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14. ABSTRACT:
This is the fifth annual report under the no cost extension period. In this period we have completed the BoNT/B light chain (full length) structure and the manuscript is ready for submission. The virtual screening of inhibitors with BoNT/E light chain was reported in the last report and we are continuing on that. Due to lack of personnel, our plan to continue the same with BoNT/B and A light chains got stalled. However, we have started this work now. The work on the C fragment of BoNT/A will be continued in addition to structures of small molecules with holo BoNT/B.

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Clostridium, botulinum, neurotoxin, zinc chelators, inhibitors, macromolecular crystallography, 3D structure

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1. Reprint of paper published in J. Mol. Biol.

Structural Studies on Intact *Clostridium botulinum* Neurotoxins Complexed with Inhibitors Leading to Drug Design
Annual Report for the Period ending January 2007

Introduction

The major goal of this project is to identify counter measures for botulinum neurotoxins. Our major aim in this is to identify potential small molecule inhibitors to block the toxicity of botulinum neurotoxins. The conventional drug design is based on determining the structures of potential inhibitors in complex with the toxin or the relevant target and then modifying the inhibitor chemically for better inhibition which is done iteratively. Botulinum neurotoxins follow a four-step process, viz., binding to neuronal cells, internalization into vesicles, translocation of catalytic domain into cytosol through the endosomal membrane and the cleavage of one of the three target proteins responsible for neurotransmission. Blocking anyone of these steps will deactivate the protein. In our project we have chosen two of them: blocking the binding site on the binding domain and shielding the active site to prevent catalytic action. A two-pronged approach is being used. We are trying either small molecules which could chelate the active-site zinc or peptide mimics of the substrates to block the active site residues. The general approach is to study the crystal structure of the toxin in complex with a potential inhibitor via x-ray crystallography and then analyze the interactions between the inhibitor and the protein.

Body

(1) Studies on the binding domain of C. neurotoxins

The binding domains of *C.* neurotoxins are attractive targets for blocking the toxicity of botulinum since it will block the first stage of botulinum action, namely binding to the neuronal cells. The advantage here is that the inhibitor need not traverse through the membrane wall to inactivate the protein residing in the cytosol. In the last annual report, we had reported the progress on our work on the crystal structure of the binding domain of botulinum neurotoxin type B. This work was done in collaboration

with Drs Len Smith and Syed Ahmed of USAMRIID. Since the ganglioside binding site is essentially the same as in the holotoxin, this structure essentially gives an opportunity to study inhibitors which would block the activity. Our idea was to combine virtual screening with macromolecular crystallography to identify small molecules.

In a similar manner, we wanted to continue this study with the binding domain of *C.* botulinum neurotoxin type A. We have cloned and expressed the protein (aa 871-1295) and have set up crystallization screens to identify crystallization conditions.

Though it is not part of this project we are also working on the binding domain of *C.* botulinum neurotoxin type E in collaboration with Dr. Lance Simpson of Jefferson Medical College, Philadelphia.

These structures will provide an opportunity to study three serotypes which might yield a common inhibitor. The problems in the progress are addressed in bottleneck section later in the report.

(2) Studies with C. neurotoxin catalytic domains

In this section we will describe our work on the catalytic domain of various serotypes of botulinum neurotoxins. This was done to understand the mode of action of all serotypes which have different substrates. The study showed that the active site geometry has a common pattern and it may be possible to develop a common inhibitor to all of them. In this regard we have determined the structures of light chains of BoNT/A (unpublished), BoNT/B, BoNT/D (unpublished), BoNT/E *(1)*, BoNT/F *(2)* and tetanus toxin *(3)*. In addition, we have studied number of mutants of BoNT/E-LC to understand the catalytic mechanism. Most of these results have been published (the reprints were attached in the last annual report). These structures have highlighted the commonness of the active site geometry which could be exploited in designing a common inhibitor for all of them even though each one has different substrate specificity.

In these studies we made a major discovery that the C terminal region of B light chain completely changed its conformation from beta strand to alpha helical on separation from holotoxin *(4)*. This change in conformation was not observed in the reported structure of BoNT/B LC since it lacked the C terminal region *(5)*.

(3) Virtual Screening of small molecule database:

Initially, we considered ZINC database (Zinc Is Not Commercial) for virtual screening *(6)*. Though it contains more than 72,000 compounds, we restricted to 50,000 compounds for our preliminary studies. At first we checked virtual screening procedure with BoNT/E-LC. BoNT/E-LC was prepared for DOCK program *(7)* with suitable charges, etc. This was done with local scripts written in our laboratory and we used SGI origin 300 for computations. Since the code that we used was not parallelized, it took more than 30 days to analyze 50,000 compounds with BoNT/E-LC. We chose this serotype since we had a high-resolution x-ray structure. This procedure filtered out about 31,000 compounds and only 19,000 could be docked with reasonable energies. Thus the virtual screening procedure had filtered out 62% of the compounds in the database. Further work is on progress.

Bottlenecks and problems in this reporting period

This period, Jan 2005 to Jan 2006, was a no-cost extension period to complete the tasks undertaken. Unfortunately, the scientists involved in the research had to move on to other projects and the PI had to look for new personnel to complete the task. Though a person was selected early, he could not join because of visa restrictions and the work suffered. This is the main reason that the progress was stalled. Now a new person has joined and the requested no-cost extension for one more year (already approved) will be productively used.

Key Research Accomplishments

- Crystal structure of BoNT/B catalytic domain has been determined helping us to understand the commonality in the mechanism of action of clostridial neurotoxins. A manuscript describing the structure is being submitted for publication.
- A subset of small molecules that may inhibit the catalytic activity of BoNT/E has been identified and the work is continuing.
- BoNT/A binding domain has been cloned and expressed.

Reportable outcomes

S. Swaminathan (PI) was invited to contribute a chapter in a book titled “Treatments from Toxins”.

1. S. Swaminathan and R.C. Stevens. Three-dimensional protein structures of light chains of botulinum neurotoxin serotypes A, B and E and tetanus neurotoxin. In, *"Treatments from Toxins: The therapeutic potential of clostridial neurotoxins"*. Ed. K.A. Foster, P. Hambleton and C.C. Shone. CRC Press, 2006, 19-45.

2. S. Sikorra, T. Henke, S. Swaminathan, T. Galli and T. Binz.Identification of the amino acid residues rendering TI-VAMP insensitive toward botulinum neurotoxin B. *J. Mol. Biol.*, 2006, **357**, 574-582.

Conclusions

In our studies we have shown that there is common architecture at the active site of clostridial neurotoxins which may be exploited to design a common drug for all serotypes. We have also shown residues important for the catalytic activity of BoNT/E and the possibility of blocking these residues to inhibit them. Some of the mutants of BoNT/E which are completely devoid of activity, may be used as genetically modified vaccines. We have identified a subset of small molecules as potential inhibitors for BoNT/E via virtual screening.

Plans for the next year:

We will complete the virtual screening of BoNT/E with Dock program. Crystallize and determine the structure of BoNT/A binding domain. Also structural work on BoNT/B-inhibitor complex will be continued.

Personnel in the Project

1. S. Swaminathan (PI)	Scientist	30% effort

Reference:

1. Agarwal, R., Binz, T., and Swaminathan, S. (2005) Analysis of active site residues of botulinum neurotoxin E by mutational, functional and structural studies: Glu335Gln is an apoenzyme, *Biochemistry 44*, 8291-8302.
2. Agarwal, R., Binz, T., and Swaminathan, S. (2005) Structural analysis of botulinum neurotoxin serotype F light chain: implications on substrate binding and inhibitor design, *Biochemistry 44*, 11758-65.

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6. Irwin, J. J., and Shoichet, B. K. (2005) ZINC - A free database of commercially available compounds for virtual screening, *J. Chem. Inf. Model 45*, 177-182.
7. Kuntz, I. D. (1992) Structure-based strategies for drug design and discovery, *Science 257*, 1078-1082.

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Identification of the Amino Acid Residues Rendering TI-VAMP Insensitive toward Botulinum Neurotoxin B

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Botulinum neurotoxins types B, D, F, and G, and tetanus neurotoxin inhibit vesicular fusion *via* proteolytic cleavage of VAMP/Synaptobrevin, a core component of the membrane fusion machinery. Thus, these neurotoxins became widely used tools for investigating vesicular trafficking routes. Except for VAMP-1, VAMP-2, and Cellubrevin, no other member of the VAMP family represents a substrate for these neurotoxins. The molecular basis for this discrepancy is not known. A 34 amino acid residue segment of VAMP-2 was previously suggested to mediate the interaction with botulinum neurotoxin B, but the validity of the data was later questioned. To check whether this segment alone controls the susceptibility toward botulinum neurotoxin B, it was used to replace the corresponding segment in TI-VAMP. The resulting VAMP hybrid and VAMP-2 were hydrolysed at virtually identical rates. Resetting the VAMP-2 portion in the hybrid from either end to TI-VAMP residues gradually reduced the cleavability. A hybrid encompassing merely the VAMP-2 segment 71–80 around the Gln76/Phe77 scissile bond was still hydrolysed, albeit at a ~tenfold lower cleavage rate. The contribution of each non-conserved amino acid of the whole 34-mer segment to the interaction was investigated employing VAMP-2. We find that the eight non-conserved residues of the 71–80 segment are all necessary for efficient cleavage. Mutation of an dditional six residues located upstream and downstream of this segment affects substrate hydrolysis as well. *Vice versa*, a readily cleavable TI-VAMP molecule requires at the least the replacement of Ile158, Thr161, and the section 165–174 by Asp64, Ala67, and the 71–80 segment of VAMP-2, respectively. However, the insensitivity of TI-VAMP to botulinum neurotoxin B relies on at least 12 amino acid changes *versus* VAMP-2. These are scattered along an interface of 22 amino acid residues in length.

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Keywords: botulinum neurotoxin; VAMP; Synaptobrevin; TI-VAMP; membrane fusion

Introduction

The individual members of the vesicle-associated membrane protein (VAMP)/Synaptobrevin, synaptosomal-associated protein of 25 kDa (SNAP-25), and Syntaxin families constitute the core components of the vesicular fusion machinery and are thus indispensable for the various intracellular vesicular transport routes. They are collectively termed soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs).[1] The isoforms Syntaxin-1A (Syx-1A), SNAP-25, and VAMP-2 are the most thoroughly investigated. They accomplish the release of neurotransmitters, i.e. the fusion of synaptic vesicles with the presynaptic membrane, whereas tetanus neurotoxin (TeNT)-insensitive VAMP (TI-VAMP, also known as VAMP-7)[2,3] mediates membrane fusion processes of secretory vesicles with the plasma membrane *via* the formation of complexes with plasmalemmal syntaxins and SNAP-23 or SNAP-25.[4] It is assumed that membrane merging is driven by the formation

Abbreviations used: BoNT, botulinum neurotoxin; CNTs, clostridial neurotoxins; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors; TeNT, tetanus neurotoxin; VAMP, vesicle-associated membrane protein..

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of a coiled-coil four-helix bundle, for which the vesicular SNARE protein provides one, and two or three target membrane SNAREs provide together three helices.[5]

Clostridial neurotoxins (CNTs), i.e. TeNT and seven serotypes of botulinum neurotoxins (BoNTs A to G) are well-established tools for studying vesicular trafficking routes, as they compromise the function of certain members of the three SNARE families.[6,7] They are synthesised by various bacteria of the genus *Clostridium* and consist of a catalytic domain (designated L chain), a translocation domain that transfers the L chain subsequent to receptor-mediated endocytosis across the membrane of the endosomal compartment, and a cell-binding subunit, that mediates the selective binding to neural cell membranes. Upon delivery to the cytosol, the L chain becomes separated from the rest of the molecule by reduction of the disulphide bridge by which it is tethered to the translocation domain to allow for the attack of their intracellular substrates. The L chains of each serotype act as zinc endoproteases and exhibit individual substrate specificity. BoNT/C hydrolyses Syntaxins, BoNT/A, C, and E cleave SNAP-25 family members, and any other clostridial neurotoxins proteolyse VAMPs. Furthermore, except for BoNT/B and TeNT, they all hydrolyse different peptide bonds.[8] Hydrolysis occurs in each case inside the coiled-coil-forming helical domain of the substrate. CNT L chains feature a peculiarity that distinguishes them from other proteases. They require an extended substrate segment for optimal catalytic activity as indicated by studies employing truncated substrates.[9–14] Interestingly, despite sharing the same scissile bond in VAMPs, even TeNT and BoNT/B appear to interact with different substrate sites.[9,15] Short helical segments, called SNARE recognition motifs, present in multiple copies in substrate SNAREs, were due to their capacity to interfere with the cleavage reaction suggested to function as a further recognition site.[16] Amino acid substitutions[13,15,17–19] and a recently determined co-crystal structure[20] confirmed that remote substrate sites upstream and downstream of the scissile peptide bonds do in fact interact with the L chains.

The application of CNTs as tools to study intracellular transport is limited to routes employing neurotoxin-susceptible SNAREs. BoNT/B, D, F, G, and TeNT, for example, can merely proteolyse the closely related VAMP-1, VAMP-2, and Cellubrevin (VAMP-3), whereas the remaining VAMP family members, exhibiting about 40% sequence identity with to the former within the coiled-coil domain, resist cleavage.[3,21]

For functional studies, however, it would be desirable to have cleavable variants of the non-substrate VAMPs at hand. As a first step toward creating such mutants, it is essential to fathom the molecular basis for their resistance. The published co-crystal structure of the BoNT/B L chain bound to its substrate VAMP-2[22] appeared to provide an ideal starting basis for an analysis, though the validity of the determined structure was later questioned due to the absence of sufficient electron density of the substrate.[20,23] In the present study, we therefore assessed the impact of each residue within the proposed interacting segment toward cleavage of VAMP-2 by BoNT/B. We individually replaced all non-conserved residues of VAMP-2 with the corresponding residues of a resistant VAMP, TI-VAMP, and determined the effect on the cleavage rate. *Vice versa*, TI-VAMP residues that significantly decreased the cleavage rate of VAMP-2 were stepwise substituted by the corresponding residues of VAMP-2. A replacement of ten TI-VAMP residues was required to turn this protein into a readily cleavable substrate.

Results

The resistance of TI-VAMP relies on the segment Leu155 to Met181

TI-VAMP is a clostridial neurotoxin-resistant VAMP family member.[3] Unlike cleavable VAMPs, it exhibits an N-terminal extension of ~100 residues, a so-called longin domain.[24] Therefore, in order to check whether this domain renders TI-VAMP inaccessible for CNTs, we treated an N-terminally truncated version of TI-VAMP lacking the longin domain (residues 1–93) with CNT L chains. However, removal of this extension did not affect cleavage by any VAMP-specific CNT (data not shown). In a second approach toward understanding the molecular basis for its resistance, we asked whether swapping a segment of 34 amino acid residues in length of the genuine substrate VAMP-2 (amino acid residues 55–88), which is supposed to mediate the interaction with BoNT/B based on a co-crystallisation study,[22] for the corresponding region of TI-VAMP, would confer full susceptibility to TI-VAMP. The resulting VAMP hybrid, TI-VH34, was generated by *in vitro* transcription/translation and incubated in separate reactions with the L chains of all VAMP-specific CNTs. As shown in Figure 1, TI-VH34 was cleaved with similar efficiency as VAMP-2 by the L chains of BoNT/B and G, whereas it largely resisted BoNT/D, F and TeNT L chains, although the latter shares the scissile peptide bond with BoNT/B. This finding agrees with previous results on interacting regions of the substrate VAMP-2. In the case of TeNT, besides the actual cleavage site, amino acid residues of the SNARE recognition motif V1 (Figure 1), which is located N-terminal of the swapped region, were recognised as being essential for optimal cleavage. In contrast, residues of the SNARE recognition motif V2 were shown to be critically involved in substrate recognition by BoNT/B and G.[9,15] The failure of BoNT/F and BoNT/D to cleave this hybrid molecule matches the fact that V1 and V2 motif amino acid residues

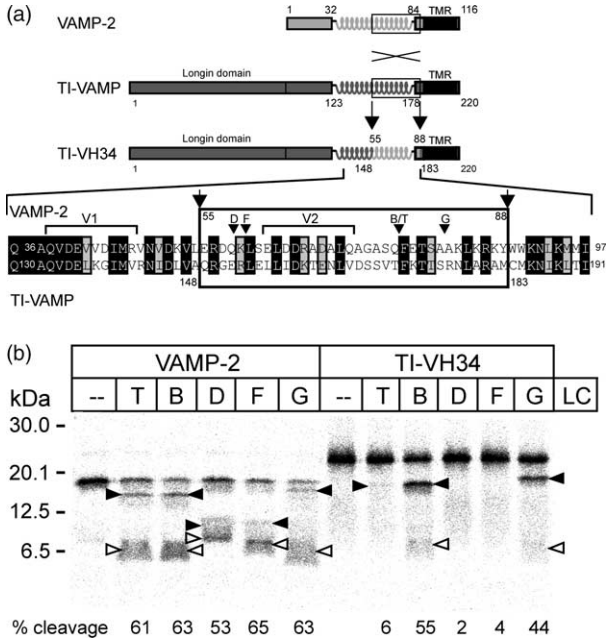

Figure 1. Effects of CNTs on TI-VH34. (a) Schematic representation of TI-VAMP (grey), the BoNT/B substrate VAMP-2 (light grey), and the VAMP hybrid, TI-VH34. The coiled-coil domains mediating the interaction with partner SNAREs are displayed. Numbers below or above specify amino acid border positions of VAMP domains or interfaces between VAMP-2/TI-VAMP portions in the TI-VH34 protein. V1 and V2 denote SNARE recognition motifs. B, D, F, G, and T designate the peptide bonds hydrolysed by BoNT/B, BoNT/D, BoNT/F, BoNT/G, and TeNT. The boxed regions were swapped between VAMP-2 and TI-VAMP. (b) SDS-PAGE analysis of CNT-treated [^{35}S]methionine-labelled VAMP-2 and TI-VH-34. VAMP-2 and TI-VH-34 were incubated with various CNT L chains for 1 h at 37 °C in toxin assay buffer. Cleavage was assayed employing 15% gels and phosphorimaging. The CNT L chains were used at the following final concentrations: TeNT, 20 nM; BoNT/B, 20 nM; BoNT/D, 0.2 nM; BoNT/F, 2 nM; and BoNT/G, 300 nM. These concentrations roughly correspond to L chain doses that yield 60% cleavage of VAMP-2 at the given conditions. N and C-terminal cleavage products are marked by filled or open arrowheads, respectively. The actual extent of substrate cleavage of this representative experiment is depicted below.

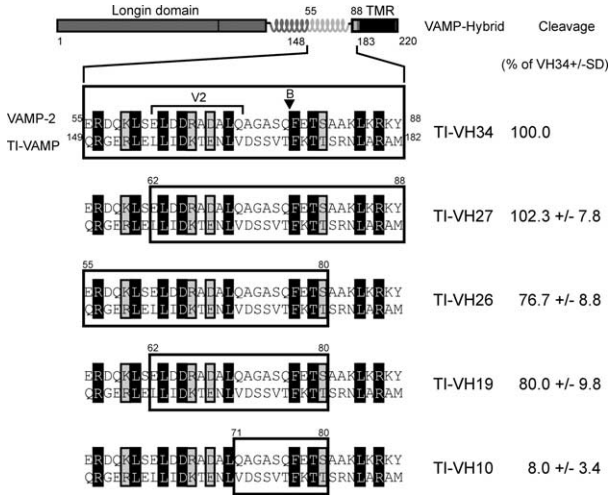

Figure 2. Effects of BoNT/B on various VAMP hybrids. Left panel: Alignment of the 34 amino acid residues encompassing the region of VAMP-2 supposed to mediate the interaction with BoNT/B with the corresponding region of TI-VAMP. VAMP-2-derived segments of the various generated hybrids are boxed. Identical residues are indicated by white letters on a black background. Conserved residues are shown in boxes on a grey background. The bond attacked by BoNT/B is marked B, the SNARE recognition motif V2. Right panel: Hybrid proteins were radiolabelled by *in vitro* transcription/translation and incubated for 1 h in the presence of 20 nM BoNT/B L chain. Samples were analysed by SDS-PAGE using 15% gels. Values represent the percentage of cleavage *versus* TI-VH34±SD of five to ten independent experiments each performed in duplicate.

are required for their optimal activity in addition to the cleavage site.[17]

In order to narrow the interacting region, we next reset the VAMP-2 portion from either end to TI-VAMP residues, resulting in the hybrid constructs TI-VH27, TI-VH26, TI-VH19, and TI-VH10, in which the number specifies the size of the remained VAMP-2 segment (see Figure 2). Reversion of the N-terminal seven residues to those found in TI-VAMP had no effect on cleavability, whereas replacement of the C-terminal eight residues with those of TI-VAMP yielding TI-VH26 and accordingly TI-VH19, slightly reduced the cleavability. The hybrid TI-VH10, with yet a shorter stretch of VAMP-2-specific residues comprising merely the 10-mer segment 71–80 of VAMP-2, was still hydrolysed, albeit at an at least tenfold lower cleavage rate compared to TI-VH19 (Figure 2). These results suggest that apart from the cleavage site, including roughly the five residues surrounding the scissile bond on either side, especially amino acid side groups of the region Glu62 to Ala69, constituting largely the SNARE recognition motif V2, appear to be critically involved in substrate recognition by BoNT/B.

Substitution of 14 of the 25 non-conserved amino acid residues within the VAMP-2 segment Glu55 to Tyr88 affects the cleavage rate

A systematic complementary approach was performed next to identify each individual VAMP-2 residue that contributes to the interaction with BoNT/B but is not conserved in TI-VAMP. For this purpose 25 non-conserved VAMP-2 residues were individually mutated to those present in the corresponding position of TI-VAMP. Mutated VAMP-2 proteins were generated by *in vitro* transcription/translation and incubated with BoNT/B L chain. The extent of hydrolysis was determined subsequent to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) by phosphorimaging. Amino acids, whose

substitution resulted in an at least 10% diminished cleavage rate were considered important for substrate cleavage. According to this, presetting 14 of the 25 non-conserved residues within the segment Glu55 to Tyr88 proved to be important (Figure 3), of which five reduced cleavability by over 60%. Except for Ser61 all of the critical residues are scattered within the VH19 region, and four of the five mutations causing strong effects localise to the VH10 peptide region. In order to exclude that the observed effects do not appear at higher substrate concentration, cleavage rates were also analysed employing recombinant VAMP-2 mutants purified from *Escherichia coli*. The results of this analysis corroborate the previous observations. The mutations Gly73Ser, Ala74Ser, Ser75Val, and Lys87Ala showed even a slightly more pronounced effect (see Supplementary Data Figure 1). Together, the results are largely compatible with the preceding mapping experiment, which predicted critical residues to reside in the VH19 segment.

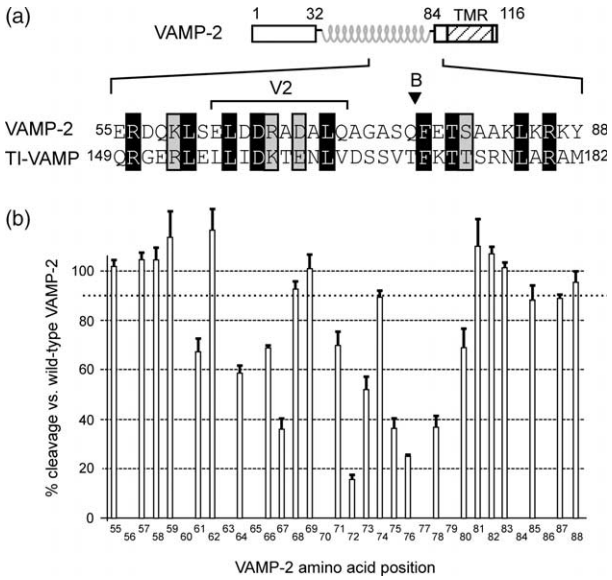

Figure 3. Cleavage analysis of various VAMP-2 point mutants. (a) Schematic representation of VAMP-2 and alignment of the amino acid region supposed to bind to BoNT/B with the corresponding region of TI-VAMP. Identical residues are indicated by white letters on a black background. Conserved residues are shown in boxes shaded grey. The bond attacked by BoNT/B is marked B. The SNARE recognition motif V2 is also indicated. (b) Each non-conserved amino acid of VAMP-2 was individually replaced by the corresponding amino acid of TI-VAMP in order to determine the effect on hydrolysis by BoNT/B. VAMP-2 mutants were radiolabelled by *in vitro* transcription/translation and incubated for 1 h in the presence of 20 nM BoNT/B L chain. Samples were analysed by Tris/Tricine-PAGE using 15% gels. Columns represent percentages of cleavage *versus* the wild-type VAMP-2. Data represent the means ± SD of four to six independent experiments. The dotted line specifies the threshold of 10% reduction in cleavability. Of the 25 non-conserved amino acid residues, 14 were found to reduce the cleavage rate by more than 10% and were thus considered essential for optimal cleavage.

Replacement of 11 TI-VAMP amino acid residues with the corresponding ones of VAMP-2 creates a TI-VAMP variant with full sensitivity toward BoNT/B

Next we set about creating a cleavable TI-VAMP variant containing as little as possible of VAMP-2-derived residues. To this end, VAMP-2 residues crucial for cleavage were introduced in corresponding positions in TI-VAMP. None of these mutations, even when analysed as multiple mutations, e.g. the triple mutant TI-VAMP-Val165Gln/Thr170Gln/Lys172Glu, yielded a BoNT/B-sensitive TI-VAMP (not shown). Since all VAMP-2 residues present in TI-VH10 (71-80) appeared to be important for cleavage by BoNT/B (Figure 3), we consequently chose TI-VH10 as source to assemble those VAMP-2 amino acids, positioned outside this 10-mer segment and previously recognised as being critical for optimal cleavage of VAMP-2, alone or in various combinations in TI-VAMP. The resulting mutants, TI-VH10-1 to TI-VH10-11, were tested with respect to cleavability (Figure 4). In line with the results of the preceding single amino acid replacement experiment in VAMP-2, apart from replacing Ala181 with lysine, all of the additional single amino acid substitutions improved cleavability. The Ile158Asp and Thr161Ala exchanges caused the strongest effects (Figure 4). A combination of these two VAMP-2 residues in the TI-VH10 background led to a readily cleavable molecule (TI-VH10-8). In order to create a TI-VAMP variant that is as good a substrate for BoNT/B as VAMP-2, lysine was incorporated into TI-VH10-8 to replace Ala179, as the segment 175–182 was recognised to contribute to the L chain-substrate interaction as well (see Figure 2) and since this residue appeared to be the most important one within this segment (see Figure 3; cf. TI-VH10-5 and TI-VH10-6). In fact, the resulting TI-VH10-10 displayed a significantly increased cleavability even exceeding that of TI-VH34. Interestingly, jointly fitting all VAMP-2 residues, whose mutation reduced the cleavage rate of VAMP-2, in TI-VAMP (TI-VH10-11) raised the sensitivity even further. Anyway, the assembly of aspartate, alanine, and lysine in positions 158, 161, and 179, respectively, in TI-VH-10 created a hybrid VAMP that possesses merely 11 amino acid residues originating from VAMP-2, but exhibits full sensitivity to BoNT/B (Figure 4). Therefore this hybrid, TI-VH10-10, was referred to as BBs-TI-VAMP for BoNT/B-sensitive TI-VAMP and further analysed. BBs-TI-VAMP could represent a useful tool for further studies on the cellular function of TI-VAMP.

BBs-TI-VAMP forms stable SNARE complexes *in vitro*

The introduction of the mutations present in the BBs-TI-VAMP cDNA into the wild-type TI-VAMP gene could be an attractive future goal in order to generate, for example, a transgenic animal model

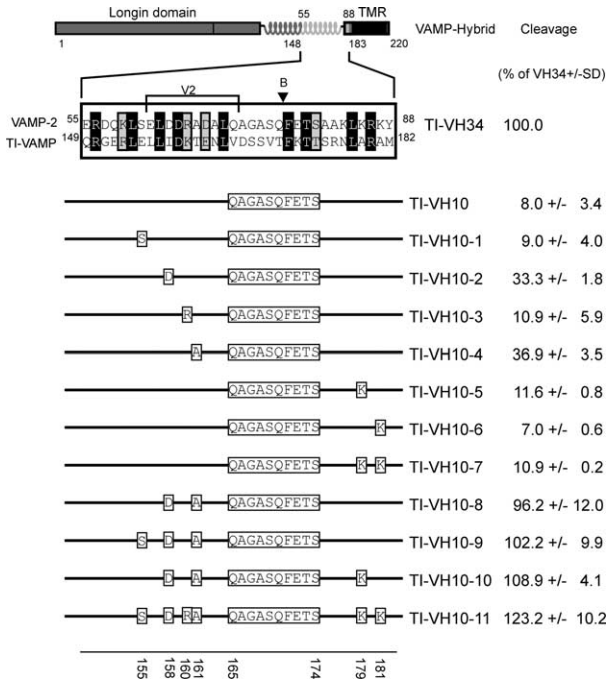

Figure 4. Generation of BoNT/B-sensitive TI-VAMP variants. Left panel: Alignment of the 34 amino acid residues encompassing the region of VAMP-2 supposed to mediate the interaction with BoNT/B with the corresponding region of TI-VAMP. Identical residues are indicated by white letters on a black background. Conserved residues are shown in boxes on a grey background. B denotes the scissile peptide bond for BoNT/B, V2 the SNARE recognition motif 2. Numbers below specify mutated TI-VAMP residues. Right panel: Mutated TI-VAMP variants were radiolabelled by *in vitro* transcription/translation and incubated for 1 h in the presence of 20 nM BoNT/B L chain. Cleavage was determined subsequent to SDS-PAGE (15% gels) and phosphorimaging. Values represent the percentage of cleavage *versus* TI-VH34 ± SD of four to eight independent experiments.

that exhibits a conditional loss of function phenotype upon expression of BoNT/B L chain or administration of BoNT/B. A prerequisite for this work is to demonstrate an unaffected physiological capacity of the mutant TI-VAMP. As a first step to assess whether BBs-TI-VAMP could functionally replace wild-type TI-VAMP, we investigated its ability to form SDS-resistant complexes with partner SNAREs and its stability at elevated temperatures. Both TI-VAMP and the genuine BoNT/B substrate VAMP-2 were previously shown to form SDS-resistant ternary complexes with Syx-1A and SNAP-25.[25] Thus, this target membrane binary SNARE complex is suitable for a comparative binding study. Recombinant GST-Syx-1A/SNAP-25 binary complexes were pre-assembled on glutathione-Sepharaose beads and incubated with *in vitro* translated [^{35}S]methionine-labelled VAMP-2, TI-VAMP, or BBs-TI-VAMP. Figure 5(a) demonstrates that BBs-TI-VAMP can form SDS-resistant complexes with Syx-1A/SNAP-25 like wild-type TI-VAMP.

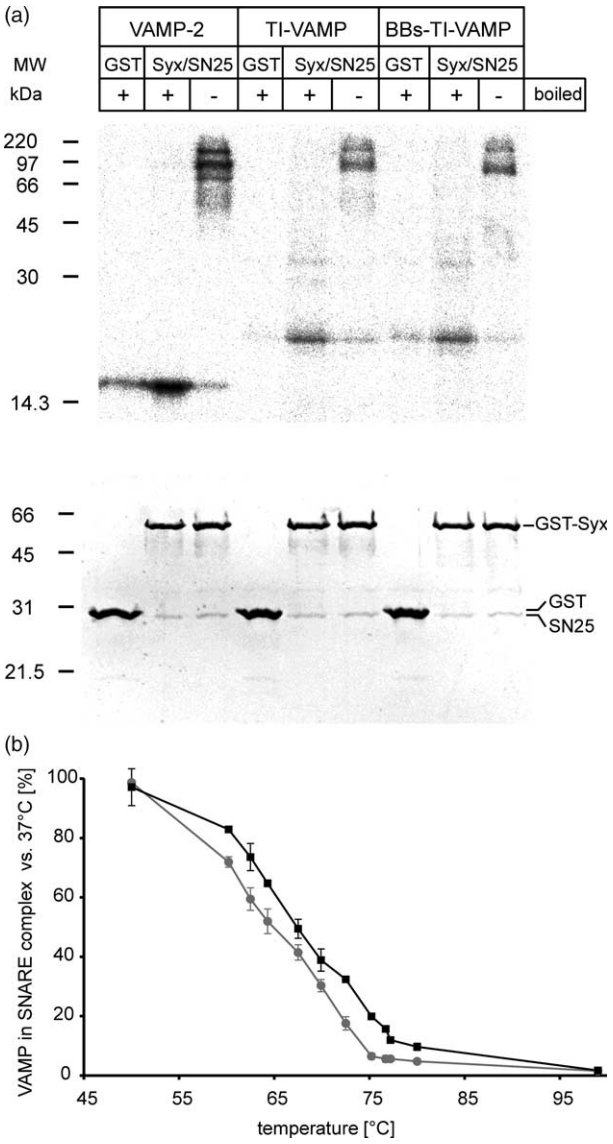

Figure 5. Analysis of the capacity of BBs-TI-VAMP to form SNARE complexes *in vitro*. (a) GST and binary GST-Syx-1A/SNAP-25His6 (SN25) complexes pre-assembled on GT-Sepharose beads were incubated overnight with radiolabelled VAMP-2, TI-VAMP, or BBs-TI-VAMP. Bead-bound protein was analysed by SDS-PAGE upon treatment in SDS sample buffer for 20 min at 37 °C or boiling for 2 min. Upper panel: Radiolabelled protein was visualised following gel drying by phosphorimaging. Lower panel: Commassie blue staining of the same gel to control for the amount of GST, GST-Syx-1A, and SNAP-25His6. (b) Ternary Syx/SNAP-25/TI-VAMP and Syx/SNAP-25/BBs-TI-VAMP complexes bound to GT-Sepharose were set up during 16 h of incubation. After washing, aliquots of the complexes were exposed to different temperatures and the relative intensities of complex integrated and free TI-VAMP or BBs-TI-VAMP were determined by phosphorimaging. The portions of complex integrated TI-VAMP (black square) or BBs-TI-VAMP (grey circles) are depicted as the percentage *versus* complex integrated protein after treatment at 37 °C. Data represent the means ± SD of three independent experiments. Notably, complexes containing BBs-TI-VAMP exhibit a slightly lower stability compared to respective TI-VAMP complexes.

We next assessed the stability of the complexes, as their thermal stabilities could provide hints toward possible effects on the *in vivo* function. SNARE complexes containing BBs-TI-VAMP proved to be thermoresistant but displayed an approximately 2 deg.C lower melting temperature (the temperature at which 50% of complexes are disassembled) than complexes containing the wild-type TI-VAMP (Figure 5(b)). At present it is unclear whether or to what extent this difference in thermostability affects the vital function of TI-VAMP, the zippering into SNARE complexes, which is believed to drive membrane fusion.

Discussion

Clostridial neurotoxins are, due to their ability to proteolyse certain SNARE proteins, well-established tools for investigating intracellular vesicular trafficking routes and studying the mechanism of membrane fusion processes. Their application is, however, limited to the subset of neurotoxin-sensitive SNARE proteins. To date coherent information that explains why many SNARE proteins are not hydrolysed by CNTs is lacking, but this would be a prerequisite if one wants to engineer corresponding sensitive SNARE molecules. As a first step toward this goal we took the non-substrate TI-VAMP as an example and pinpointed the amino acid residues governing its insensitivity toward BoNT/B. This was achieved by two complementary approaches, the individual replacement of all VAMP-2 amino acid residues that are not conserved among VAMP-2 and TI-VAMP in a 34-residue segment previously suggested to mediate the interaction of VAMP-2 and BoNT/B[9,22,26] and *vice versa* the assembly of all VAMP-2 residues in TI-VAMP found in the first analysis to be critical for hydrolysis by BoNT/B.

Results of the first approach indicated that the resistance of TI-VAMP cannot be ascribed to single amino acid changes in TI-VAMP, as all VAMP-2 mutants were still relatively good substrates. This is in contrast to an earlier finding with BoNT/D. Here, the single amino acid change of Met46 to isoleucine in VAMP-2 resulted in a 500-fold decline in sensitivity.[18] When drawing a threshold at 10% reduction of the VAMP-2 cleavage rate, 14 amino acid substitutions in the 34-mer appear to mediate the insensitivity of TI-VAMP toward BoNT/B. Four of these amino acid residues account for the region around the putative scissile peptide bond. The mutants Ser75Val, Gln76Thr, and Glu78Lys (mutations of the P_2, P_1, and P'_2 according to the nomenclature of Schechter and Berger[27]) each decreased the cleavage rate to less than 40% of wild-type VAMP-2, agreeing well with earlier results on other mutations of these amino acids employing synthetic VAMP-2 peptides.[26] The V2 SNARE recognition motif (Glu62-Leu-Asp-Asp-Arg-Ala-Asp-Ala-Leu-Gln71) has previously been suggested to function as another recognition site for BoNT/B,[16] and mutation of Asp64, Asp65, and Asp68 to either serine or asparagine was later shown to drastically reduce the cleavage rate.[15,28] In line with these data, VAMP-2-Asp64Ile exhibited a significantly lower susceptibility, whereas the conservative replacement of Asp68 to glutamate had almost no effect. A novel finding is that Arg66, Ala67, and Gln71 of the V2 SNARE recognition motif do considerably contribute according to our data to the substrate cleavage process as well. Notably, the two amino acid residues linking the V2 SNARE recognition motif and the site around the scissile bond (P_3 to P'_3; i.e. Ala74 to Thr79) were identified as further residues being essential for substrate hydrolysis. Addition of a carboxyl group to Ala72 had the strongest effect of all mutations analysed. A bulkier substituent in position 73 (Gly73Ser) also had a major effect on the cleavage rate. Moderate effects on substrate cleavage were also seen when Ser61, Ser80, Lys85, and Lys87 were mutated. Of the residues conserved among VAMP-2 and TI-VAMP, Asp65 and Phe77 are probably also involved in the substrate hydrolysis process[15,26] and we are aware that this could apply to the remaining conserved residues as well. The characterisation of their contribution was however outside the scope of the present study.

Summing up, the results are in striking conflict with several predictions for interacting residues of the co-crystal structure of VAMP-2 bound to BoNT/B L chain.[22] On the one hand the mutations Glu62Leu, Asp68Glu, and Tyr88Met do not affect the cleavage rate, though these residues were suggested to stay in interaction with the enzyme *via* their side-chains. On the other hand, change of several amino acid residues not considered to be involved in the substrate–enzyme interaction did severely interfere with substrate cleavage: Arg66Lys, Ser61Glu, Ala72Asp, Gly73Ser, Ser75Val. As assessed by computer-based structural analyses employing the Insight II software, the effects of the latter four mutations are probably not due to their larger space requirements or incompatibility of the newly introduced charges (not shown).

In a second approach we swapped TI-VAMP residues for corresponding VAMP-2 residues, whose mutation significantly affected the cleavage rate. Starting from TI-VH10, which covers the VAMP-2 residues of the cleavage region and those linking this region to the V2 SNARE recognition motif residues, six further VAMP-2 amino acid residues were tested for their ability to increase the sensitivity of TI-VH10 to BoNT/B. In agreement with the prior analysis, swapping Ile158 for Asp64 of VAMP-2 or Thr161 for Ala67 drastically improved the cleavability, whereas Ala179 for Lys85 caused a slight increase, and Ala181 for Lys87 had no effect. The results on the latter two constructs are compatible with moderate effects caused by the reverse mutation in VAMP-2. In contrast, the fact that changes of Glu155 (TI-VH10-1) and Lys160 (TI-VH-10-3) did not result in a significant improvement of cleavability was

unexpected. This could, for example, be explained if an unfavourable intramolecular interaction of these side-chains was the reason for the decreased cleavage rate in the mutated VAMP-2. This would not affect TI-VAMP hybrids exhibiting the reverse mutations, as those intramolecular interactions were unlikely to occur there due to the absence of the respective interacting groups. In line with the remarkable sensitivity of TI-VH10-2 and TI-VH10-4, incorporation of both these VAMP-2 residues that drastically increased cleavability, i.e. Asp64 and Ala67, again enhanced the cleavability reaching almost the level of TI-VH34. The assembly of all VAMP-2 residues putatively involved in the interaction with BoNT/B L chain in TI-VAMP (TI-VH10-11) yielded an even better substrate than TI-VH34 and thus VAMP-2, as TI-VH34 and VAMP-2 are cleaved with comparable efficiency. This finding reveals that wild-type VAMP-2 is possibly not the perfect substrate for BoNT/B. Altogether, the two complementary experimental approaches suggest that the insensitivity of TI-VAMP toward BoNT/B is attributable to changes of at least 11 residues. Eleven VAMP-2-derived amino acid residues were assembled in BBs-TI-VAMP, a TI-variant that exhibits a comparable sensitivity to the genuine BoNT/B substrate VAMP-2. As learned from the effects of replacing Glu155 and Lys160 with serine and arginine, respectively (see above), it is possible that a BBs-TI-VAMP residue residing in the VAMP-2 derived 10-mer segment 71–80 is not involved in the interaction with BoNT/B. It could thus be worth individually back-mutating the eight non-conserved residues of this segment in future studies in order to render the number of VAMP-2-derived amino acid residues yet smaller. If further VAMP-2 residues were dispensable, the likelihood of a fully functional TI-VAMP variant that represents a substrate for BoNT/B would increase.

It is imperative to know if, and to what extent, the substitutions affect the functional efficiency of BBs-TI-VAMP. Amino acid residues in heptad repeat positions mediate the interaction between partner SNAREs.[29] Three of the nine heptad positions in the swapped 34-mer segment are not conserved in TI-VAMP. These are threonine in position 161 and serine in positions 168 and 175, the first two being replaced by alanine in BBs-TI-VAMP. As we observed a slightly reduced thermal stability of the Syx-1A/SNAP-25/BBs-TI-VAMP complex, it can presently not be excluded that one or both of these residues are important for the *in vivo* function of TI-VAMP. The remaining nine changes are located at the surface of the SNARE complex. Two of them, Val169Ser and Lys172Glu, could be important for the functionality of TI-VAMP, since mutation of their counterparts in VAMP-2 Ser75 and Glu78 as the triple mutation Ser75Ala/Glu78Ala/Thr79Ala reduced exocytosis in chromaffin cells.[30] It remains open, whether the amino acid residues in these positions of TI-VAMP are indispensable and whether the remaining seven surface changes for example do interfere with the interaction of accessory proteins, which control SNARE complex assembly, or the regulation of SNARE complex activity.

In conclusion, we report here a TI-VAMP mutated in 11 positions that exhibits full sensitivity toward the clostridial neurotoxin BoNT/B. This TI-VAMP variant may serve for establishing a conditional loss of function phenotype model and thus be useful for further functional studies on TI-VAMP. The present study also adds comprehensive information to the understanding of the substrate–CNT interaction.

Materials and Methods

Plasmid constructions

The cytosolic portion (amino acid residues 1–97) encoding a segment of the wild-type rat VAMP-2 gene was inserted into the pET15b vector (Merck Biosciences GmbH, Schwalbach Ts., Germany). The open reading frame for human TI-VAMP was subcloned in pSP72 (Promega, Mannheim, Germany). For the generation of the TI-VAMP/VAMP-2 hybrid (TI-VH34), the VAMP-2 portion was generated by PCR using the forward primer 5′-TCTCTCAGATCTGGTAGCTGAGCGAGACCAGAAGC TATC-3′ and the reverse primer 5′-TCTCTCAAGCTT GAGGTTCTTCATACAGTATTTGCGCTTGAGCTTGGC-3′. In order to introduce a HindIII site at the downstream linkage site, the 3′-portion of the TI-VAMP cDNA was also generated by PCR using the oligonucleotide 5′-TCTCTCAAGCTTACTATTATCATCATCATCGTATC-3′ and a T7 promoter primer. The VAMP-2-specific PCR product was cleaved with BglII and HindIII and the TI-VAMP-specific PCR product with HindIII and EcoRV. The two fragments were ligated with pSP72-TI-VAMP from which the BglII-EcoRV fragment was previously removed. The resulting hybrid (TI-VH34) encodes amino acid residues 1–148 of TI-VAMP, followed by amino acid residues 55–88 of VAMP-2 and TI-VAMP amino acid residues 183–220 (see Figure 1). Starting from TI-VH34 and using PCR primers that introduced suitable restriction sites, the hybrids TI-VH27, TI-VH26, and TI-VH19, and TI-VH10 were constructed. Point mutations in pET15b-VAMP-2 and pSP72-VH10 were introduced by oligonucleotide primer-based PCR mutagenesis using Pwo-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany).

Expression and purification of recombinant CNT L chains and SNARE proteins

The *E. coli* strain M15pREP4 (Qiagen GmbH, Hilden, Germany) was transfected with L chain-encoding plasmids of BoNT/B, BoNT/D, BoNT/F, BoNT/G, and TeNT. Plasmids encoding the different VAMP-2 variants were transfected into the *E. coli* strain BL21-DE3 (Stratagene Europe, Ebsdorfergrund, Germany). Recombinant proteins were produced during 3 h of induction at 21 °C and purified on Ni^{2+}-nitrilotriacetic acid-agarose beads according to the manufacturer's instructions. Fractions containing recombinant proteins were dialysed against toxin assay buffer (150 mM K^+ glutamate, 10 mM Hepes-KOH, pH 7.2), frozen in liquid nitrogen, and kept at −70 °C.

GST, GST-Syx-1A (rat), and SNAP-25His6 (rat) were affinity purified on GT-Sepharose (Amersham

Biosciences, Freiburg, Germany) or Ni^{2+}-nitrilotriacetic acid-agarose beads and finally dialysed against 10 mM Tris–HCl (pH 7.5) buffer supplemented with 100 mM NaCl.

In vitro transcription and translation

VAMP-2, TI-VAMP, and their derivatives were generated by *in vitro* transcription/translation using the above described plasmids, the SP6/T7 coupled TNT reticulocyte lysate system (Promega), and [^{35}S]methionine (275 kBq, 37 TBq/mmol, Amersham) according to the manufacturer's instructions.

Cleavage assays

Cleavage assays contained 1 μl of the transcription/translation mixture of [^{35}S]methionine-labelled VAMP-2 or TI-VAMP variants and purified L chain and were incubated for 60 min at 37 °C in a total volume of 10 μl of toxin assay buffer. Alternatively VAMP-2 variants (5 μM final concentration) were incubated in a total volume of 100 μl of toxin assay buffer in the presence of BoNT/B L chain (10 nM final concentration), and 15 μl aliquots were taken after different time periods. Reactions were stopped by the addition of an equal volume of double-concentrated sample buffer (120 mM Tris–HCl (pH 6.75), 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 20% (w/v) glycerol, 0.014% (w/v) bromphenol blue), or Tris/Tricine sample buffer in the case of VAMP-2 samples (100 mM Tris–HCl (pH 6.8), 200 mM 1,4 dithiothreitol, 8% (w/v) SDS, 30% (w/v) glycerol, 0.04% (w/v) Coomassie brilliant blue G-250). Samples were boiled for 2 min and subjected to SDS-PAGE using 15% gels. Samples of VAMP-2 cleavage assays were run on Tris/Tricine 15% gels in Tris/Tricine/SDS electrophoresis buffer (100 mM Tris, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.3, Bio-Rad-Laboratories GmbH, Munich, Germany). Subsequently, gels were dried and radiolabelled proteins were visualised employing a BAS-1500 phosphorimager (Fuji Photo Film, Co., Ltd., Tokyo, Japan) or stained using Coomassie blue. Quantification of radiolabelled proteins and fragments thereof was done with the Tina 2.09 software (Raytest Isotopenmessgeräte GmbH, Berlin, Germany). Coomassie-stained proteins were quantified by means of the LAS-3000 imaging system (Fuji Photo Film, Co., Ltd) and the AIDA 3.51 program.

SNARE complex assembly and stability assays

GST-Syx-1A (0.1 nmol each) prebound to 12.5 μl of GT-Sepharose beads was incubated for 90 min at 4 °C in 400 μl of PBS containing 2 mM EDTA and 0.1% NP-40 with 0.3 nmol of SNAP-25His6. Following four times washing with 400 μl of Tris/NaCl buffer (10 mM Tris–HCl (pH 7.5), 100 mM NaCl), 2.5 μl of radiolabelled VAMP-2, TI-VAMP, and BBs-TI-VAMP, as generated by *in vitro* transcription/translation, were added to a total of 200 μl of Tris/NaCl buffer, and the incubation was continued for 16 h. The beads were then collected by centrifugation and washed three times each with 400 μl of Tris/NaCl buffer. Washed pellet fractions were analysed together with the supernatant fractions by SDS-PAGE and fluorography.

In stability assays SNARE complexes were set up by incubating GST-Syx, SNAP-25His6 and radiolabelled TI-VAMP/BBs-TI-VAMP for 16 h at 4 °C in Hepes/NaCl buffer (4 mM Hepes–NaOH (pH 7.4), 100 mM NaCl, 3.5 mM $CaCl_2$, 3.5 mM $MgCl_2$, 1 mM EDTA, 0.1% NP-40). Beads were washed three times (50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.1% NP-40) and subsequently resuspended in sample buffer. Aliquots were then exposed for 10 min to different temperatures and run on SDS-PAGE gels. Relative intensities of complex integrated and free TI-VAMP or BBs-TI-VAMP were determined by phosphorimaging of dried gels. The portions of complex integrated TI-VAMP or BBs-TI-VAMP were calculated as the percentage *versus* complex integrated protein after treatment at 37 °C.

Molecular modelling

Molecular modelling was done using the Insight II 2003.L software (Accelrys, San Diego, USA).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2005.12.075

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