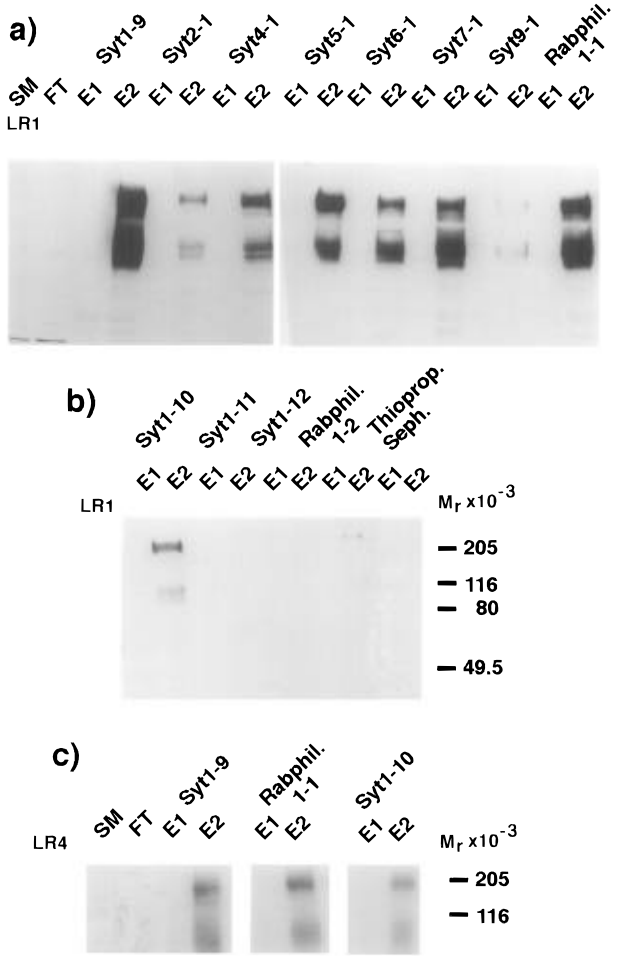

FIGURE 2: COOH-terminal domains of multiple synaptotagmins enrich neurexins. Chromatography was as described under Experimental Procedures. Solubilized brain membranes (SM) (50 μg), 50 μg of flowthrough (FT), and 50 μg of the first (E1) and second (E2) eluate from chromatographies using affinity columns of synthesized peptides were run on SDS−7.5% polyacrylamide gels, transferred to nitrocellulose, and blotted with LR1 antibody. (a) Fractions from the COOH-terminal domain of multiple synaptotagmins enrich neurexins. (b) Only peptides containing both Leu-X-His-Trp and Trp-His-X-Leu motifs enrich neurexins. (c) Neurexins enriched from COOH-terminal peptides are recognized by multiple neurexin antibodies.

(Perin, 1994), chromatography of solubilized brain membranes on columns of the carboxyl terminus of synaptotagmin 1 (syt1-9) results in extensive enrichment in the E2 eluate of 200-kDa protein(s) immunoreactive with neurexin antibodies. LR1 and LR4 antibodies also recognize a prominent 110-kDa apparent proteolytic fragment. This band is not recognized by preimmune sera, immunoreactivity of this band is blocked by incubation with peptide and a similar-sized band is evident in old or protease-treated samples of purified neurexins [Perin (1994) and data not shown]. The carboxyl termini of synaptotagmin 2, 4, 5, 6, 7, and 9 and rabphilin 3A (syt2-1, syt3-1, syt4-1, syt5-1, syt6-1, syt7-1, syt9-1, and rabphil1-1) were also capable of enrichment of 200-kDa protein(s) immunoreactive with neurexin antibodies (Figure 2). The 200-kDa bands enriched on these columns were immunoreactive to four different antibodies to extracellular and cytoplasmic domains of neurexin 1a (Figure 2 and data not shown). These results clearly demonstrate that the 200-kDa proteins eluted off these columns are neurexins. To determine the relative ability of each column to enrich neurexins, similar blots were incubated with neurexin antibodies and subsequently incubated with ^{125}I-labeled goat anti-rabbit antibodies. After exposure, the area surrounding the 200-kDa band was excised and counted. Counts were normalized to those from elutate 2 (E2) off the carboxyl terminus of synaptotagmin 1. The carboxyl terminus of rabphilin 3A was as efficient as the carboxyl terminus of synaptotagmin 1 (1.1 normalized counts). The carboxyl terminus of synaptotagmin 5 was the next most efficient (0.59 normalized count), followed by synaptotagmin 4 (0.51 normalized count), synaptotagmin 7 (0.4 normalized count), synaptotagmin 6 (0.35 normalized count), synaptotagmin 2 (0.3 normalized count), and synaptotagmin 9 (0.1 normalized count). It was surprising that the carboxyl terminus of rabphilin 3A (rabphil1-1) was as effective in enriching neurexin-immunoreactive 200-kDa protein(s) even though this domain is only 34% identical between these two proteins. When columns were eluted with a salt gradient, neurexin immunoreactive protein eluted between 200 and 600 mM salt, similar to elution from syt1-9 columns (data not shown). Like the enrichment of neurexins on synaptotagmin 1, the enrichment of neurexins on syt2-1, 4-1, 5-1, 6-1, 7-1, and 9-1 and rabphil1-1 is calcium-independent. This is seen in the lack of elution of neurexins from these columns with EDTA. In addition, neurexins can be enriched by chromatography on rabphil1-1 columns in the absence of calcium (all solutions containing 10 mM EDTA; data not shown). One note is that the peptide syt4-1 contains three cysteines (one amino-terminal introduced cysteine and the cysteines 409 and 423 of synaptotagmin 4) and syt6-1 and rabphil1-1 each contain two cysteines. We do not know if these cysteines form disulfide bonds or if such formation affects neurexin binding although, as coupled, syt4-1, syt6-1, and rabphil1-1 were still capable of enriching neurexins.

As these results suggest that the conserved motifs in the synaptotagmins and rabphilin 3A mediate interaction with the neurexins, I investigated whether truncation of these domains would alter the ability to bind neurexins and enrich them from solubilized brain membranes. For this, two smaller synaptotagmin 1 peptides were synthesized. Syt1-10 contains residues 388−408 of synaptotagmin 1. Syt1-11 contains residues 403−421. Syt1-10 is missing sequence downstream of the Trp-His-X-Leu motif. Syt1-11 is missing the first motif and the next 12 residues. As shown in Figure 2, Syt1-10 is still capable of purifying neurexins, although at a reduced level compared to the full synaptotagmin 1 carboxyl-terminal peptide. Syt1-11 does not appear to purify neurexins. I also synthesized a synaptotagmin 1 carboxyl-terminal peptide (syt1-12) where Trp 404 and His 405 were replaced by alanines. Syt1-12 did not enrich neurexins. We also synthesized a truncation of the rabphilin 3A carboxyl terminus (rabphil1-2). Rabphil1-2 contains residues 678−704 of rabphilin 3A and lacks the first Leu-X-His-Trp domain. This peptide purifies little neurexin compared to rabphil1-1. Therefore, both the Leu-X-His-Trp and Trp-His-X-Leu motifs are critical for neurexin enrichment.

Chromatography of solubilized brain membranes on columns of the rabphil1-1 peptide results in the enrichment of neurexins. It also results in a calcium-dependent enrichment of a 17−20-kDa protein in eluate E1 (Figure 3a). This protein was not enriched with chromatography in the absence of calcium, unlike the calcium-independent enrichment of neurexins (data not shown). The 17−20-kDa protein shifts in apparent molecular weight when electrophoresed in the

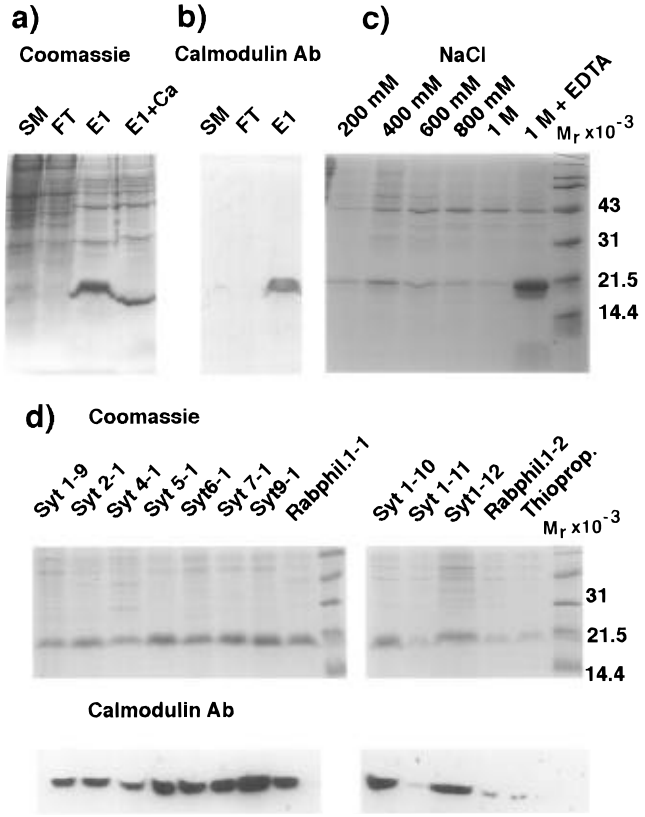

FIGURE 3: Calmodulin is enriched by chromatography on the COOH-terminal domain of multiple synaptotagmins and rabphilin 3A. (a) Samples (25 μg) of solubilized brain membranes (SM) and flowthrough (FT) and $^1/_{30}$ volume of eluate 1 (E1) of rabphil 1-1 column alone or with the addition of 1 mM free $CaCl_2$ were run on 12% gels. Gels were stained with Coomassie blue or (b) transferred and blotted with antibody to calmodulin. (c) Solubilized brain membranes were chromatographed over rabphil 1-1 column in the presence of 1 mM free $CaCl_2$ and eluted with a step gradient of NaCl in 1 mM $CaCl_2$ and a final elution of 1 M NaCl/10 mM EDTA. Each eluate ($^1/_{50}$ volume) was run on a 12% gel and stained with Coomassie blue. (d) Equivalent chromatographies of solubilized brain membranes were run on syt1-9, syt2-1, syt4-1, syt5-1, syt6-1, syt7-1, syt9-1, rabphil 1-1, syt1-10, syt1-11, syt1-12, rabphil 1-2, and uncoupled thiopropyl-Sepharose columns, and $^1/_{375}$ volume of eluate 1 from each column was run on a 12% gel and stained with Coomassie blue or transferred and blotted with antibody to calmodulin. The last lane contains standards for apparent molecular weight.

presence of calcium (Figure 3a), suggesting that it is a high-affinity calcium binding protein. Sequencing of CNBr-derived peptides of this protein revealed it to be calmodulin (MARKMKDTDSEE and KDTDSEEEIREAFRVFDKDG, corresponding to residues 71−82 and 76−96 of calmodulin). This 17−20-kDa protein was immunoreactive with anti-calmodulin antibody (Figure 3b). Elution of rabphil1-1 columns with a salt step gradient in the presence of calcium elutes little calmodulin even using 1 M NaCl (Figure 3c). In contrast, elution with EDTA removes virtually all bound calmodulin. When rabphil1-1 columns were eluted with different calcium concentrations, calmodulin eluted from the column at calcium concentrations between 2 μM and 100 nM (data not shown), a range of calcium concentrations at which calmodulin interacts with other proteins (Means et al., 1991). I further investigated this calcium-dependent enrichment of calmodulin for the other peptide columns (Figure 3d). I observed substantial enrichments of calmodulin on syt1-9, syt2-1, syt4-1, syt5-1, syt6-1, syt7-1, syt9-1, and rabphil1-1 columns as shown by Coomassie blue staining and reactivity with anti-calmodulin antibody (Figure 3d). Gels were loaded with an equal percentage of each eluate for comparison. For chromatographies on syt1-9, syt2-1, syt4-1, syt5-1, syt6-1, syt7-1, syt9-1, and rabphil1-1, greater than 50% of the protein in the E1 elution consisted of calmodulin. This enrichment is approximately 10-fold greater for these columns than for thiopropyl-Sepharose as judged by dilution necessary to yield similar staining as thiopropyl-Sepharose fractions (data not shown).

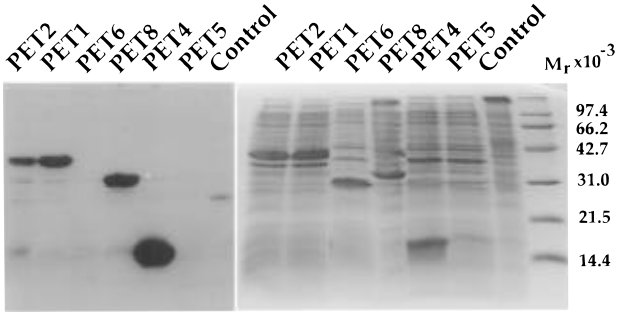

FIGURE 4: Recombinant pieces of synaptotagmin containing the carboxyl-terminal sequence bind ^{125}I-calmodulin. Induced *E. coli* BL21(DE3) cells expressing recombinant cytoplasmic domains of human and rat synaptotagmin 1 (PET 2, PET 1) or pieces of the cytoplasmic domain of rat synaptotagmin 1 (PET 8 spanning both C2 domains and C-terminal sequence, PET 6 spanning linking and C2A domains, PET 4 spanning C2B and C-terminal sequences, and PET 5 spanning the linking domain) were analyzed by SDS-PAGE (25 μg of protein/lane) followed by Coomassie blue staining (left panel) or blotting with ^{125}I-labeled calmodulin (right panel). The blot was exposed for 12 h to film with an intensifying screen.

Previous studies had suggested that calmodulin binds to synaptotagmin 1 as synaptotagmin 1 can be retained on calmodulin−Sepharose columns and binds iodinated calmodulin in calmodulin overlay blots (Fournier & Triforo, 1988). Using solubilized synaptic vesicles, I repeated these experiments and also found that synaptotagmin 1 is retained on calmodulin columns in the presence of calcium (data not shown). To determine whether the enrichment of calmodulin on synaptotagmin carboxyl-terminal peptide represents direct binding, we tested whether recombinant pieces of the cytoplasmic domain of synaptotagmin 1 were capable of binding ^{125}I-calmodulin in overlay blots (Figure 4). Bacteria expressing recombinant pieces of synaptotagmin 1 were solubilized with SDS and electrophoresed on SDS−polyacrylamide gels. Gels were stained with Coomassie blue or transferred to PDVF membranes and incubated with ^{125}I-calmodulin, washed, and exposed to film. The bacterially produced recombinant proteins tested included the full cytoplasmic domain of human and rat synaptotagmin 1 (PET 2 and PET 1), protein initiating at the first C2 domain containing both C2 domains and the carboxyl-terminal sequence (PET 8), protein initiating at the linking domain and containing the first C2 domain (PET 6), protein initiating at the second C2 domain and containing the carboxyl-terminal sequence (PET 4), protein containing only the linking domain (PET 5), and bacteria not expressing synaptotagmin protein (control). PET 2, PET 1, PET 8, and PET 4 bound ^{125}I-calmodulin with high affinity in blot overlays (binding easily detected at ~60 pM calmodulin). All these proteins contain the carboxyl-terminal sequence. PET 5 and PET 6, which do not contain the carboxyl-terminal sequence, did not bind ^{125}I-calmodulin in blot overlays.

Because calmodulin binds to domains with basic and hydrophobic elements (Means et al., 1991), I also tested whether calmodulin was enriched on columns lacking or containing modifications of the motifs implicated in neurexin binding. Deletion of the first motif in rabphilin 3A (rabphil1-2) or deletion of the first motif and the next 12 residues of synaptotagmin 1 (syt1-11) yields greatly reduced enrichment of calmodulin (Figure 3). Enrichment of calmodulin on these columns is comparable to the enrichment of calmodulin on uncoupled thiopropyl-Sepharose. I also tested whether calmodulin was enriched by chromatography on syt1-10 (containing both motifs) and syt1-12 (where the Trp and His of the second motif are changed to Ala). Both columns greatly enriched calmodulin. Together this suggests that the first Leu-X-His-Trp motif is critical for calmodulin enrichment.

The interaction of neurexins with the carboxyl termini of multiple synaptotagmins raises the possibility that this binding may mediate docking at active sites or a direct modulation of neurotransmitter release. As such, the binding of synaptotagmins and neurexins may form a scaffold for subsequent interactions of protein components of the docking/fusion apparatus. I have previously shown that one component of the docking/fusion apparatus, syntaxin, is not enriched by chromatography on the carboxyl terminus of synaptotagmin 1. With our chromatographies on the carboxyl termini of multiple synaptotagmins, I tested whether other components of the docking/fusion apparatus may bind directly to the carboxyl termini of synaptotagmins. For this, I assayed by immunoblotting whether chromatography of solubilized brain membranes on these carboxyl-terminal peptide columns enriched other putative components of the docking/fusion apparatus. The components tested included syntaxin, rab3A (data not shown), synaptobrevin, SNAP25, NSF, and α-SNAP. Although these proteins showed differential adherence to these columns, none of these proteins are enriched beyond their starting abundances by chromatography on these columns (Figure 5). This suggests either that none of these proteins bind to the carboxyl termini of synaptotagmins or that such interactions are of a lower affinity than the interaction with neurexins, which are enriched greater than 100-fold.

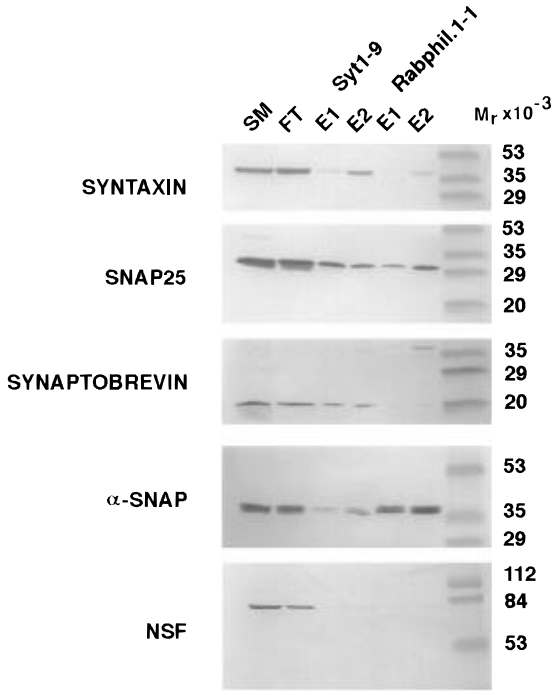

FIGURE 5: Multiple proteins of the docking/fusion complex are not enriched by chromatography on the COOH-terminal domains of synaptotagmin 1 and rabphilin 3A. Samples (25 μg) of solubilized brain membranes (SM), flowthrough (FT), and eluates 1 (E1) and 2 (E2) from both chromatographies on the COOH-terminal peptides of synaptotagmin 1 (syt 1-9) and rabphilin 3A (rabphil 1-1) were run on 10% and 12% gels, transferred and blotted with antibodies to syntaxin, SNAP25, synaptobrevin, α-SNAP, and NSF.

DISCUSSION

Several different avenues of experimentation have suggested that synaptotagmin 1 interacts with the presynaptic proteins, the neurexins. This included the copurification of synaptotagmin 1 and neurexins on α-latrotoxin columns (Petrenko et al., 1991), the demonstration that the carboxyl terminus of synaptotagmin 1 can enrich neurexins (Perin, 1994), and the observation that the carboxyl termini of neurexins can mediate an interaction with synaptotagmin 1 (Hata, 1993). I have investigated whether the carboxyl termini of other synaptotagmins and the synaptotagmin-like protein rabphilin 3A can also interact with neurexins. Chromatography of solubilized brain membranes on columns of the carboxyl-terminal domains of these proteins results in the substantial enrichment of neurexins, suggesting that multiple synaptotagmins and even the synaptotagmin-like protein rabphilin 3A can interact with neurexins at the nerve terminal. The conservation between these divergent synaptotagmins suggests motifs important for this interaction. These motifs include symmetrical domains of Leu-X-His-Trp and Trp-His-X-Leu that are separated by 13 residues.

The homologies between the carboxyl-terminal domains of synaptotagmins raised the possibility that these domains may mediate similar interactions, but it has been suggested that because of lower homologies to synaptotagmin 1, the carboxyl termini of synaptotagmins 3−9 and rabphilin 3A would not interact with neurexins (Südhof, 1995). Purification of neurexins on columns of the carboxyl termini of synaptotagmins 2−9 and on the carboxyl terminus of rabphilin 3A suggest that the neurexins can interact with multiple synaptotagmins. The substantial purification of neurexins on rabphilin 3A columns is particularly dramatic. Immunoreactivity to neurexins in brain membranes is barely detectable by Western blotting (Figure 2) but after chromatography is easily detectable. This enrichment (like that on syt1−9 columns) results in a greater than 100-fold enrichment of neurexins as measured by the dilution of eluate necessary to get a comparable staining to starting membranes (data not shown). This enrichment (again like that on syt1−9 columns) is calcium-independent in that neurexins are not eluted with EDTA and can bind to rabphil1−1 columns in the absence of calcium. The lower homology between the carboxyl termini of rabphilin 3A and synaptotagmin 1 puts the focus on conserved residues for potentially mediating the interaction with neurexins. The symmetrical motifs Leu-X-His-Trp and Trp-His-X-Leu are particularly apparent, although the leucine of the first motif is not required as the Syt1-9 peptide that has a cysteine replacing this leucine can greatly enrich neurexins. Strikingly, a central sequence (Asp-Met-Leu-Ala-Asn-Asp-Pro-Arg-Arg-Pro-Ile-Ala-Gln, resi-

dues 392−403 of synaptotagmin 1) that is well-conserved between synaptotagmins shows only three identical residues with the same stretch in rabphilin 3A. This stretch in rabphilin 3A does have a similar polybasic stretch that replaces arginines with lysines. Given that rabphil1-1 enriches neurexins, the exact sequence within this domain may not be critical for neurexin interaction.

Carboxyl-terminal peptides of synaptotagmins 2, 4, 5, 6, 7, and 9 were all capable of enriching neurexins to some extent. The carboxyl-terminal peptides of synaptotagmins 2 and 9 were the least effective in enriching neurexins immunoreactive to antibodies LR1, LR2, LR3, and LR4 (Figure 2 and data not shown). This is surprising as synaptotagmin 2 has the highest homology to synaptotagmin 1 in this domain (78% identity) and synaptotagmin 9 is 58% identical. Although Syt2-1 and Syt9-1 enrich neurexins immunoreactive to LR1,2 (cytoplasmic domain antibodies) and LR3,4 (antibodies to extracellular domains of neurexin 1a), it is possible that these antibodies might not recognize other neurexin-like proteins. This is an intriguing possibility in that synaptotagmin 1 and 2 are present in different neurons (Li et al., 1995) and it is likely that all neurons contain either synaptotagmin 1 or 2 to mediate the calcium activation of neurotransmitter release (Li et al., 1995).

The sequence conservation between the carboxyl termini of synaptotagmin 1 and rabphilin 3A suggest that the Leu-X-His-Trp and Trp-His-X-Leu motifs are important for neurexin binding. The Leu-X-His-Trp domain of rabphilin 3A appears to be important, as its removal greatly reduces neurexin enrichment. Residues after the Trp-His-X-Leu motif may be less important for neurexin binding, as they are not conserved between synaptotagmin 1 and rabphilin 3A, are missing in synaptotagmins 4 and 7, and are deleted in the peptide syt1-10. The Leu of the Leu-X-His-Trp motif is apparently not critical, as we replaced it with Cys in Syt1-9 and synaptotagmins 4, 5, 6, and 7 have Gly, Gly, Arg, and Val, respectively, at this position. The Trp-His-X-Leu motif is important, as substitution of Ala for Trp and His in Syt1-13 eliminated neurexin binding. We have not tested the importance of the spacing of these two motifs, but in all synaptotagmins and rabphilin 3A these motifs are separated by 13 amino acids and this arrangement is not found in any other characterized proteins. It is interesting to note that all three motifs are present (if one allows substitution of the leucine in the first motif) in synaptotagmins 6, 7, 8, and 9 that are expressed in nonneuronal tissues. This raises the possbility that there are nonneuronal or ubiquitously expressed neurexin-like proteins. It is likewise possible that the carboxyl-terminal domain in synaptotagmins and rabphilin 3A may also interact with other neurexin-like proteins in neurons.

The existence of multiple synaptotagmins and synaptotagmin-like proteins such as rabphilin 3A within individual neurons raises the question of the cellular function of these proteins. In flies, worms, and mice lacking synaptotagmin 1, there is residual neurotransmitter release (Littleton et al., 1993; DiAntonio et al., 1993; Nonet et al., 1993; Geppert et al., 1994). It is possible that the interaction of rabphilin 3A or of other synaptotagmins with neurexins could allow docking and subsequent calcium activation of residual neurotransmitter release. As rabphilin 3A also interacts with rab3A, rabphilin 3A−neurexin interaction could mediate a rab3A regulation of docking.

The substantial purification of calmodulin on these columns also suggests that synaptotagmins can interact with calmodulin. Synaptotagmin 1 has been previously shown to be retained on calmodulin columns in the presence of calcium (Fournier & Trifaro, 1988), although the existence of a physiological interaction has been questioned (Brose et al., 1992). The demonstration that columns of the carboxyl termini of the synaptotagmins can greatly enrich calmodulin puts an additional focus on potential calmodulin regulation of synaptotagmins by locating a calmodulin binding site on synaptotagmins and rabphilin 3A and suggests a potential function for this binding. This binding site for calmodulin appears to be centered on the Leu-X-His-Trp motif. Deletion of this site in carboxyl-terminal peptides of synaptotagmin 1 or rabphilin 3A greatly reduces calmodulin enrichment whereas substitution of Ala for Trp and His in the Trp-His-X-Leu domain of the carboxyl terminal peptide of synaptotagmin 1 has little effect. Calmodulin binding sites are composed of hydrophobic and positively charged elements. If one represents the first motif and the next 13 residues of the carboxyl terminus of synaptotagmin 1 and rabphilin 3A as an α helix in a helical wheel representation, these sequences show a hydrophobic side (synaptotagmin 1 residues L387, W390, L394, and I401 and rabphilin 3A residues L674, W677, L681, and D685) and a positively charged side (synaptotagmin 1 residues R388, S391, A395, and R398 and rabphilin 3A residues K675, Y678, K682, and K686). Tryptophan is prominent in the first motif and is present in the hydrophobic sides of many calmodulin binding amphipathic helixes (Dedman et al., 1993). It should be noted that the linker domain that connects the transmembrane sequence and the two C2 domains has an extensive potential amphipathic α-helix (Perin et al., 1991) but does not contain tryptophans, and this domain does not bind calmodulin (PET5,6; Figure 4). Clearly the presence of a positively charged and hydrophobically sided α-helix is not sufficient for calmodulin binding. The C2A domain and by analogy the C2B domain form compact structures composed of β-sheet (Sutton et al., 1995). It should be noted that as yet we have not been able to demonstrate, by NMR, α-helical structure for the syt1-9 peptide in solution (data not shown). This suggests that peptide needs to be anchored, or becomes structured upon interaction with other proteins like neurexins or calmodulin.

The requirement of the first motif for neurexin binding raises the question of potential overlap of neurexin and calmodulin binding. The enrichment of neurexins on synaptotagmin columns does not require calcium, and calmodulin binding to these columns does not inhibit neurexin binding. One possibility is that the first motif is required for neurexin binding but the second motif is the neurexin binding site. Deletion of the first motif would therefore affect the presentation of the neurexin binding site. It is also possible that this column purification assay does not replicate nuances of the interaction between synaptotagmins, calmodulin, and neurexins in the neuron. The overlap or close proximity of the calmodulin binding site with the neurexin binding site suggests the possibility of calcium-calmodulin regulation of the interaction between synaptotagmins and neurexins. One functional role of this could be the calcium regulation of docking, although we and other groups have found little evidence for the calcium regulation of the interaction of synaptotagmins with neurexins (Perin, 1994;

Hata et al., 1993). Alternatively, the interaction of calmodulin with the carboxyl termini of synaptotagmins may be independent of neurexin binding and serve a separate purpose. This calmodulin binding site is localized near the second C2 repeat of the synaptotagmins and rabphilin 3A. As such, it could regulate potential activities of this domain such as multimerization (Sugita et al., 1996), binding inositol polyphosphates (Fukuda et al., 1994), binding AP2 (Zhang et al., 1994), and regulating endocytosis (Artalejo et al., 1996) or regulating interactions with other proteins such as syntaxin (Bennett et al., 1992; Li et al., 1995). Consequently, these may represent other calcium-dependent steps of synaptic vesicle traffic. An additional purpose of calmodulin binding could involve regulation of synaptotagmin function by facilitating phosphorylation. Synaptotagmin 1 has been shown to be phosphorylatable by casein kinase II (Davletov et al., 1993; Bennett et al., 1993) and rabphilin 3A has been shown to be phosphorylated by calcium calmodulin kinase II (Fykse et al., 1995; Kato et al., 1994). Clarification of these issues will likely require an *in vivo* or genetic approach to study the interaction of synaptotagmins with neurexins and calmodulin.

Multiple lines of evidence, including work on clostridial neurotoxins, work on biochemical interactions, and genetic studies, suggest that a series of proteins including synaptotagmin 1 form complexes that mediate the docking, activation, and fusion of synaptic vesicles [reviewed in Südhof (1995)]. The existence of such complexes raises the possibility of a plethora of different protein interactions that may be mediated by different domains of each protein. The lack of enrichment on synaptotagmin carboxyl-terminal columns of any of the other currently identified proteins of the docking/fusion apparatus suggests that proteins like SNAP25, syntaxin, synaptobrevin, NSF, and α-SNAP do not directly interact or interact poorly with the carboxyl termini of synaptotagmin 1, 2, 4, 5, 6, 7, or 9 or rabphilin 3A. This fits with data that the C2 domains interact with syntaxin (Li et al., 1995; Kee & Scheller, 1996), that SNAP25 interacts with synaptobrevin and syntaxin, and that NSF interacts with synaptobrevin, syntaxin, and SNAP25 [reviewed in Südhof (1995)]. I have suggested that the interaction of synaptotagmins and neurexins mediates an initial step in recognition of the active site. Precise localization of neurexins to active-site complexes would greatly strengthen this hypothesis. Other components of the fusion/docking complex are not restricted to active sites. Synaptobrevin is present on all the synaptic vesicles, NSF and the α- and γ-SNAPs are cytosolic proteins, and syntaxin 1 and SNAP25 are distributed along the entire presynaptic membrane. Some proportion of syntaxin 1 and SNAP25 even becomes distributed to organelles that participate in synaptic vesicle recycling (Walch-Solimena et al., 1995). This suggests that some protein or complex of proteins constitutes the active site and that other fusion/docking proteins are recruited to this site. The neurexins are still ideal candidates for proteins to mediate this initial interaction based on their structures and interaction with specific motifs of synaptotagmins. Hopefully these studies will lead to more direct demonstrations of the role of the neurexins in docking or modulation of neurotransmitter release. The structure and diversity of neurexins also raise the possibility of their involvement in neuronal recognition, synapse formation, or the correct alignment of release sites with arrays of postsynaptic receptors. Direct tests of this hypothesis await immunocytological localization of neurexins to the active sites and evidence of neurotransmission defects in animals lacking one or several neurexin genes.

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