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Mixed and Non-cognate SNARE Complexes

CHARACTERIZATION OF ASSEMBLY AND BIOPHYSICAL PROPERTIES*

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Assembly of soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) proteins between two opposing membranes is thought to be the key event that initiates membrane fusion. Many new SNARE proteins have recently been localized to distinct intracellular compartments, supporting the view that sets of specific SNAREs are specialized for distinct trafficking steps. We have now investigated whether other SNAREs can form complexes with components of the synaptic SNARE complex including synaptobrevin/VAMP 2, SNAP-25, and syntaxin 1. When the Q-SNAREs syntaxin 2, 3, and 4, and the R-SNARE endobrevin/VAMP 8 were used in various combinations, heat-resistant complexes were formed. Limited proteolysis revealed that these complexes contained a protease-resistant core similar to that of the synaptic complex. All complexes were disassembled by the ATPase *N*-ethylmaleimide-sensitive fusion protein and its cofactor α-SNAP. Circular dichroism spectroscopy showed that major conformational changes occur during assembly, which are associated with induction of structure from unstructured monomers. Furthermore, no preference for synaptobrevin was observed during the assembly of the synaptic complex when endobrevin/VAMP 8 was present in equal concentrations. We conclude that cognate and non-cognate SNARE complexes are very similar with respect to biophysical properties, assembly, and disassembly, suggesting that specificity of membrane fusion in intracellular membrane traffic is not due to intrinsic specificity of SNARE pairing.

SNAREs[1] represent a protein superfamily that is thought to play a key role in all intracellular membrane fusion events within eukaryotes (1–6). They possess a homologous domain of approximately 60 amino acids referred to as the SNARE motif (7). The best characterized SNAREs are those mediating exocytosis of synaptic vesicles in neurons. They include the vesicle protein synaptobrevin (also referred to as VAMP) and the membrane proteins SNAP-25 and syntaxin 1. *In vitro*, these proteins form a stable ternary complex that is reversibly dissociated by the soluble ATPase NSF in conjunction with soluble cofactors termed SNAPs (8, 9). Assembly and disassembly of SNAREs has recently been investigated in detail by several laboratories (5, 6, 10–12). It is generally believed that it is the formation of a ternary complex between complementary SNAREs residing on the membranes destined to fuse ("trans" complexes) that drives the fusion reaction. After fusion, the complexes are disassembled by NSF and SNAPs and thus re-energized for another round of membrane fusion.

According to the original SNARE hypothesis (1), each fusion step in membrane trafficking would be mediated by a unique set of SNAREs. These would function only in one fusion step and be excluded from others. This specificity was thought to be caused by the intrinsic affinity of SNAREs for each other, *i.e.* only cognate SNAREs were thought to bind to each other. Recently, however, it has become clear that at least some SNAREs can function in multiple trafficking steps such as the yeast proteins Sed5p and Vti1p (13–15). Furthermore, these proteins apparently participate in the formation of several different SNARE complexes, suggesting that they are able to pair with more than one set of partners.

SNARE complex assembly is mediated by the SNARE motifs of the participating proteins which form a protease-resistant core domain (16, 17). The transmembrane regions of syntaxin and synaptobrevin are directly adjacent to the SNARE motifs, aligned at one end of the core domain (18, 19). A dramatic increase in α-helical content is associated with SNARE complex formation, showing that major conformational changes occur during assembly. (12, 20–23). These features, together with the heat stability of the complex (21), led to the proposal that the SNAREs "zipper up" during assembly, forcing the transmembrane domains into close proximity and thus pull the fusing membranes together (3, 4). The energy released during assembly would thus be used to overcome the energy barrier separating the two membranes (21).

The central domain of the synaptic SNARE complex is represented by a 12-nm-long bundle consisting of four parallel α-helices that are wound around each other (24). The interacting amino acids form distinct layers perpendicular to the axis of the four helix bundle, which are similar to those found in typical coiled-coils. These layers are formed by hydrophobic amino acid side chains with the exception of an ionic layer in the middle which consists of three glutamine residues, contributed by syntaxin and the two SNARE-motifs of SNAP-25, and one arginine residue, contributed by synaptobrevin (24). The striking conservation of the glutamine (Q) and arginine (R) throughout the entire SNARE superfamily led us to reclassify SNAREs into Q- and R-SNAREs (25). The hydrophobic layers in the four helix bundle are also conserved whereas residues

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF132812.

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[1] The abbreviations used are: SNARE, SNAP receptor; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNAP-25, synaptosomal-associated protein of 25 kDa; BoNT, botulinum neurotoxin; GST, glutathione *S*-transferase; CD, circular dichroism; MALLS, multi-angle laser light scattering; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Tricine, *N*-tris(hydroxymethyl) methylglycine.

exposed at the surface are much more variable. The ability to form four helix bundles is probably the essential feature of the SNAREs that is conserved among the entire superfamily (25).

Given the high degree of core residue conservation the question arises if SNARE pairing is as specific as previously assumed. Numerous side-chain interactions were observed at the surface of the synaptic SNARE complex, particularly between SNAP-25 and syntaxin (24), which involve non-conserved residues (25). It is not known to which extent these interactions contribute to the overall stability of the SNARE complex or to the kinetics of SNARE assembly. Since the overall sequence homology between more distant members of the SNARE superfamily is relatively low, such interactions may contribute to pairing specificity. If, however, the surface interactions of the SNARE complex do not exert a decisive influence on complex assembly and stability, one would predict that complexes form not only between cognate but also between non-cognate SNAREs. If complex formation is indeed indiscriminatory, then one must look elsewhere for explaining the specificity of membrane fusion events.

In order to address this issue, we have investigated whether different SNAREs can form complexes, and if so, whether these complexes resemble the neuronal SNARE complex. In our experiments, we have focused on complexes containing either closely related or distant relatives of the neuronal SNAREs. Our results show that all SNAREs investigated here can be combined in arbitrary composition to yield complexes of very similar biophysical properties. All complexes have a high α-helical content, contain a protease-resistant core domain, are heat-stable, and are disassembled by NSF. Furthermore, the distant synaptobrevin relative, endobrevin/VAMP 8, is not discriminated from synaptobrevin upon assembly with the neuronal SNAREs SNAP-25 and syntaxin 1.

EXPERIMENTAL PROCEDURES

Materials—NSF and α-SNAP in pQE-9 plasmids encoding for His_6-tagged fusion proteins were kindly provided by S. Whiteheart and J. E. Rothman (Memorial Sloan-Kettering Cancer Center, New York). Syntaxin 1A (residues 1–265) in the pET22b vector encoding for a factor Xa cleavable COOH-terminal His_6 fusion protein was kindly provided by A. T. Brünger (Yale University, New Haven, CT). The recombinant protein fragments were derived from cDNAs encoding for rat synaptobrevin 2 and rat syntaxin 1A (kindly provided by R. H. Scheller, Stanford University School of Medicine, Stanford, CA) and for SNAP-25A (kindly provided by T. C. Südhof, University of Texas Southwestern Medical Center, Dallas, TX). Recombinant light chain of botulinum neurotoxin E (BoNT/E) was a generous gift of H. Niemann (Institut für Biochemie, Medizinische Hochschule Hannover, Hannover, Germany).

Molecular Cloning of cDNA Encoding for Rat Endobrevin—Rat endobrevin was amplified by PCR using primers annealing outside of the coding region based on sequence information from Expressed Sequence Tag data base. cDNA from rat liver, lung, and kidney was used as a template. The PCR product was subcloned into pBS vector and sequenced. All constructs derived from the three different tissues were identical. The rat endobrevin amino acid sequence was 99% identical to mouse endobrevin (26, 27).

Immunoprecipitation—PC12 cell homogenates were prepared by passing the cell suspension 10 times through a ball cracker. Postnuclear supernatant was generated by centrifugation at $1000 \times g$ for 10 min and solubilized in extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 1% (v/v) Triton X-100) at a final protein concentration of 0.5 mg/ml. Lysates were clarified by centrifugation at $200{,}000 \times g$ for 60 min. After transfer of the supernatant to a fresh tube, immunoprecipitations were conducted for 2 h at 4 °C with a monoclonal antibody against synaptobrevin (69.1) (28) or a serum against endobrevin (residues 1–74) that was raised in rabbits. The serum against endobrevin had been affinity-purified. Antibodies were bound to Protein G-Sepharose beads (Amersham Pharmacia Biotech) for 30 min, sedimented, and washed eight times with extraction buffer. The supernatants were precipitated according to Wessel and Flügge (29). The immunoprecipitates and 10% of the precipitated supernatants were analyzed by SDS-PAGE and immunoblotting using the antibody described above for endobrevin, 69.1 for synaptobrevin, and the monoclonal antibody HPC-1 for syntaxin-1 (30).

Generation of Recombinant Fusion Proteins—Coding sequences were amplified by PCR using primers with appropriate restriction sites for subsequent subcloning into the desired plasmid. The sequence encoding for the cytoplasmic region of rat endobrevin (residues 1–74) was subcloned into pGEX-KG (Amersham Pharmacia Biotech) via *Bam*HI and *Eco*RI restriction sites resulting in a fusion protein with glutathione *S*-transferase (GST). The cytoplasmic domains of rat syntaxin 2 (1–265), syntaxin 3 (1–260), and syntaxin 4 (1–273) were subcloned into the pHO2c vector (21) via *Nde*I and *Eco*RI restriction sites resulting in fusion proteins carrying a carboxyl-terminal His_6 tag. In order to obtain better expression, rat SNAP-25A was subcloned into the vector pET28a (Novagen) via *Nhe*I and *Xho*I restriction sites resulting in an amino-terminal His_6 tag. In addition, four cysteines (Cys-84, -85, -90, and -92) were replaced by serines by the overlapping primer method of Higuchi (31). No difference in structural and binding properties to the cysteine containing SNAP-25 construct (21) was observed (data not shown).

Protein Purification—GST-endobrevin (residues 1–74) was purified by affinity chromatography on glutathione-Sepharose beads essentially as described (20). After purification, the GST tag was cleaved by thrombin. Fusion proteins containing His_6 tags (syntaxin 1 (1–265), pET22b; syntaxin 1 (180–262), pET28a (24); syntaxin 2 (1–265), pHO2c; syntaxin 3 (1–260), pHO2c; syntaxin 4 (1–273), pHO2c, SNAP-25A, pET28a; synaptobrevin 2 (1–96), pET28a (24)) were purified by Ni^{2+}-Sepharose as described (16, 21). After elution from the affinity matrices, all recombinant proteins were dialyzed against standard buffer and further purified by ion exchange chromatography using Mono-Q or Mono-S columns on an FPLC system (Amersham Pharmacia Biotech). After loading, the proteins were eluted with a linear gradient of NaCl in 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (standard buffer). The peak fractions were pooled and dialyzed against standard buffer containing 100 mM NaCl. The eluted proteins were about 95% pure, as determined by SDS gel electrophoresis. All binary and ternary complexes were purified using a Mono-Q column (Amersham Pharmacia Biotech) after overnight assembly of the purified monomers. Protein concentrations were determined by absorption at 280 nm and the Bradford assay (32).

Limited Proteolysis—The purified ternary complexes were subjected to limited digestion in standard buffer containing 100 mM NaCl using proteinase K in a ratio of 1:100 (w:w) protease:protein complex at 25 °C for 5 min. For analysis by SDS-PAGE, PMSF-containing SDS sample buffer was added. For analysis by size-exclusion chromatography on a HR-10/30 Superdex-200 column (Amersham Pharmacia Biotech) followed by multi-angle laser light scattering (MALLS), the reaction was stopped by adding 1 mM PMSF and placing the samples on ice.

Disassembly Reaction—Ternary complexes were disassembled by addition of 3 μM NSF, 11 μM α-SNAP, 2 mM $MgCl_2$, 2.5 mM ATP in standard buffer for 40 min at 30 °C in the presence of 1.5 μM BoNT/E light chain. The reaction was stopped by heating the samples for 5 min at 95 °C in SDS-sample buffer. As controls, the reaction was carried out either in absence of NSF and α-SNAP, or the ATPase activity of NSF was abolished by replacing $MgCl_2$ with 10 mM EDTA. As an assay for disassembly, cleavage of SNAP-25 by BoNT/E was monitored using Tricine electrophoresis for fragment separation. For immunodetection of SNAP-25, the monoclonal antibody Cl 71.1 (33) was used.

CD Spectroscopy—Far UV CD spectra were obtained by averaging over 5–50 scans using steps of 0.2 nm with a scan rate of 50 nm/min on a Jasco model J-720 upgraded to a J-715U equipped with a 6-Position Peltier Effect Cell Changer. Measurements were performed in Hellma quartz cuvettes with path lengths of 0.1 cm. All CD spectra were recorded after reaching equilibrium following an overnight incubation at 4 °C in the standard buffer. To evaluate changes of the CD spectrum attributable to complex formation, the spectra were compared with the theoretically noninteracting sum of the individual spectra using the equation $[\theta]_{sum} = \Sigma_i c_i n_i [\theta]_i / \Sigma_i c_i n_i$, where c_i are the respective concentrations of the proteins, n_i are the respective numbers of amino acid residues, and $[\theta]_i$ are the mean residue ellipticities of the individual proteins. For thermal melts, the ellipticity at 220 nm was measured between 25 and 95 °C with a temperature increment of 30 °C/h.

Electrophoretic Procedures—Routinely, SDS-PAGE was carried out as described by Laemmli (34). When testing for SDS resistance, samples were solubilized in SDS sample buffer (final concentrations: 60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 3% β-mercaptoethanol) and incubated at room temperature (not boiled) or 95 °C (boiled) for 5 min before analysis on a 15% polyacrylamide gel. For analysis of the constituents of the protease-resistant core complex, Tricine gel electrophoresis (16.5% T, 6% C) was used (35).

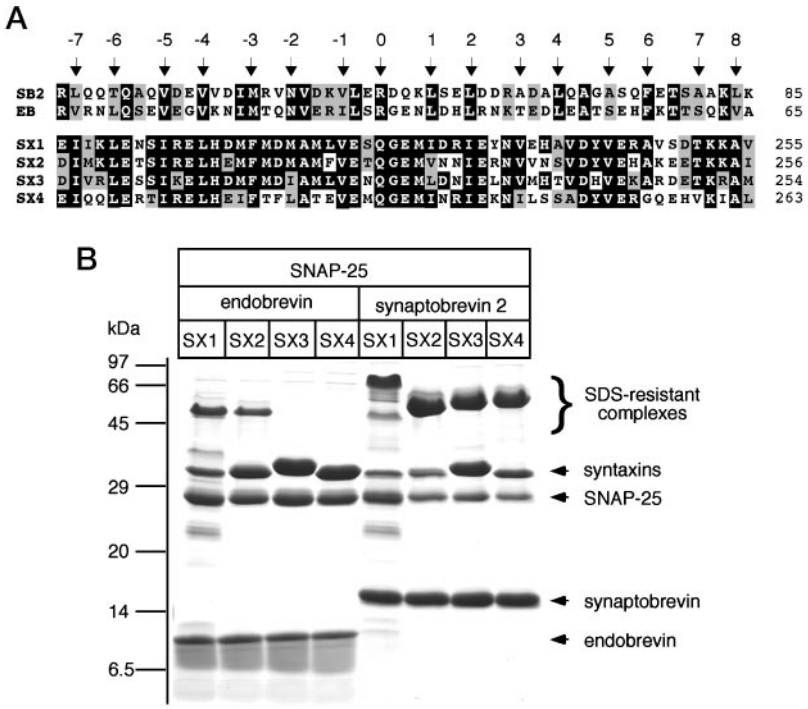

FIG. 1. **Formation of SDS-resistant complexes between various SNARE proteins.** *A*, sequence alignment of the subset of syntaxin and synaptobrevin homologs used for complex formation. Alignment is restricted to the region encompassing the 16 interacting layers of the synaptic fusion complex. The residues participating in the layers are indicated by *arrows* and *numbered* as in Refs. 24 and 25. Identical and conserved amino acids are *darkly* and *lightly shaded*, respectively. GenBank™ accession numbers are as follows: synaptobrevin 2 (M24105), endobrevin/VAMP 8 (pending), syntaxin 1A (D45208), syntaxin 2 (L20823), syntaxin 3 (L20820), and syntaxin 4 (L20821). *B*, approximately equal molar ratios of purified SNARE proteins were mixed, incubated overnight, and subjected to SDS-PAGE without boiling of the sample. After the run, the gel was stained with Coomassie Blue. Synaptobrevin forms SDS-resistant complexes with syntaxin 1–4 (*SX1*, *2*, *3*, and *4*, respectively), whereas endobrevin only forms SDS-resistant complexes with syntaxin 1 and 2.

Multi-angle Laser Light Scattering—Size-exclusion chromatography was performed on a HR-10/30 Superdex-200 column (Amersham Pharmacia Biotech) in standard buffer containing 150 mM NaCl at a flow rate of 0.5 ml/min. The elution profiles were monitored by UV absorption at 280 nm, light scattering at 632.8 nm, and differential refractometry. Light scattering and differential refractometry were carried out using the Dawn and Optilab instruments, respectively, of Wyatt Technology Corp. Analysis was carried out as described by Astra software (36). For each sample, 100 μl of protein solution (between 0.5 and 1 mg/ml protein) was loaded. The *dn/dc* value (change of solution refractive index with respect to a change in concentration of the molecules being investigated) is fairly constant for proteins (37) and was set to 0.189 for the analysis of the light scattering data.

RESULTS

Biochemical and Biophysical Properties of Mixed SNARE Complexes—Four different SNARE proteins were examined for their ability to form complexes with the SNAREs involved in neuronal exocytosis. These included syntaxin 2, syntaxin 3, and syntaxin 4, three relatives of syntaxin 1 that exhibit a similar domain structure (38), and are significantly homologous within the SNARE motifs (Fig. 1*A*). The homology is not limited to the amino acids participating in core interactions but includes residues on the surface. Despite their differential tissue distribution and intracellular localization, these syntaxins are probably involved in fusions of transport vesicles with the plasma membrane and thus may interact physiologically with the neuronal SNAREs in certain cell types. As the fourth example, we chose the R-SNARE endobrevin/VAMP 8, a distant relative of synaptobrevin, which is localized to endosomal compartments (26, 27). Within the SNARE motif, only the amino acids of the core layers are partially conserved, with much less similarity in the rest of the sequence (Fig. 1*A*).

For the binding experiments, all proteins were expressed in *Escherichia coli* and purified (see "Experimental Procedures" for details). The proteins were mixed in various combinations and then analyzed by SDS-PAGE for the formation of SDS-resistant complexes. It was shown previously that the synaptic SNARE complex is resistant to SDS, a feature widely used for monitoring complex formation (39). Of the eight SNARE combinations, six formed SDS-resistant complexes, as demonstrated by the appearance of protein bands with apparent molecular masses corresponding to ternary complexes (Fig. 1*B*). No SDS-resistant complex was observed with the combinations SNAP-25/endobrevin/syntaxin 3 and syntaxin 4, respectively. However, complex formation was detected when the samples were analyzed by non-denaturing PAGE or size exclusion chromatography (data not shown, see also below).

For further analysis, we chose three representative complexes including endobrevin/syntaxin 1/SNAP-25, synaptobrevin 2/syntaxin 4/SNAP-25, and endobrevin/syntaxin 3/SNAP-25. These complexes were purified by ion exchange chromatography and then subjected to limited proteolysis. Previously, it was shown that the core domain of the synaptic SNARE complex, *i.e.* the region of the interacting SNARE motifs, is protease-resistant, whereas non-interacting SNARE motifs are efficiently cleaved (16, 17). As shown in Fig. 2*a*, digestion of all three complexes with proteinase K yielded a fragment migrating at about 16 kDa and a group of bands migrating between 8 and 14 kDa. When the digests of the endobrevin/syntaxin 1/SNAP-25 and synaptobrevin 2/syntaxin 4/SNAP-25 complexes were not heated prior to electrophoresis, a single SDS-resistant band was visible instead of the group of 8–14-kDa bands whereas the 16-kDa band remained unchanged (Fig. 2*B*). This result precisely corresponds to the observations made previously for the synaptic SNARE complex (16) and indicates that the 16-kDa represents the NH_2-terminal domain of the respective syntaxins, whereas the 8–14-kDa bands represent the complex-forming SNARE motifs. These

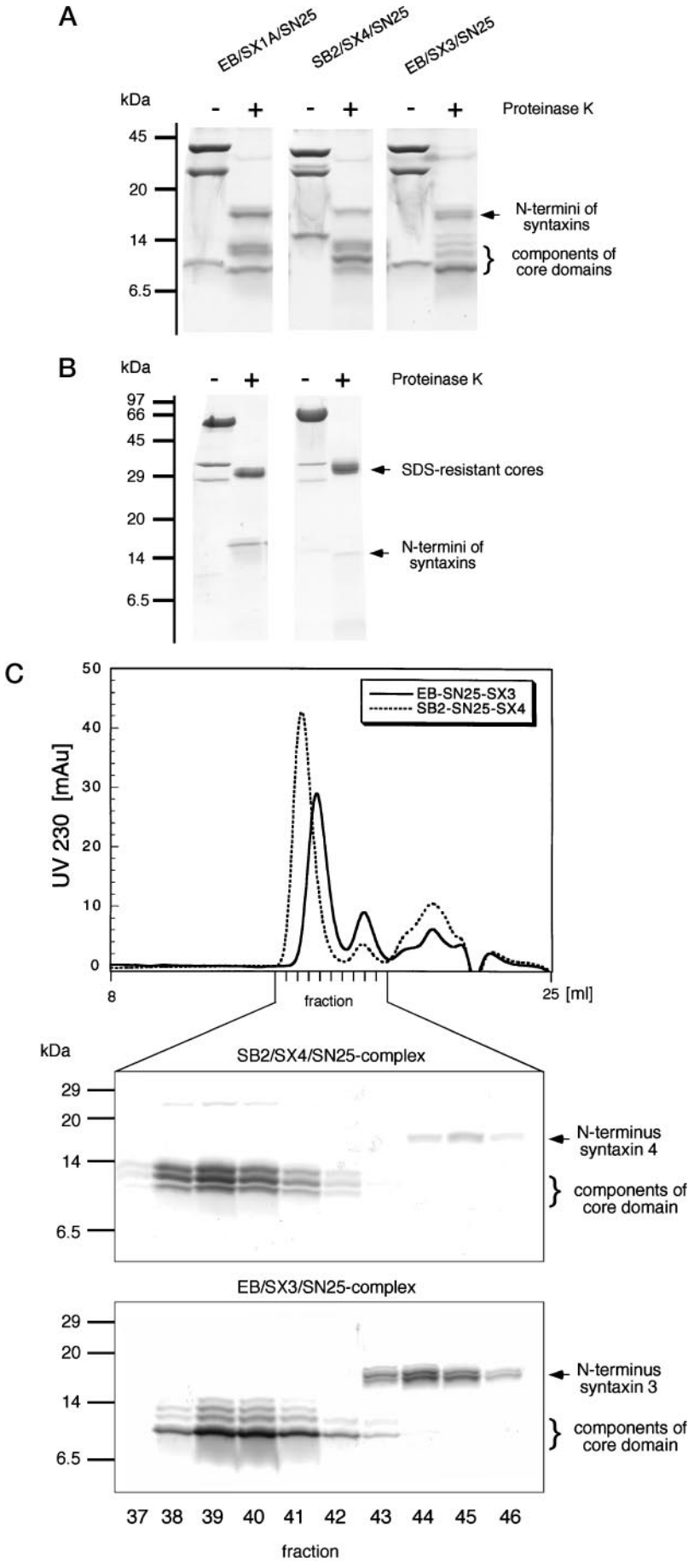

FIG. 2. **Limited proteolysis reveals a similar domain structure of the non-cognate SNARE complexes to the neuronal SNARE complex.** The following non-cognate SNARE complexes were purified and subjected to limited proteolysis by proteinase K: endobrevin/syntaxin1/SNAP-25 (*EB/SX1/SN25*), synaptobrevin 2/syntaxin 4/SNAP-25 (*SB2/SX4/SN25*), and endobrevin/syntaxin 3/SNAP-25 (*EB/SX3/SN25*). *A*, after digest all samples were boiled in SDS-containing sample buffer and analyzed by the Tricine variant of SDS-PAGE (35) followed by Coomassie Blue staining. The positions of the NH_2-terminal domains of the respective syntaxins, and of the fragments contributing to the core domains, are indicated. *B*, same as in *A* but without boiling of the sample. An SDS-resistant core SNARE domain is separated from a proteolytic fragment of the NH_2-terminal region of the respective syntaxin as indicated. *C*, after proteolysis, the complexes endobrevin/syntaxin 3/SNAP-25 (*EB/SX3/SN25*), which does not form an SDS-resistant complex, and synaptobrevin 2/syntaxin 4/SNAP-25 (*SB2/SX4/SN25*) were objected to size exclusion chromatography on a Superdex 200 column. Fractions containing proteolytic fragments were separated by Tricine gel electrophoresis. For both complexes, the core SNARE domain consisting of several small proteolytic fragments with an apparent molecular mass between 14 and 8 kDa is separated from the NH_2-terminal domain of the respective syntaxin.

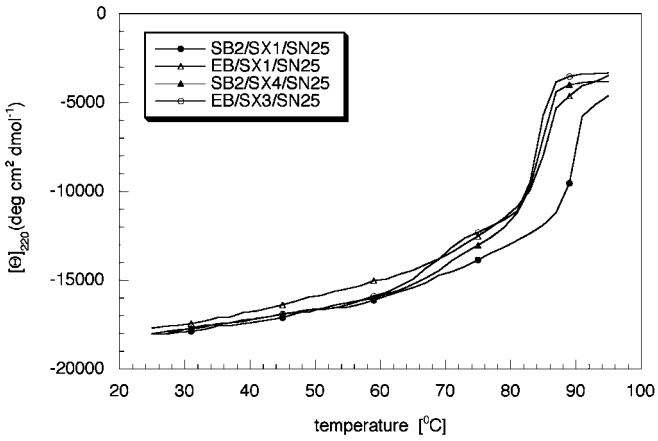

FIG. 3. **Thermal stability of SNARE complexes.** The following SNARE complexes were purified by ion exchange chromatography: synaptobrevin 2/syntaxin 1/SNAP-25 (*SB2/SX1/SN25*), endobrevin/syntaxin 1/SNAP-25 (*EB/SX1/SN25*), synaptobrevin 2/syntaxin 4/SNAP-25 (*SB2/SX4/SN25*), and endobrevin/syntaxin 3/SNAP-25 (*EB/SX3/SN25*). Thermal denaturation of these complexes was monitored by circular dichroism (*CD*) spectroscopy. The change in the mean residue ellipticity [θ] at 220 nm of the purified SNARE complexes was measured in standard buffer containing 100 mM NaCl.

findings suggest that protease-resistant core domains are formed that are very similar to that of the synaptic complex.

No such SDS-resistant band containing the 8–14-kDa SNARE motifs was observed with the endobrevin/syntaxin 3/SNAP-25 complex (data not shown). Therefore, we have analyzed the digest of this complex by size-exclusion chromatography. Fig. 2*C* shows that a single major peak eluted from the column which contained a group of 8–14-kDa bands (presumably representing the core complex forming SNARE motifs). The 16-kDa band (presumably representing the NH_2-terminal domain of syntaxin 3) was well separated and eluted at a position corresponding to a smaller molecular mass (Fig. 2*C*). The first peak eluting from the column was further analyzed by MALLS, a procedure allowing for a direct determination of the molecular mass irrespective of the shape of the complex (36). The procedure resulted in a molecular mass of 41.3 ($\pm$ 0.7) kDa, which is similar to that of the core of the synaptic SNARE complex (16). A similar elution profile was obtained when the digest of the SDS-resistant synaptobrevin 2/syntaxin 4/SNAP-25 complex was separated (Fig. 2*C*). This indicates that these complexes contain the four SNARE motifs in a 1:1:1:1 stoichiometry.

As outlined above, the synaptic complex is represented by an extended bundle of four α-helices yielding a characteristic α-helical spectrum in CD measurements. CD-spectroscopy of the three non-cognate complexes analyzed here resulted in similar spectra (data not shown), further suggesting that the structure of all complexes is very similar. Using CD spectroscopy as a means to monitor unfolding, we next examined the thermal stability of the complexes. Previous work has shown that the synaptic complex is remarkably resistant to thermal denaturation, a feature believed to be a hallmark of SNARE complexes (21). As shown in Fig. 3, the thermal denaturation curves of the three complexes are virtually superimposable. All three complexes are almost as stable as the neuronal complex (Fig. 3) and clearly more stable than the exocytotic SNARE complex of *Saccharomyces cerevisiae* (22). Together these data show that the features of all complexes are very similar and suggest that differential sensitivities to SDS do not reflect major differences in the biochemical and biophysical properties.

Disassembly of Mixed SNARE Complexes—The structural

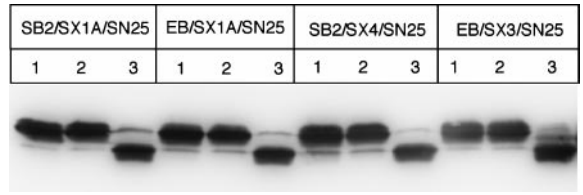

FIG. 4. **Disassembly of SNARE complexes.** The purified SNARE complexes (see Fig. 3) were incubated with BoNT/E alone (*lanes 1*), or with BoNT/E, NSF, and α-SNAP, in the presence of EDTA (*lanes 2*) or Mg^{2+} (*lanes 3*). NSF-driven disassembly rendered SNAP-25 susceptible to cleavage by the BoNT/E light chain that was visualized by Tricine gel electrophoresis and immunoblotting using the monoclonal antibody Cl 71.1 (33).

similarities between the various SNARE complexes prompted us to investigate whether these complexes are "functional" with respect to the action of the disassembly chaperone NSF. With a few specialized exceptions, NSF is thought to operate on all SNARE complexes (1, 15). NSF binds to SNARE complexes in the presence of α-SNAP and ATP. When ATP hydrolysis is permitted, the complexes reversibly disassemble into their monomeric constituents. This reaction is currently thought to be responsible for the regeneration of active SNAREs after fusion is complete.

Purified SNARE complexes were incubated in the presence of recombinant NSF and α-SNAP under conditions either allowing or prohibiting ATP hydrolysis by the ATPase NSF. To measure disassembly, we monitored the cleavage of SNAP-25 by the light chain of botulinum neurotoxin E (BoNT/E). Botulinum neurotoxins cleave the neuronal SNAREs only in the disassembled state, whereas the ternary complex is toxin-resistant (39–42). Fig. 4 (*lanes 1*) shows that not only the neuronal complex (*left panel*) but also the heterologous SNARE complexes are resistant to BoNT/E. When ATP hydrolysis by NSF was permitted, SNAP-25 was efficiently cleaved in each case (Fig. 4, *lanes* 3), demonstrating that all complexes can be disassembled by NSF and α-SNAP.

Further Characterization of the Non-cognate Endobrevin/Syntaxin 1/SNAP-25 Complex—In the last series of experiments, we investigated whether structural and kinetic properties of the assembly reaction are changed when synaptobrevin is replaced by its distant relative endobrevin in the synaptic complex. The cognate SNARE partners of endobrevin are not yet known, but its localization and its tissue distribution make it highly unlikely that it interacts with the synaptic SNAREs in intact cells. To confirm that endobrevin does not form complexes with the neuronal SNAREs, endobrevin and synaptobrevin 2 were coimmunoprecipitated from detergent extracts of PC12 cells. As shown in Fig. 5, syntaxin 1 coprecipitated with synaptobrevin 2 but not with endobrevin even though precipitation of endobrevin was almost quantitative.

As in our previous work (16, 20, 21), we used CD spectroscopy to monitor structural changes during assembly. We had shown before that synaptobrevin is unfolded as a monomer but assumes an α-helical conformation upon assembly (21). Similarly, the CD spectrum of monomeric endobrevin was typical for unfolded proteins (data not shown) (43). However, a large increase in α-helical content is observed upon formation of the endobrevin/syntaxin 1/SNAP-25 complex (Fig. 6), which is comparable to that observed during the formation of the synaptobrevin/syntaxin 1/SNAP-25 complex (21).

These data show that the assembly of the endobrevin complex involves structural changes remarkably similar to that of the genuine synaptic SNARE complex. However, despite these structural and thermodynamic similarities, it cannot be ruled out that there is a kinetic preference for the formation of the native complex. To test for this possibility, we monitored for-

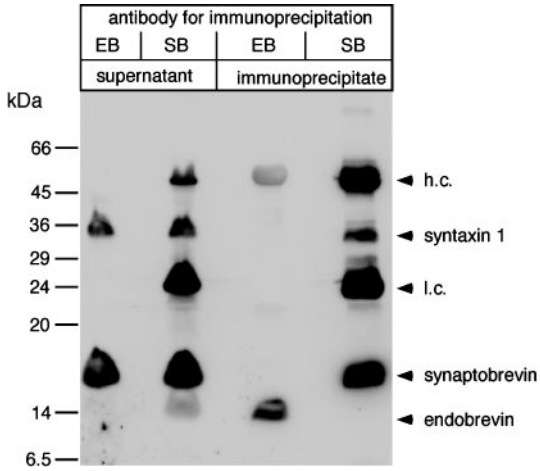

FIG. 5. **Endobrevin does not form a complex with syntaxin 1 in PC12 cells.** Immunoblot analysis of immunoprecipitations from Triton X-100 solubilized PC12 cells using an affinity-purified polyclonal antibody for endobrevin (*EB*) or the 69.1 (*28*) monoclonal antibody for synaptobrevin 2 (*SB*). The immunoprecipitate and supernatant were analyzed with the endobrevin and synaptobrevin antibodies used for precipitation and the monoclonal HPC-1 antibody for syntaxin 1 (30). *h.c.* and *l.c.* indicate the positions of the IgG heavy and light chains, respectively.

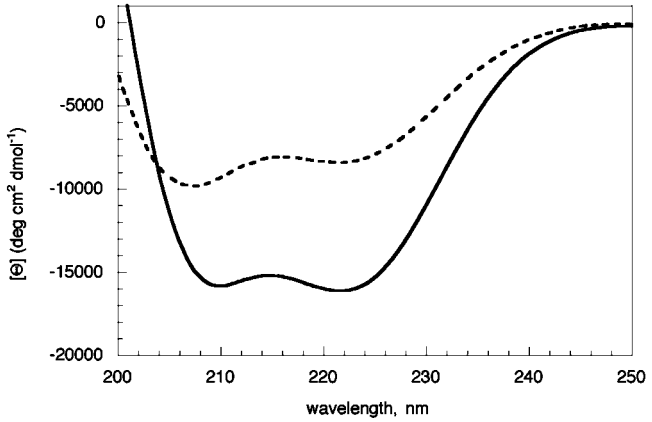

FIG. 6. **Structural changes upon formation of a non-cognate SNARE complex between endobrevin, SNAP-25, and syntaxin 1.** Upon mixing of endobrevin with the neuronal SNAREs SNAP-25, and syntaxin 1, a major increase in α-helical content was observed. *Dotted lines* represent the theoretically noninteracting mean residue ellipticities calculated from the observed spectra of the individual proteins. The CD spectrum of the combined components was measured in standard buffer containing 100 mM NaCl at 25 °C after overnight incubation of the proteins.

mation of ternary complexes in the presence of about equal concentrations of endobrevin and synaptobrevin. The syntaxin 1/SNAP-25 binary complex was purified and incubated either with synaptobrevin alone, endobrevin alone, or with a mix of both proteins. Parallel experiments were carried out in which only the SNARE motif of syntaxin (SX180–262) was present in the binary complex with SNAP-25. Complex formation was monitored by the appearance of SDS-resistant complexes, which were distinguishable due to their different apparent molecular masses. As shown in Fig. 7, about equal amounts of each of the ternary complexes formed when synaptobrevin and endobrevin were present, demonstrating that there is no kinetic preference for the cognate *versus* the non-cognate R-SNARE regardless of whether intact or truncated syntaxin was used.

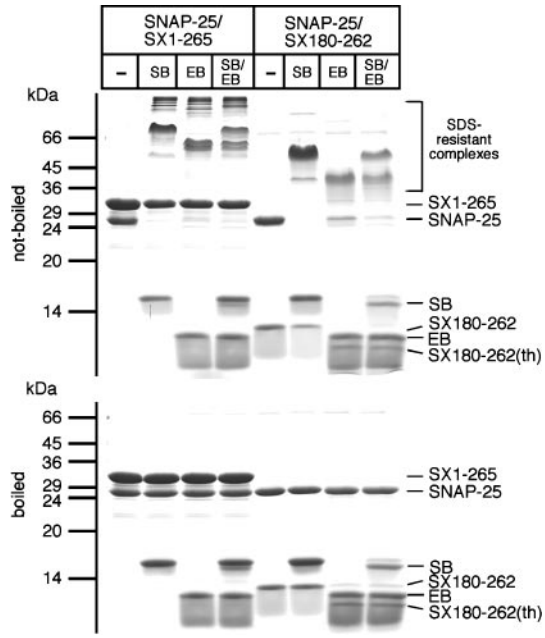

FIG. 7. **Endobrevin and synaptobrevin 2 are equally efficient in binding to the synaptic SNAREs syntaxin 1 and SNAP-25.** Purified binary complexes consisting of SNAP-25 and either the whole cytoplasmic region of syntaxin (*SX1–265*) or a fragment of syntaxin containing the SNARE motif (*SX180–262*) (16, 21) were incubated with either the R-SNAREs synaptobrevin (*SB*), endobrevin (*EB*), or a mixture of both at about equal concentrations. After 2 h of incubation at 25 °C, the samples were analyzed by SDS-PAGE with (*lower panel*) or without (*upper panel*) boiling in SDS sample buffer followed by Coomassie Blue staining. Both R-SNAREs were able to form SDS-resistant ternary SNARE complexes. When incubated simultaneously, about equal amounts of SDS-resistant SNARE complexes containing either synapto- or endobrevin were formed. Note that, due to carryover of residual thrombin in the endobrevin-containing sample, the His_6 tags of synaptobrevin, SX180–262, and SNAP-25 were cleaved, giving rise to band multiplicity. The thrombin-cleaved band of the syntaxin fragment is indicated by *SX180–262 (th)*.

DISCUSSION

In the present study, we have shown that complex formation between SNARE proteins is less specific than previously assumed (1, 44). Using four different syntaxins and a distant relative of synaptobrevin, we found that promiscuous SNARE complexes can be formed in arbitrary combination. The features of these complexes are remarkably similar to those of the genuine synaptic complex with respect to assembly, disassembly, and biophysical properties, strongly suggesting that they are, at least *in vitro*, functionally interchangeable.

The crystal structure of the core domain of the synaptic SNARE complex allowed the identification of layers of interacting amino acids in the core of the four helix bundle (24). The data presented here strongly support the view that these interactions are indeed essential in defining the features of SNARE complexes. Modeling showed previously that syntaxin 1 can be replaced with syntaxin 4 without major steric and electrostatic penalties (25), a hypothesis now supported by experimental evidence. However, the degree of promiscuity in complex formation between distantly related SNAREs was surprising. The sequence identity between endobrevin and synaptobrevin is low (33%) (26, 27), but the amino acids forming the core layers are either identical or at least similar (Fig. 1*A*).

Several conclusions can be drawn from these observations. First, it is becoming clear that the amino acids in the core are the essential residues for SNARE complex formation with the residues on the surface of the complex being less important. Second, the features of these complexes are virtually indistinguishable with respect to domain structure, stability, confor-

mational change, and disassembly. These remarkable similarities indicate that at least the complexes investigated here form four-helix bundles supporting our previous hypothesis that all SNARE complexes exhibit this basic structure (25). Apparently, "drifts" in these features were not tolerated during evolution, even though overall sequences are highly variable. This lends strong support to the idea that it is these features that are required for function, in full agreement with the current "zipper" model of SNARE function in membrane fusion (3). We conclude that no intrinsic property prevents "false" SNAREs from forming complexes with each other, disproving one of the original tenets of the SNARE hypothesis (1).

These arguments suggest that one needs to look elsewhere in order to explain the indisputable specificity in SNARE interactions. As outlined in the Introduction, there is evidence that individual SNAREs participate in multiple interactions in yeast (14, 15). However, SNAREs are remarkably specific with respect to their subcellular localization (27, 45). Unfortunately, there is presently no reliable biochemical method for discriminating cognate from non-cognate SNARE complexes, although lack of co-immunoprecipitation is generally regarded as evidence for non-interacting SNAREs. We were unable to coprecipitate syntaxin 1 together with endobrevin from PC12 cells, *i.e.* a cell line in which both the synaptic SNAREs and endobrevin are highly expressed. This supports the view that they do not interact with each other in intact cells. Despite localization to different subcellular compartments, however, such non-cognate SNAREs may pass through the same organelle during membrane recycling. Thus, sorting to different compartments is probably insufficient to prevent non-cognate SNAREs from forming complexes. Rather, control proteins must exist that regulate individual SNAREs with a higher degree of specificity than they display among each other. Such regulators may involve one of the many additional proteins that specifically interact with individual SNARE proteins, *e.g.* the Munc18/Sec1p protein family that binds to the NH_2-terminal domain of syntaxin, which, as recently suggested (46) and further confirmed here, appears to be a separately folded domain in the syntaxins 1–4.

In addition, the surface of individual SNARE complexes may carry information that is selectively recognized by specific regulatory proteins. For instance, proteins such as synaptotagmin or complexin have been shown to interact with the synaptic SNARE complex, but it is not yet known whether they would also bind to any of our non-cognate complexes. The role of proteins binding to fully assembled SNARE complexes remains to be elucidated. They may regulate SNARE function at a late step in the fusion reaction in a positive or negative manner. Discrimination between individual SNARE complexes would ensure that regulation by such proteins is highly specific.

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Note Added in Proof—After submission of this paper, non-selective SNARE protein interactions were also described by another group (47).

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