Complexins: Cytosolic Proteins That Regulate SNAP Receptor Function

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Summary

A family of proteins called complexins was discovered that compete with α-SNAP, but not synaptotagmin, for SNAP receptor binding. Complexins I and II are highly homologous hydrophilic proteins that are tightly conserved, with 100% identity among mouse, rat, and human complexin II. They are enriched in neurons where they colocalize with syntaxin and SNAP-25; in addition, complexin II is expressed ubiquitously at low levels. Complexins bind weakly to syntaxin alone and not at all to synaptobrevin and SNAP-25, but strongly to the SNAP receptor–core complex composed of these three molecules. They compete with α-SNAP for binding to the core complex but not with other interacting molecules, including synaptotagmin I, suggesting that the complexins regulate the sequential interactions of α-SNAP and synaptotagmins with the SNAP receptor during exocytosis.

Introduction

Recent studies have revealed an astounding parallelism between membrane fusion in different types and parts of eukaryotic cells. Similar proteins appear to function in diverse intracellular fusion events, ranging from the nuclear envelope to the synapse, and from yeast to human (reviewed by Takizawa and Malhotra, 1993; Bennett and Scheller, 1994; Ferro-Novick and Jahn, 1994; Rothman, 1994). In particular, the central roles of two classes of cytosolic proteins, N-ethylmaleimide-sensitive factor (NSF) and α, β, and γ types of soluble NSF attachment proteins (SNAPs), in intracellular membrane traffic provided convincing evidence for a conserved molecular scaffold in membrane fusion (Beckers et al., 1989; Diaz et al., 1989; Wilson et al., 1989; DeBello et al., 1995; Morgan and Burgoyne, 1995). However, the mechanisms by which these proteins act are largely unclear.

Synaptic vesicle traffic is one of the best-studied systems to probe membrane fusion. The high degree of specialization of synaptic vesicles for the uptake and exocytosis of neurotransmitters and their relatively small size have made it possible to characterize their components exhaustively (reviewed by Südhof, 1995). Genetic studies in mice, Drosophila, and Caenorhabditis elegans coupled

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to biochemical and electrophysiological analyses revealed actions of several important proteins in synaptic vesicle fusion and suggested hypotheses for their functions (for example, see Geppert et al., 1994; Jorgensen and Nonet, 1995; Schulze et al., 1995). A model for the molecular basis of synaptic vesicle exocytosis has emerged from these studies that postulates cascades of binding reactions leading to fusion (Südhof, 1995). This model suggests that during or after vesicle docking, the plasma membrane proteins syntaxin and SNAP-25 and the synaptic vesicle protein synaptobrevin/VAMP assemble into a tight, trimeric, SDS-resistant complex referred to as the core complex (Söllner et al., 1993; Hayashi et al., 1994). The core complex then serves as a SNAP receptor (or SNARE). SNAP binding leads to ATP-dependent binding of NSF, which subsequently catalyzes the disruption of the complex (Söllner et al., 1993; McMahon and Südhof, 1995). Finally, synaptotagmin triggers the last step in the fusion reaction, possibly via its Ca^{2+}-dependent interaction with syntaxin (Geppert et al., 1994; Li et al., 1995).

In spite of the identification of key proteins in membrane fusion, it is unclear how the interactions leading to the assembly and disruption of the core complex are regulated and how they relate to synaptotagmin and the fusion of phospholipid membranes. For example, at most synapses more than five synaptic vesicles are docked at the active zone, apparently ready for fusion at any given time, yet on the average fewer than one vesicle fuses upon Ca^{2+} influx (Allen and Stevens, 1994). These and other findings suggest a tight control over the fusion reaction at the synapse that goes beyond the acute regulation of exocytosis by Ca^{2+}. In the current study, we have searched for regulatory mechanisms in the SNAP receptor–core complex. A family of evolutionarily conserved, ubiquitous proteins called complexins was identified that competes with α-SNAP for binding to the SNAP receptor, suggesting a role in regulating SNAP receptor function during membrane fusion.

Results

To investigate mechanisms that regulate SNAP receptor–core complex function, we used syntaxin antibodies to immunoprecipitate the core complex and associated proteins from rat brain homogenates (Figure 1). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analyses confirmed coprecipitation of SNAP-25 and synaptobrevin with syntaxin (Söllner et al., 1993). In addition, Munc18, α-SNAP, and synaptotagmin were bound to syntaxin as expected (Bennett et al., 1992; Yoshida et al., 1992; Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994). Furthermore, synaptophysin was unexpectedly coprecipitated with syntaxin, but other abundant synaptic proteins were not (Figure 1; data not shown).

The core complex of syntaxin, synaptobrevin, and SNAP-25 in brain is SDS resistant. This results in a shift of the apparent molecular masses of syntaxin, SNAP-25, and synaptobrevin on SDS–polyacrylamide gels from mo-

A

Antibody	I 378		PIS	
Boiling	+	-	+	-

Munc 18
*
Syntaxin
SNAP25*
Syb 2

— 106
— 80
— 50
— 34
— 25
— 18

$M_r \times 10^{-3}$

B

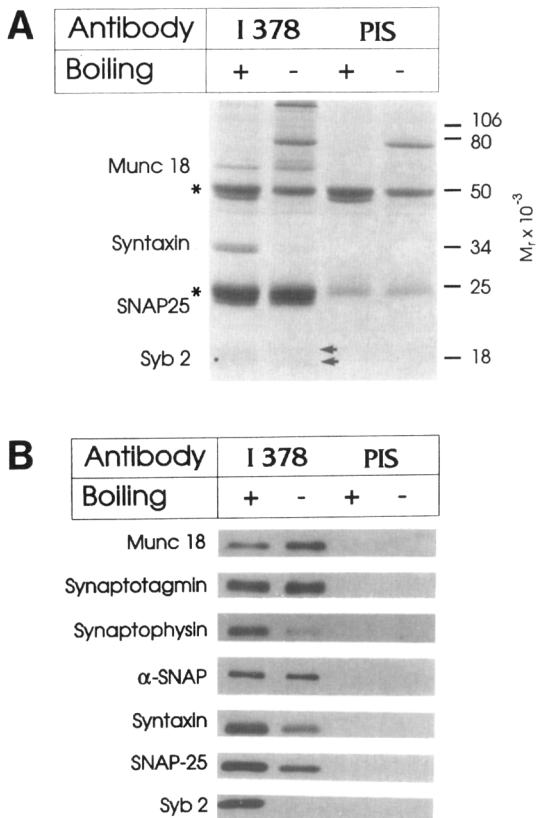| Antibody	I 378		PIS	
Boiling	+	-	+	-

Munc 18
Synaptotagmin
Synaptophysin
α-SNAP
Syntaxin
SNAP-25
Syb 2

Figure 1. Immunoprecipitation of Syntaxin I from Brain

Immunoprecipitates with a polyclonal antibody to syntaxin 1A (I378) or with preimmune serum (PIS) were analyzed by SDS–PAGE with or without boiling. Gels were stained with Coomassie blue (A) or analyzed by immunoblotting (B). No monomer synaptobrevin is present in non-boiled samples, allowing unequivocal identification of the 18 and 19 kDa complexins (arrows in [A]; dot identifies synaptobrevin, and asterisks indicate heavy and light chains of antibodies).
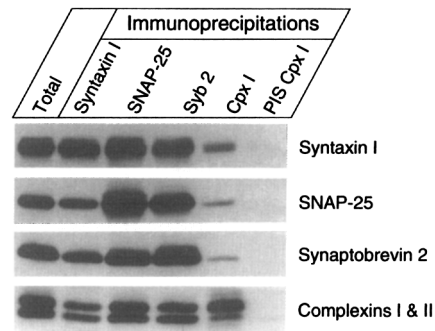

Figure 2. Immunoprecipitations of the Synaptic Core Complex from Brain with Antibodies to Syntaxin I, SNAP-25, Synaptobrevin 2, and Complexin I

Immunoprecipitates from rat brain homogenates with the indicated antibodies were analyzed by immunoblotting with the antibodies shown on the right. The right lane contains immunoprecipitates with complexin I preimmune serum as a negative control (PIS Cpx I).

nomers to multimers if samples are not boiled prior to electrophoresis (Hayashi et al., 1994; Figure 1A). Synaptobrevins were identified as major 18 kDa proteins in the syntaxin immunoprecipitates by immunoblotting of boiled samples. In nonboiled samples, monomeric synaptobrevins were not detectable at 18 kDa because they are present in the high molecular weight core complex (Figure 1B). Unexpectedly, however, Coomassie blue staining revealed proteins of 18 and 19 kDa in the syntaxin immunoprecipitates that were equally present in boiled and nonboiled samples, suggesting that they are not synaptobrevins (arrows in Figure 1A). Because of their binding properties to the core complex (see below), we have named these proteins complexin I (18 kDa) and complexin II (19 kDa).

Although complexins do not stain well because of their small size, Coomassie-based quantitation of the relative ratios of complexins, synaptobrevins, and syntaxins from five immunoprecipitations indicated a 1:1 molar ratio for both complexins together to syntaxins, with an approximate 2- to 4-fold excess of complexins over synaptobrevins (data not shown). Five lines of evidence suggest that the coimmunoprecipitation of complexins with the synaptic core complex is specific. First, comparisons of preim-

mune and immune sera using two independent syntaxin antibodies showed that immune but not preimmune sera precipitated complexins and the core complex (Figure 1). Second, as described above, quantitations of the approximate ratios of the precipitated proteins demonstrated nearly stoichiometric concentrations. Third, immunoblotting with antibodies to a series of abundant synaptic proteins (synapsins, GDI, dynamin) revealed that they were not coprecipitated with syntaxin (data not shown). Fourth, immunoprecipitations of the core complex with SNAP-25 or synaptobrevin antibodies also coprecipitated complexins together with the components of the core complex (Figure 2). Fifth, immunoprecipitations with complexin I antibodies but not with preimmune serum also coprecipitated the core complex (Figure 2). The complexin I antibodies did not coprecipitate the core complex as efficiently as the syntaxin, SNAP-25, and synaptobrevin antibodies, possibly because excess free complexins are present in brain or because the antibody preferentially recognized noncomplexed complexins. Together these data suggest that complexins are major components of the synaptic fusion complex in brain.

To determine the primary structure of the complexins, we purified them from the immunoprecipitates by SDS–PAGE of nonboiled samples. Peptide sequences from purified complexins were used to design primers for polymerase chain reactions (PCRs) and library screens, resulting in the isolation of rat cDNAs encoding complexins I and II (Figure 3A). Their sequences showed that the two complexins are highly homologous to each other (84% identity), suggesting that they represent isoforms. Database searches did not reveal significant homologies to identified proteins, although distant similarities to acidic segments in caldesmon, myosin, and troponin T were noticed (data not shown).

Complexins are small, highly charged proteins of 134 amino acids. Aspartate, glutamate, lysine, and arginine account for 44%–47% of their residues. In addition to rat cDNAs, human cDNAs and mouse genomic clones encoding complexin II were characterized. Surprisingly, the
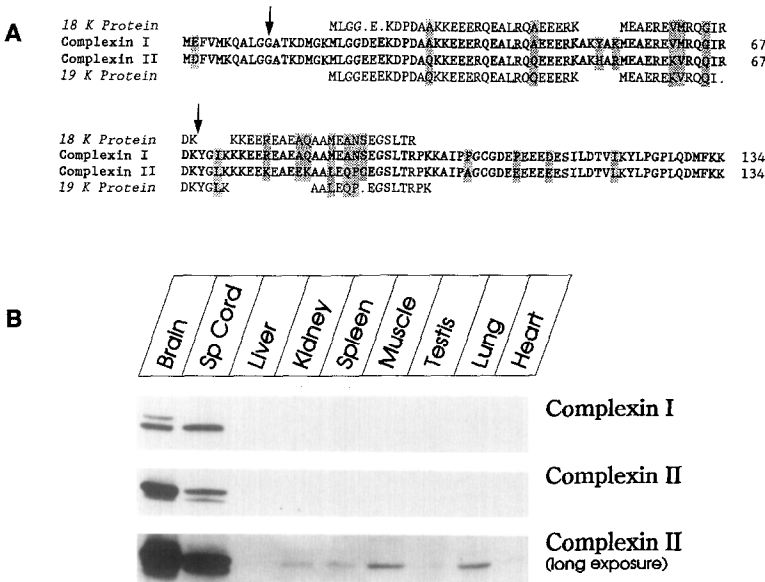
A

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18 K Protein                    MLGG.E.KDPDAAKKEEERQEALRQAEEERK        MEAEREVHRQGIR
Complexin I    MEFVMKQALGGATKDMGKMLGGDEEKDPDAAKKEEERQEALRQAEEERKAKYARMEAEREVHRQGIR    67
Complexin II   MDFVMKQALGGATKDMGKMLGGEEEKDPDAQKKEEERQEALRQQEEERKAKHARMEAEREKVRQGTR   67
19 K Protein                  MLGGEEEKDPDAQKKEEERQEALRQQEEERK        MEAEREKVRQGI.
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18 K Protein   DK    KKEEREAEAQAAMEANSEGSLTR
Complexin I    DKYGIKKKEEREAEAQAANEANSEGSLTRPKKAIPPGCGDEPEEEDESILDTVIKYLPGPLQDMFKK  134
Complexin II   DKYGLKKKEEKEAEEKAALEQPGEGSLTRPKKAIPACCGDEPEEEESILDTVIKYLPGPLQDMFKK  134
19 K Protein   DKYGLK        AALEQP.EGSLTRPK
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B

Complexin I

Complexin II

Complexin II
(long exposure)

Figure 3. Structure and Tissue Distribution of Complexins I and II

(A) Primary structure of complexins. The amino acid sequences of complexins I and II deduced from rat cDNA sequences are aligned with each other and with peptide sequences obtained from the immunoprecipitated 18 kDa and 19 kDa proteins. Residues that differ between complexins I and II are stippled. Equivocal residues in the peptide sequences are indicated by periods. The human cDNA sequence for complexin II and the coding sequences for the mouse complexin II gene were also analyzed; their translated amino acid sequences are identical with rat complexin II. The positions of the two introns in the complexin II gene are indicated by arrows.

(B) Rat tissue homogenates (30 µg protein/lane) were immunoblotted with the complexin I and II antibodies. Bands were visualized by ECL, and filters were exposed to film for 10 s (top and middle) or 1 min to detect lower levels of complexin II in nonneural tissues (bottom).

amino acid sequences for mouse, rat, and human complexin II are 100% identical, although the nucleotide sequences of their mRNAs diverge considerably (Figure 3A; data not shown). Two introns interrupt the coding region of the mouse complexin II gene (arrows in Figure 3A). The sequence conservation of complexin II in the three mammalian species that are separated evolutionarily by millions of years includes the residues that differ between complexins I and II, suggesting that the sequence differences between the two complexins may be functionally important. Thus, complexin II belongs to the rare class of proteins that are completely conserved in several mammalian species.

RNA blots showed that the mRNAs for both complexins are highly enriched in brain (data not shown). In addition, low levels of complexin I mRNAs were observed in testis, and complexin II mRNAs were found at low abundance in all tissues. To confirm this bimodal tissue distribution, we raised antibodies against recombinant complexins and tested them on COS cells transfected with complexin I and II expression vectors. Each antibody reacted preferentially with the complexin against which it was raised, but both antibodies cross-reacted with the other complexin as expected from their sequence homology (Figure 3; data not shown). Immunoblots of rat tissues confirmed that both complexins were primarily expressed in brain and that low but significant levels of complexin II were also present in all tissues tested (Figure 3B).

The antibodies were used to localize complexins in brain by immunocytochemistry using rat brain sections (Figure 4) and cultured hippocampal neurons (Figure 5). A survey of brain areas revealed neuronal expression of complexins I and II in most regions of the CNS in a differential pattern (demonstrated for the hippocampus in Figures 4A and 4B). Complexin staining was primarily concentrated over neuronal cell bodies and synaptic layers, as evidenced by the staining of the stratum pyramidale by complexin I antibodies and the mossy fiber terminals by complexin II antibodies. High levels of complexin I were also observed in the cytoplasm of scattered neurons throughout the hippocampus similar to the nonuniform distribution of SNAP-25 (arrowheads in Figure 4A; Duc and Catsicas, 1995). Higher magnifications revealed enrichment of complexin I in central synapses (shown for the cerebral cortex in Figure 4C) and in the neuromuscular junction (arrows in Figure 4D). However, comparison with the punctate-like synapsin stain (Figure 4E) showed that only a subset of central synapses contained high levels of complexins. In addition to synapses, labeling of cell bodies, axons, and dendrites was observed (Figure 4C). Control experiments using antigen blocking and preimmune serum confirmed the specificity of the staining (Figure 4F; data not shown).

The nonuniform distribution of complexins between neurons and their localization both in and out of synapses are different from that of synapsins (compare Figures 4C and 4E) but similar to that reported for syntaxin I and SNAP-25 (Barnstable et al., 1983; Duc and Catsicas, 1995; Koh et al., 1993). To compare the relative distributions of complexins, SNAP-25, and syntaxin I, we analyzed cultured hippocampal neurons by double immunofluorescence labeling (Figure 5). Similar to the pattern observed in sections, the staining intensity for complexins varied greatly between cells. Comparable differences in staining intensity were found for syntaxin and SNAP-25 that mostly, but not always, correlated with the complexin staining. Complexins, syntaxin, and SNAP-25 were largely colocalized. However, dendrites appeared to have relatively more complexins, and different from the tissue sections, no enrichment of complexins in synapses was evident, possibly because of special properties of cultured neurons. Specificity of the labeling and the quality of the neurons were confirmed using immunocytochemistry with other antibodies, single labeling experiments, and preimmune sera (data not shown).

The complexins were identified by virtue of their association with the synaptic core complex (see Figure 1), suggesting a role in membrane traffic. To determine with which component of the core complex the complexins in-
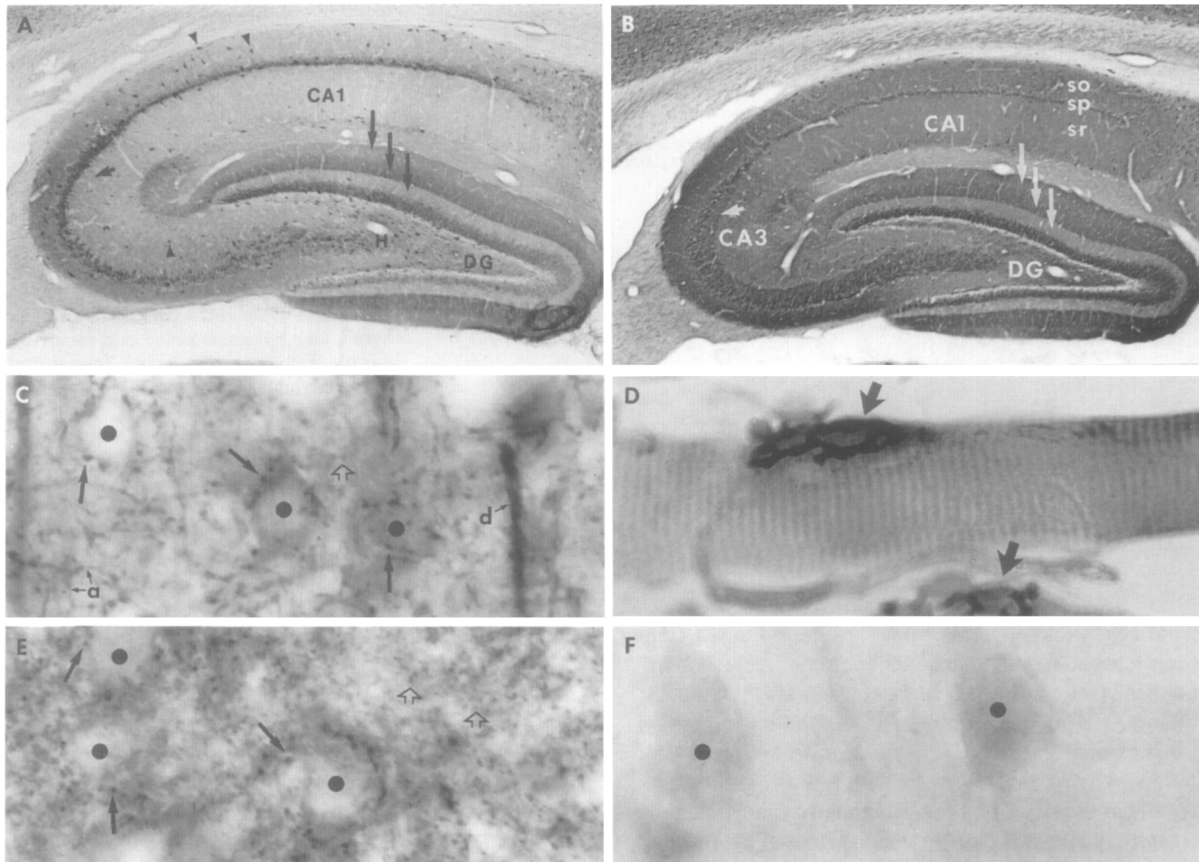
Figure 4. Immunolocalization of Complexins in Rat Brain and Muscle Sections Using Peroxidase Labeling

Cryostat sections from hippocampus (A and B), cerebral cortex (C, E, and F), and anterior tibial muscle (D) were reacted with polyclonal sera against complexin I (A, C, D, and F), complexin II (B), or synapsins (E). For (F), the antibody was preabsorbed with recombinant complexin I as a representative example of the controls used. In (A) and (B), landmarks are identified by the following abbreviations: DG, dentate gyrus; so, sp, sr, and sg, stratum oriens, pyramidale, radiatum, and granulare, respectively. Complexins I and II are differentially distributed between different synaptic layers, as indicated by arrows pointing to mossy fiber terminals (small left arrows) or synaptic layers in the dentate gyrus (larger right arrows). In (C), (E), and (F), closed circles identify cell bodies, and open and closed arrows point to synapses in the neuropil and on cell bodies, respectively. Note that complexin I and synapsin antibodies both stain synapses, but only complexin antibodies label axons (labeled a) and dendrites (labeled d). In (D), presynaptic nerve terminals at the neuromuscular junction are identified by arrows. Approximate final magnifications: (A and B) 11×; (C, E, and F) 342×; (D) 273×.

teract, we studied these interactions using recombinant proteins. Complexin fusion proteins with glutathione S-transferase (GST) bound to glutathione–agarose beads were incubated with recombinant syntaxin I, synaptobrevin 2, and SNAP-25 expressed in COS cells. Beads were washed, and bound proteins were analyzed by immunoblotting (Figure 6). When incubated with syntaxin, synaptobrevin, or SNAP-25 individually, complexins only bound to syntaxin, suggesting that complexins, similar to Munc18 and synaptotagmin, only interact with syntaxin. However, upon addition of synaptobrevin and SNAP-25 to syntaxin, complexin binding increased dramatically (Figure 6), raising the possibility that complexin binding to syntaxin is enhanced by assembly of the core complex as was shown for α-SNAP (McMahon and Südhof, 1995). No differences between the two complexins were observed. We therefore quantitated the binding of syntaxin I to GST–complexin I at different syntaxin concentrations in the presence or absence of a fixed amount of synapto-

brevin and SNAP-25 (Figure 7). Complexin I binding to syntaxin increased dramatically and reached nanomolar affinities in the presence of SNAP-25 and synaptobrevin. These data demonstrate that the core complex constitutes a cellular receptor not only for SNAPs but also for complexins.

α-SNAP and synaptotagmin I compete for binding to the core complex, indicating a possible sequential interaction (Söllner et al., 1993). We therefore tested whether α-SNAP and synaptotagmin I competed not only with each other but also with complexins for syntaxin binding. When α-SNAP was added to immobilized GST–complexin I, syntaxin I binding was completely abolished, suggesting that α-SNAP competes with complexins for binding (Figure 8, lanes 1 and 2). Addition of SNAP-25 or of SNAP-25 and synaptobrevin enhanced syntaxin binding to GST–complexin I, but again α-SNAP displaced syntaxin (Figure 8, lanes 3–6). The competition between α-SNAP and complexins for syntaxin binding was confirmed by syntaxin immunoprecipita-
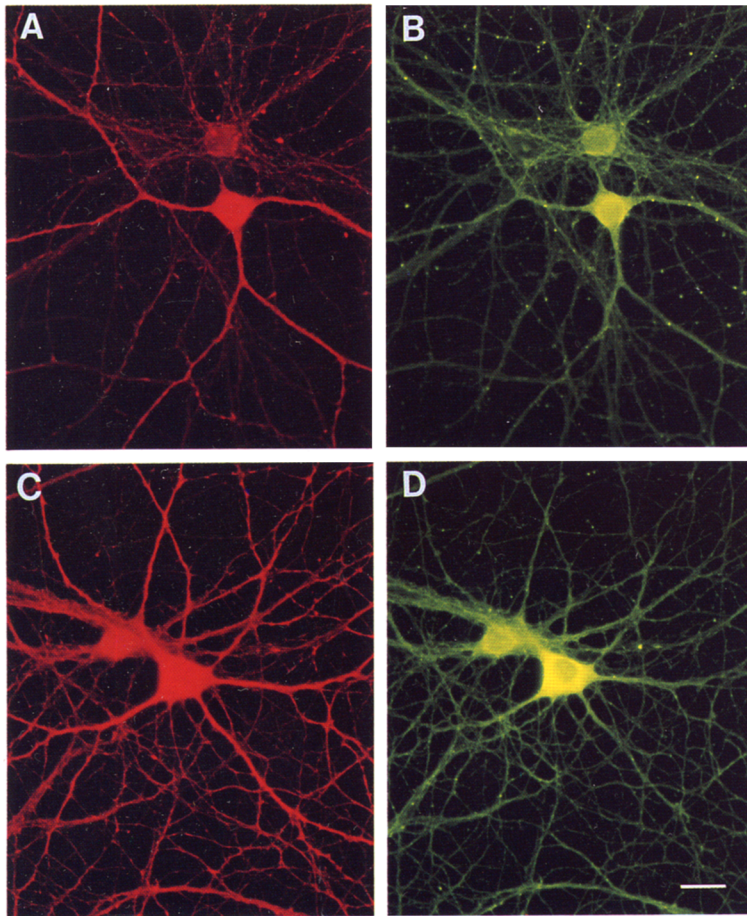
Figure 5. Colocalization of Complexins with Syntaxin and SNAP-25 in Cultured Hippocampal Neurons

Cultured neurons (22 days in vitro) were double labeled by immunofluorescence using polyclonal and monoclonal antibodies, respectively, to complexin I and syntaxin I (A and B) or to complexin II and SNAP-25 (C and D). Note the differences between neurons in staining intensity for complexins, SNAP-25, and syntaxin I. Scale bar in (D), which applies to all panels, is 40 μm.

tions as an independent method. These also demonstrated that addition of excess α-SNAP selectively interfered with the binding of complexins and of synaptotagmin to syntaxin whereas the levels of other coprecipitated proteins did not change (data not shown). Thus, complexins and α-SNAP specifically compete for syntaxin binding.

Similar to complexins, synaptotagmin I competes with α-SNAP for syntaxin binding (Söllner et al., 1993). Do complexins also compete with synaptotagmin I for syntaxin binding, or do these molecules bind to distinct sites that are both contacted by α-SNAP? To test this question, we performed immunoprecipitations of synaptotagmin I from brain. These demonstrated that complexins coprecipitate with synaptotagmin (Figure 9A), suggesting that synaptotagmin I and complexins interact simultaneously with syntaxin and do not compete with each other. In contrast with complexins, α-SNAP was not present in the synaptotagmin I immunoprecipitates (data not shown). The coprecipitation of syntaxin I and complexins with synaptotagmin I was disrupted by exogenous α-SNAP in a concentration-dependent manner, confirming that the coprecipitation of complexins with synaptotagmin is mediated via an interaction with syntaxin (Figure 9A, lane 3). Addition of exogenous complexin, on the other hand, had no effect on the binding of synaptotagmin to syntaxin, and parallel immunoprecipitations of syntaxin confirmed these interactions

(Figure 9A, lanes 4–7). These data suggest that complexins compete for syntaxin binding with α-SNAP but not with synaptotagmin.

Is the interaction of complexins with syntaxin regulated by Ca^{2+} similar to the interaction of synaptotagmins with syntaxins (Li et al., 1995)? To investigate this question, we studied the binding of syntaxin to immobilized GST–fusion proteins of synaptotagmin and complexin as a function of Ca^{2+} and α-SNAP (Figure 9B). Ca^{2+} greatly enhanced syntaxin binding by the first C_2 domain of synaptotagmin but not by complexin; α-SNAP only competed with synaptotagmin for syntaxin binding in the absence of Ca^{2+}, whereas α-SNAP effectively competed with complexin both in the presence and absence of Ca^{2+} (Figure 9B). Thus, complexin binding to syntaxin is Ca^{2+} independent and resembles that of α-SNAP in all of its characteristics.

Discussion

At the synapse, syntaxin, synaptobrevin/VAMP, and SNAP-25 form a complex that plays an essential role in synaptic vesicle exocytosis. The components of the complex are substrates for botulinum and tetanus toxins, which inhibit exocytosis and impair assembly of a functional complex that bridges synaptic vesicle and plasma membranes (reviewed by Hayashi et al., 1994; Südhof,
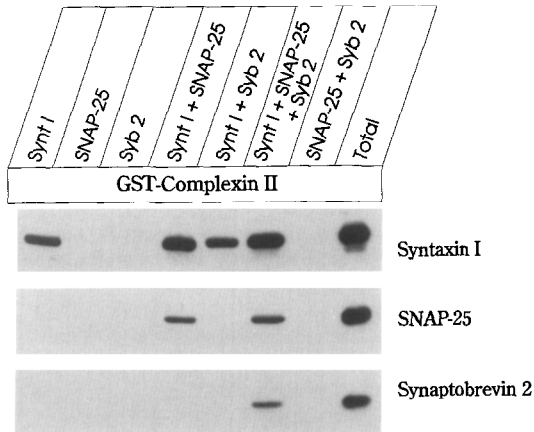
Figure 6. Binding of Complexins to Syntaxin and the Synaptic Core Complex

GST–complexin II bound to glutathione–agarose beads was incubated with extracts from COS cells transfected with syntaxin I (Synt I), SNAP-25, or synaptobrevin 2 (Syb 2) or with combinations thereof. Beads were washed, and proteins bound were analyzed by immunoblotting. Lanes designated total were loaded with the equivalent total amount of protein present in the respective incubations. Identical results were obtained with GST–complexin I (data not shown).

1995). The complex serves as a high affinity receptor for SNAP and NSF and is enzymatically disrupted by NSF, a protein required for many membrane fusion reactions (Söllner et al., 1993). However, the exact function of the core complex in the fusion of phospholipid membranes, the regulation of its assembly and disruption, and its relation to synaptotagmin (which is involved in the final Ca^{2+}-regulated fusion step; Geppert et al., 1994) are unknown. We have identified a family of proteins named complexins
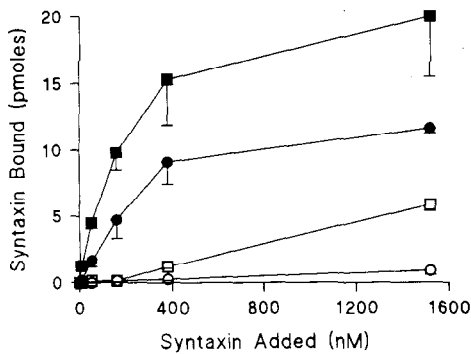

Figure 7. Binding of Increasing Amounts of Syntaxin to Immobilized GST–Complexin I in the Presence or Absence of Synaptobrevin and SNAP-25

Recombinant syntaxin 1A from transfected COS cells was quantitated using purified recombinant syntaxin standards and ^{125}I-labeled secondary antibodies. The indicated amounts of full-length syntaxin were incubated with approximately 9 pmol (circles) or 40 pmol (squares) of immobilized GST–complexin I in the presence (closed symbols) or absence (open symbols) of approximately 30 pmol of SNAP-25 and synaptobrevin produced in transfected COS cells in a 0.2 ml volume. Binding was quantified using ^{125}I-labeled secondary antibodies and is expressed in means ± SD (n = 3).

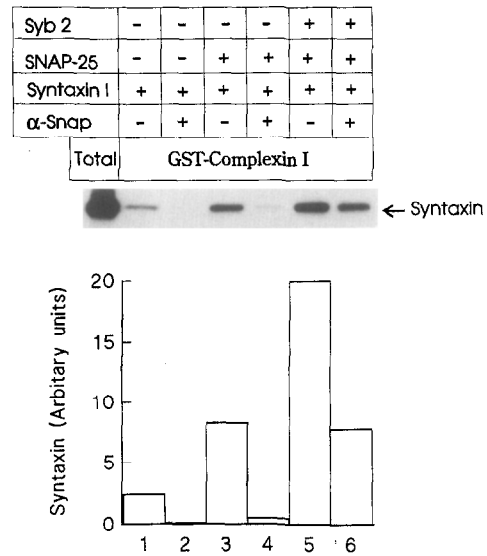| Syb 2	–	–	–	–	+	+
SNAP-25	–	–	+	+	+	+
Syntaxin I	+	+	+	+	+	+
α-Snap	–	+	–	+	–	+

Figure 8. α-SNAP Displaces Complexin from Syntaxin

Immobilized GST–complexin I was incubated with recombinant syntaxin from transfected COS cells with or without recombinant SNAP-25, synaptobrevin 2 (Syb 2), and α-SNAP. Bound syntaxin was visualized by immunoblotting using ^{125}I-labeled secondary antibodies and quantified. Similar results were obtained with syntaxin immunoprecipitations (data not shown).

that stoichiometrically bind to the core complex and compete with α-SNAP but not synaptotagmin for binding, suggesting that they function in exocytosis.

Complexins are abundant brain proteins that are stoichiometrically bound to the synaptic fusion complex in brain as revealed by immunoprecipitations. They are tightly conserved evolutionarily with 100% identity among rat, mouse, and human complexin II. The complexins are small hydrophilic proteins that are not related to known trafficking proteins. They are highly enriched in brain, where they are present in synapses and other parts of neurons; in addition, complexin II is expressed at low levels in peripheral tissues, suggesting a ubiquitous role.

The two complexins are differentially distributed in brain, and their sequence differences are evolutionarily conserved, raising the possibility that they are functionally different. However, no differences in their binding properties were observed. Similar to syntaxin and SNAP-25, complexins are present both in synapses and outside of synapses, but, different from these proteins, complexins are soluble proteins that are largely cytosolic whereas syntaxin and SNAP-25 are membrane proteins. Many brain proteins that function in synaptic vesicle traffic have non-neural isoforms that are ubiquitously distributed and have analogous functions in nonsynaptic membrane traffic; these isoforms are usually the products of distinct genes (for example, syntaxins and synaptobrevins/cellubrevins; Bennett et al., 1993; McMahon et al., 1993). In contrast with these proteins, the same isoform of the complexins appears to be used in both neural and peripheral tissues. In this regard as well as in their binding properties, com-
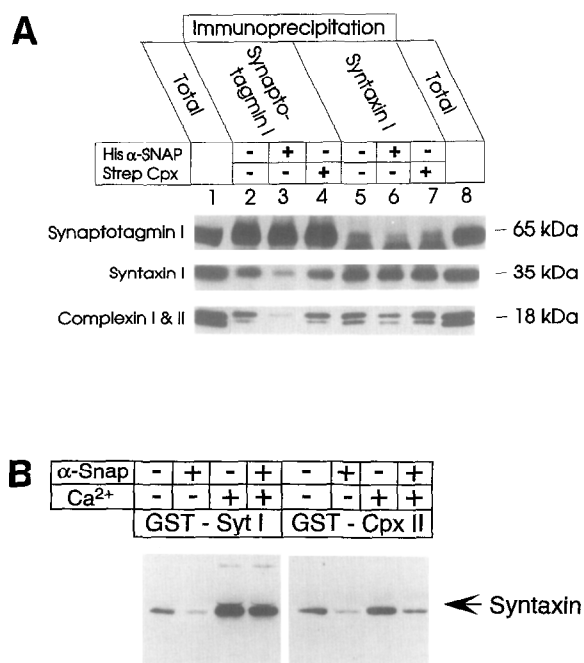
A

B

Figure 9. Synaptotagmin and Complexins Bind to Syntaxin at Non-competitive Sites with Distinct Characteristics

(A) Coimmunoprecipitation of synaptotagmin I with syntaxin and complexin: effects of recombinant α-SNAP and complexin. Synaptotagmin I (lanes 3–4) or syntaxin I (lanes 5–7) were immunoprecipitated from rat brain homogenates in the presence or absence of recombinant His-tagged α-SNAP or strep complexin (Cpx) as indicated. Proteins bound were analyzed by immunoblotting.

(B) Binding of syntaxin I from rat brain homogenates to immobilized GST–synaptotagmin and GST–complexin fusion proteins as a function of α-SNAP and Ca^{2+}. Note that syntaxin binding to the GST–synaptotagmin I fusion protein containing the first C_2 domain is greatly enhanced by Ca^{2+} (1 mM) whereas Ca^{2+} has no effect on the interaction of syntaxin with complexins or α-SNAP.

plexins are similar to α-SNAP, with which they also compete for binding. The use of the same gene products for different types of membrane traffic supports the notion of a conserved molecular scaffold that mediates diverse membrane fusion reactions.

The following evidence suggests that complexins function in regulating the SNAP receptor–core complex during membrane fusion. First, they are a component of the fusion complex as analyzed by immunoprecipitations from brain homogenates using antibodies against each of the components of the complex. Second, the complexins specifically and stoichiometrically bind to syntaxin I; the binding is dramatically enhanced if syntaxin I is assembled in the SNAP receptor–core complex, which induces a high affinity state in syntaxin for complexin and for α-SNAP. Although α-SNAPs and complexins are similar in their binding properties, they share no sequence homology. Third, complexins compete with α-SNAP for binding to the SNAP receptor–core complex. Although α-SNAP competes with both synaptotagmin I and complexins for binding to the core complex, complexins in turn only compete with α-SNAP but not with synaptotagmin I. Fourth, complexins are enriched in neurons, where they colocalize

with syntaxin and SNAP-25, but are also expressed in nonneural cells. Fifth, quantitative estimates based on immunoblots standardized with recombinant proteins suggest that in brain, syntaxin I is present at approximately 500 nmol/g protein, complexins I and II at 33 nmol, and α-SNAP at 5 nmol (data not shown). Thus, complexins and α-SNAP are present in a ratio at which they could meaningfully compete for a binding site.

Together, these findings suggest the hypothesis that complexins regulate SNAP binding to its receptor during membrane fusion and thereby contribute to determining the rate of fusion. Such a function would agree well with the striking effects of exogenous α-SNAP on exocytosis (DeBello et al., 1995; Morgan and Burgoyne, 1995). The lack of an effect of Ca^{2+} on complexin binding to syntaxin and the enormous increase in the binding affinity for complexins of the core complex over syntaxin alone suggest that complexins act before the final Ca^{2+}-dependent step in exocytosis. The fact that complexins and synaptotagmin I do not compete with each other for syntaxin binding, although each competes with α-SNAP, suggests that complexins could collaborate with synaptotagmin in acting on the fusion complex and either prevent premature action of α-SNAP and NSF or mediate the action of synaptotagmin on the fusion complex. Alternatively, complexins could regulate α-SNAP binding in preventing spontaneous fusion reactions or could drive the assembly of the core complex. Future experiments will have to test these hypotheses.

Regulation of exocytosis is particularly important for the synapse, but all types of membrane fusion are likely to be well controlled and to involve homologs of the synaptic fusion complex proteins. The high levels of complexins in neurons are compatible with the special regulatory requirements of this system whereas the low concentrations of complexin II in peripheral tissues are consistent with an analogous role in constitutive fusion reactions.

Experimental Procedures

Immunoprecipitations

Rat brains were homogenized in buffer A (0.15 M NaCl, 10 mM HEPES-NaOH [pH 7.4], 1 mM EGTA, 2 mM $MgCl_2$, 10 mg/l leupeptin, 0.1 g/l PMSF, 10 mg/l aprotonin, and 1 mg/l pepstatin A) and solubilized by addition of 1% NP-40. Insoluble material was removed by centrifugation, and the soluble extract was incubated with the respective immune or preimmune sera bound to protein A–Sepharose in the presence or absence of excess recombinant α-SNAP or complexin. After overnight incubations at 4°C, beads were washed three times in buffer A containing 1% NP-40. Bound proteins were analyzed by SDS-PAGE and Coomassie blue staining or immunoblotting. Quantitation was performed by densitometry of Coomassie-stained gels or phosphoimaging of blots reacted with iodinated secondary antibodies.

Cloning

Complexins I and II were individually purified by SDS-PAGE of unboiled samples of syntaxin immunoprecipitates and digested with endopeptidase Lys-C, and HPLC-purified peptides were sequenced as described (Hata et al., 1993). λZAPII cDNA libraries from rat brain, human temporal cortex, and human hippocampus were screened with degenerate oligonucleotides corresponding to peptide sequences KKEEERQE and VRQQIYD (Sambrook et al., 1989; Südhof et al., 1989). Seven rat complexin I and five rat complexin II clones, and one human complexin I and 14 human complexin II clones were isolated

and sequenced. A genomic Sv/129 mouse library (Stratagene) was screened with a probe from complexin I, yielding four complexin I and three complexin II clones that were also mapped and partially sequenced. All sequences are deposited in GenBank.

Expression of Proteins

The coding sequences of rat complexins I and II were PCR amplified without the initiator ATG but with flanking XbaI and HindIII sites, cloned into pGEX-KG, and expressed and purified as described previously (Guan and Dixon, 1991). Mammalian expression vectors were constructed by cloning the cDNA inserts of the rat clones p1137-15a (complexin I) and p1137-7a (complexin II) as EcoRI fragments into pCMV5. COS cell transfections of syntaxin 1A, SNAP-25, synaptobrevin 2, and complexins were performed as described previously (McMahon and Südhof, 1995). For production of streptavidin-tagged complexin I, the complexin I coding region was cloned into pASK-75 (Biometra), which creates a C-terminal fusion of complexin I with a 10 amino acid tag, and the recombinant protein was expressed and purified according to the protocol of the manufacturer. The expression vector for His-tagged α-SNAP was a gift of Dr. J. Rothman.

Protein–Protein Interaction Experiments Using Recombinant Proteins

Extracts from COS cells transfected with the various expression vectors were prepared by solubilization in buffer A containing 1% NP-40 and incubated with GST fusion proteins bound to glutathione–agarose beads in buffer A. Beads were washed three times in the same buffer and analyzed by SDS–PAGE and Coomassie blue staining or immunoblotting.

Antibodies

Polyclonal antibodies to syntaxin 1A (I378), α-SNAP (J373), Munc18 (J370), and complexins I (L669) and II (L668) were raised against purified recombinant GST–syntaxin, GST–complexins I and II, His–α-SNAP, and maltose-binding protein Munc18-1. Monoclonal antibodies to synaptobrevin (Cl 69.1), synaptophysin (Cl 7.2), syntaxin I (HPC-1), and synaptotagmin (Cl 41.1) were a gift from Dr. R. Jahn. SNAP-25 monoclonal antibody was purchased from Sternberger Monoclonals Incorporated.

Immunocytochemistry

Cryostat brain sections from perfusion-fixed adult rats and mice were stained with antibodies (serum at 1:300 dilution) using peroxidase detection and heavy metal enhancement as described previously (Rosahl et al., 1995). Neurons were cultured from rat hippocampus 6–25 days in vitro and analyzed by immunofluorescence labeling using standard procedures with antibodies at a 1:30 dilution (Banker and Cowan, 1977; Fletcher et al., 1994). Three controls were performed for all complexin immunocytochemistry experiments: use of preimmune sera, preabsorption of the antibody with the antigen, and omission of the first antibody.

Miscellaneous Procedures

SDS–PAGE was carried out as described by Laemmli (1970) using 7% or 13% gels as appropriate. Proteins were detected after blotting by either enhanced chemiluminescence (ECL) (Amersham) or ^{125}I-labeled secondary antibodies followed by quantitation on a Molecular Dynamics phosphoimager. RNA blots were purchased from Clontech and consecutively hybridized with a 0.47 kb BglII–EcoRI fragment of p1137-15a (complexin I) and a 0.66 kb EcoRI fragment of p1137-6a (complexin II).

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