Substrate Recognition Mechanism of VAMP/Synaptobrevin-cleaving Clostridial Neurotoxins*§

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Botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT) inhibit neurotransmitter release by proteolyzing a single peptide bond in one of the three soluble N-ethylmaleimide-sensitive factor attachment protein receptors SNAP-25, syntaxin, and vesicle-associated membrane protein (VAMP)/synaptobrevin. TeNT and BoNT/B, D, F, and G of the seven known BoNTs cleave the synaptic vesicle protein VAMP/synaptobrevin. Except for BoNT/B and TeNT, they cleave unique peptide bonds, and prior work suggested that different substrate segments are required for the interaction of each toxin. Although the mode of SNAP-25 cleavage by BoNT/A and E has recently been studied in detail, the mechanism of VAMP/synaptobrevin proteolysis is fragmentary. Here, we report the determination of all substrate residues that are involved in the interaction with BoNT/B, D, and F and TeNT by means of systematic mutagenesis of VAMP/synaptobrevin. For each of the toxins, three or more residues clustered at an N-terminal site remote from the respective scissile bond are identified that affect solely substrate binding. These exosites exhibit different sizes and distances to the scissile peptide bonds for each neurotoxin. Substrate segments C-terminal of the cleavage site (P4–P4’) do not play a role in the catalytic process. Mutation of residues in the proximity of the scissile bond exclusively affects the turnover number; however, the importance of individual positions at the cleavage sites varied for each toxin. The data show that, similar to the SNAP-25 proteolysis by BoNT/A and E, VAMP/synaptobrevin-specific clostridial neurotoxins also initiate substrate interaction, employing an exosite located N-terminal of the scissile peptide bond.

Clostridial neurotoxins (CNTs)2 (i.e. tetanus neurotoxin (TeNT) and seven serotypes of botulinum neurotoxins (BoNTs A–G)) interfere with neurotransmitter release. BoNTs act in extremely low doses in motoneurons at the neuromuscular junction and thereby cause muscle paralysis. TeNT operates in inhibitory interneurons of the spinal cord that down-regulate the activity of motoneurons and thus causes the opposite physiological effect of BoNTs. CNTs are synthesized as single chain protein toxins by bacteria of the genus Clostridium and later become proteolytically activated. They consist of a catalytic domain (designated the L chain (LC)), 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 nerve cells. Upon delivery to the cytosol, the L chain is released from the rest of the molecule by reduction of the disulfide bridge by which it is tethered to the translocation domain. The L chains finally attack their intracellular substrates by acting as zinc endoproteases of high individual substrate specificity. BoNT/C hydrolyzes syntaxins 1 and 3 in certain species. BoNT/A, C, and E cleave SNAP-25 (synapto-some-associated protein of 25 kDa) and SNAP-23 in certain species, and all other CNTs proteolyze several vesicle-associated membrane proteins (VAMP)s/synaptobrevins. Except for BoNT/B and TeNT, all CNTs hydrolyze unique peptide bonds in their substrates (1). The members of the VAMP/synaptobrevin, SNAP-25, and syntaxin families are collectively termed soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (2) and constitute core components of the vesicular fusion machinery. Individual sets of SNAREs are responsible for discrete intracellular vesicular fusion events, and CNTs thus became valuable tools for studying vesicular trafficking routes (3, 4). The set consisting of syntaxin 1A, SNAP-25, and VAMP-2, which are all cleaved by CNTs, is best investigated. It accomplishes the fusion of synaptic vesicles with the presynaptic membrane. Many other SNARE proteins like the TeNT-insensitive VAMP (TI-VAMP; also known as VAMP-7) (5, 6) or syntaxin 4, which together with SNAP-23 carry out membrane fusion of secretory vesicles with the plasma membrane (7), are not hydrolyzed by CNTs (6, 8–10). A peculiarity of CNT L chains which distinguishes them from conventional proteases is that they require an extended substrate segment for optimal catalytic activity, as evidenced by prior studies employing truncated substrates (8, 11–15) or amino acid substitutions (8, 16–22). The interaction of BoNT/A, C, and E with SNAP-25 has been most thoroughly analyzed. A recently determined co-crystal structure of a BoNT/A-SNAP-25 complex (23), along with structural studies on mutated L chains (24, 25) and the enzymatic characteriza-
Substrate Recognition Mode of BoNT/D, BoNT/F, and TeNT

tion of mutated substrate and L chains (26–28), provided a detailed view for the catalytic mechanism. Comparable in depth information is lacking for VAMP cleaving L chains.

Due to their extreme toxicity, the ease of production, handling, and delivery via aerosol or liquid routes, CNTs represent potential biological warfare. On the other hand BoNTs are used as therapeutics with ever expanding applications in clinical medicine to treat various neuromuscular disorders. Better understanding of the mechanisms of substrate cleavage may therefore afford valuable information for the development of counteragents against intentional misuse or for modification of the toxins to increase their properties and thus broaden the field of clinical applications.

Here we present a complete comparative analysis of the mode of substrate recognition for four of five VAMP hydrolyzing CNTs: BoNT/B, BoNT/D, BoNT/F, and TeNT. Each individual residue within the interacting segment of their substrate VAMP-2 was assessed by mutational analysis for its contribution to binding and/or hydrolysis. The data reveal that BoNT/F and TeNT require a more extended interaction interface and involve more substrate side chain contacts than BoNT/B and BoNT/D and that important interaction sites outside the cleavage site are exclusively located upstream of the respective scissile peptide bonds.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The segment encoding the cytosolic portion (amino acids 1–96) 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 full-length human TI-VAMP was subcloned in pSP72 (Promega, Mannheim, Germany). Point mutations in pET15b-VAMP-2 and pSP72-TI-VAMP were introduced by oligonucleotide primer-based PCR mutagenesis using Pwo-polynuclease (Peqlab Biotechnologie GmbH, Erlangen, Germany) or the GeneTailor site-directed mutagenesis system (Invitrogen). Hybrid VAMP-encoding plasmids (TI-VAMP hybrids; TI-VHs) were constructed in pSP72 by PCR using oligonucleotide primers that introduced suitable restriction sites and TI-VH26 (20) or the rat VAMP-2 cDNA as template. All mutations were verified by sequencing.

Expression and Purification of Recombinant VAMP-2 and CNT L Chains—The Escherichia coli strain M15pREP4 (Qiagen GmbH, Hilden, Germany) was transfected with L chain-encoding plasmids of BoNT/B, BoNT/D, BoNT/F, and TeNT. Plasmids encoding 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 Ni2+-nitrilotriacetic acid-agarose beads according to the manufacturer’s instructions. Fractions containing recombinant proteins were dialyzed against toxin assay buffer (150 mM potassium glutamate, 10 mM HEPES-KOH, pH 7.2), frozen in liquid nitrogen, and kept at −70 °C.

Protein concentrations were determined following SDS-PAGE analysis and Coomassie Blue staining by means of the LAS-3000 imaging system (Fuji Photo Film, Co., Ltd.) and the AIDA 3.51 program using various known concentrations of bovine serum albumin run as standards. A representative SDS-PAGE analysis of purified VAMP mutants is shown in Fig. S1.

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 [35S]methionine (275 kBq, 37 TBq/mmol; Amersham Biosciences) according to the manufacturer’s instructions.

Cleavage Assays—Cleavage assays contained 1 μl of the transcription/translation mixture of [35S]methionine-labeled 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. 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). Samples were incubated at 37 °C for 30 min and then subjected to SDS-PAGE using 15% Tris/Tricine gels in Tris/Tricine/SDS electrophoresis buffer (100 mM Tris, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.3; Bio-Rad). Subsequently, gels were dried, and radiolabeled proteins were visualized employing a BAS-1500 phosphor imager (Fuji Photo Film, Co., Ltd., Tokyo, Japan). Quantification of radiolabeled proteins and fragments thereof was done with the Tina 2.09 software (Raytest Isotopenmessgeräte GmbH, Berlin, Germany).

Determination of Kinetic Parameters—For the determination of the enzyme kinetic parameters of selected VAMP-2 mutants, the substrate concentration was varied between 5 and 100 μM. Each of the various substrate concentrations was endowed by the addition of 1 μl of radiolabeled His6-VAMP-2 or its mutants generated by in vitro transcription/translation. Incubation was done in a final volume of 25 μl of toxin assay buffer. After 2 and 4 min of incubation at 37 °C, aliquots of 10 μl were taken, and the enzymatic reaction was stopped by mixing with 10 μl of prechilled double-concentrated SDS-PAGE sample buffer. VAMP-2 and its cleavage products were separated by SDS-PAGE, and radiolabeled protein was visualized using a BAS-1500 phosphor imager (Fuji Photo Film). The percentage of hydrolyzed VAMP-2 was determined from the turnover of the radiolabeled substrate applying the Tina 2.09f program (Raytest Isotopenmessgeräte) and used to calculate the initial velocity of substrate hydrolysis. Km and Vmax values were derived from Lineweaver-Burk plots using the GraphPad Prism 4.03 program (GraphPad Software Inc., San Diego, CA).

Molecular Modeling—Molecular modeling was done using the Insight II 2003.L software (Accelrys, San Diego, CA).

RESULTS

VAMP Recognition by BoNT/F Depends on a More Extended Substrate Segment Compared with BoNT/D—In order to define essential substrate segments for BoNT/D and BoNT/F determining that VAMP-2 serves as substrate and TI-VAMP resists cleavage, we generated a series of hybrids that consisted of various parts of the genuine VAMP-2 substrate that replaced corresponding areas of the full-length noncleavable TI-VAMP (TI-VH; Fig. 1). In vitro translated hybrid substrates were incubated for 1 h with LC/D or LC/F (both used at a 0.2 nM final concentration). The extent of cleavage was determined by phosho-
rimaging and expressed as a percentage of cleavage of VAMP-2-(1–96). Optimal cleavage by LC/D was still observed with a VAMP hybrid that comprised only a 23-amino acid segment (Val-42 to Asp-64) of VAMP-2 (TI-VH23), whereas just TI-VH44 (VAMP-2 region Ala-21 to Asp-64) yielded optimal cleavage for LC/F. N- or C-terminal elongation of these VAMP-2 portions did not enhance the activity of LC/D or LC/F, although maximum values of 48.5 or 22.2% relative to VAMP-2-(1–96) were only achieved for LC/D and LC/F, respectively (Fig. 1). The reason for the less efficient cleavage could thus only be the presence of the TI-VAMP longin domain at the N terminus of the fusion protein or the transmembrane domain at the C terminus. Removal of the longin domain, an N-terminal extension (PROSITE: PS50859) that distinguishes members of the family of longer VAMPs (so-called longins) from classical VAMPs (29), amino acids 1–93 (e.g. in TI-VH23), indeed strongly improved cleavage by LC/D (83.8 ± 2.5%). In agreement with these data, attachment of the longin domain to VAMP-2 (TI-VH85) decreased cleavage to 50.8 ± 3.8%. Removal of the longin domain similarly improved cleavage by LC/F. It cleaved 42.6 ± 2.7% of TI-VH44 lacking residues 1–93 within 1 h of incubation. This inhibitory effect was observed for all TI-VHs and may be due to an interaction of the folded longin domain with the unfolded C-terminal part of the hybrid VAMPs. However, the presence of the longin domain did not fully explain the reduced cleavage rates of TI-VH proteins by LC/F. Therefore, cleavage of full-length VAMP-2 versus VAMP-2-(1–96) was studied. As expected, both variants were proteolyzed with comparable efficiency by LC/D (94.0 ± 4.3% of VAMP-2-(1–96)), but proteolysis of full-length VAMP-2 by LC/F was reduced to 44.4 ± 3.5%, thus indicating that the longin domain and the solvent-exposed transmembrane domain exert a negative effect on substrate recognition by LC/F. Overall, the cleavage analysis of various TI-VH proteins suggested that the resistance of TI-VAMP toward LC/D inheres in a segment of 23 amino acids, whereas that for LC/F inheres in a far longer TI-VAMP segment of 44 amino acids. Furthermore, TI-VAMP residues located C-terminal to the cleavage site (i.e. more than five residues downstream of the scissile peptide bond) do not appear to interfere with substrate recognition and cleavage by LC/D and LC/F.

Identification of Substrate Residues Involved in Formation of the Michaelis Complex and the Hydrolytic Process—Next, systematic mutagenesis of VAMP-2-(1–96) at residues 27–88 was performed to identify every individual VAMP-2 amino acid position (Val-42 to Asp-64) of VAMP-2 (TI-VH23), whereas just TI-VH44 (VAMP-2 region Ala-21 to Asp-64) yielded optimal cleavage for LC/F. N- or C-terminal elongation of these VAMP-2 portions did not enhance the activity of LC/D or LC/F, although maximum values of 48.5 or 22.2% relative to VAMP-2-(1–96) were only achieved for LC/D and LC/F, respectively (Fig. 1). The reason for the less efficient cleavage could thus only be the presence of the TI-VAMP longin domain at the N terminus of the fusion protein or the transmembrane domain at the C terminus. Removal of the longin domain, an N-terminal extension (PROSITE: PS50859) that distinguishes members of the family of longer VAMPs (so-called longins) from classical VAMPs (29), amino acids 1–93 (e.g. in TI-VH23), indeed strongly improved cleavage by LC/D (83.8 ± 2.5%). In agreement with these data, attachment of the longin domain to VAMP-2 (TI-VH85) decreased cleavage to 50.8 ± 3.8%. Removal of the longin domain similarly improved cleavage by LC/F. It cleaved 42.6 ± 2.7% of TI-VH44 lacking residues 1–93 within 1 h of incubation. This inhibitory effect was observed for all TI-VHs and may be due to an interaction of the folded longin domain with the unfolded C-terminal part of the hybrid VAMPs. However, the presence of the longin domain did not fully explain the reduced cleavage rates of TI-VH proteins by LC/F. Therefore, cleavage of full-length VAMP-2 versus VAMP-2-(1–96) was studied. As expected, both variants were proteolyzed with comparable efficiency by LC/D (94.0 ± 4.3% of VAMP-2-(1–96)), but proteolysis of full-length VAMP-2 by LC/F was reduced to 44.4 ± 3.5%, thus indicating that the longin domain and the solvent-exposed transmembrane domain exert a negative effect on substrate recognition by LC/F. Overall, the cleavage analysis of various TI-VH proteins suggested that the resistance of TI-VAMP toward LC/D inheres in a segment of 23 amino acids, whereas that for LC/F inheres in a far longer TI-VAMP segment of 44 amino acids. Furthermore, TI-VAMP residues located C-terminal to the cleavage site (i.e. more than five residues downstream of the scissile peptide bond) do not appear to interfere with substrate recognition and cleavage by LC/D and LC/F.

Identification of Substrate Residues Involved in Formation of the Michaelis Complex and the Hydrolytic Process—Next, systematic mutagenesis of VAMP-2-(1–96) at residues 27–88 was performed to identify every individual VAMP-2 amino acid position that is crucial for the interaction with CNTs. Although the mapping analysis indicated a role for the region Ala-21 to Leu-26 of VAMP-2 for substrate recognition by LC/F, it was not...
Substrate Recognition Mode of BoNT/D, BoNT/F, and TeNT

VAMP-2(1-96)

BoNT/F

BoNT/D

BoNT/B

TeNT

VAMP-2 amino acid position
Substrate Recognition Mode of BoNT/D, BoNT/F, and TeNT

FIGURE 3. Kinetic parameters of VAMP-2 cleavage by BoNT/F, BoNT/D, and TeNT. VAMP-2 or its mutants used at concentrations from 5 to 100 μM were incubated for 2 or 4 min in the presence of 0.08 nM LC/F, 0.08 nM LC/D, or 10 nM LC/T. Samples were analyzed by Tris/Tricine-PAGE using 15% gels. The percentage of hydrolyzed VAMP-2 was calculated and used to calculate the initial velocity of substrate hydrolysis. K_\text{m} and V_\text{max} values were derived from Lineweaver-Burk plots (for further details, see “Experimental Procedures”). Data represent means ± S.D. of 2–4 independent experiments. The single-letter code below the lower columns was used to identify the mutants. Black columns, wild type (wt); white columns, exosite mutants; gray columns, cleavage site mutants.

Considered in this analysis. Earlier studies had shown that truncated VAMP-2-(27–116) was an optimal substrate for LC/F (18), allowing the conclusion that the observed lower cleavage rate for TI-VH54 (Fig. 1) was due to the presence of three positively charged side groups in the corresponding TI-VAMP segment. Therefore, residues being different among TI-VAMP and VAMP-2 were mutated to those present in the corresponding position of TI-VAMP. Identical residues were replaced with alanine. VAMP-2 Ala-37, which corresponds to Ala-131 of TI-VAMP was converted to leucine. Mutated VAMP-2 proteins were initially produced as radiolabeled molecules by in vitro transcription/translation and incubated for 1 h with LC/D, LC/F, LC/T, and LC/B. The percentage of cleavage versus VAMP-2-(1–96) was calculated. Amino acids, the substitution of which resulted in an at least 33% diminished cleavage rate, were considered critical for substrate cleavage. Accordingly to this presetting, 14 amino acids within the segment Thr-27 to Tyr-88 proved to be important for LC/F. Seven mutations even reduced the cleavability by over 66%. In agreement with the results of the previous mapping experiments, significant effects were not observed for mutations downstream of Leu-60 (Fig. 2). This finding calls into question an earlier observation according to which Asp-64, Asp-65, and Asp-68 were involved in the interaction with LC/F (17). The finding that a VAMP peptide comprising Leu-32 to Asp-65 is effectively cleaved (15) clearly supports our data.

The results of our systematic mutagenesis approach do not differentiate whether mutation of a particular VAMP-2 amino acid abolished a substrate-L chain contact or bore a negative effect (e.g. through repulsion of the L chain due to insertion of a different side group). Thus, those residues whose replacement with the corresponding TI-VAMP residue had significantly decreased the cleavage rate and had either reversed or introduced charges or increased the required space for the new side group, were also mutated to alanine. Of these mutations, Q33A, Q34A, V43A, and Q58A did not affect VAMP-2 cleavage (Fig. 2, open bars), indicating that these residues are not involved in the substrate-L chain interaction but that the TI-VAMP residues in these positions are not permissive for cleavage.

Next, to find out if critical amino acid positions contribute to substrate binding or the catalytic process, kinetic parameters were determined. Mutation of residues remote from the scissile peptide bond (i.e. mutations Q33D, V39A, and V43K) considerably increased K_\text{m} but had no effect on k_\text{cat}. Vice versa, replacement of residues around the scissile bond, D57G, Q58E, K59R, and L60A, solely diminished k_\text{cat} values. Kinetic parameters of mutants that had a minor or no significant effect on cleavage, like M46A or S61E, showed wild type-like K_\text{m} and k_\text{cat} values (Fig. 3, left). Together, residues in the direct neighborhood to the scissile bond are involved in the catalytic process, whereas those remote from the cleavage site are exclusively responsible for substrate binding. The data for mutants of the cleavage site are in compliance with those of earlier work conducted with synthetic VAMP-2 peptides (15).

FIGURE 2. Cleavage analysis of various VAMP-2 point mutants. Top, schematic representation of VAMP-2 and alignment of the amino acid region that was analyzed by systematic mutagenesis 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 gray. Peptide bonds attacked by BoNT/F, D, B, or TeNT are marked F, D, B, and T. Bottom, in order to determine the effect on hydrolysis by CNT L chains, residues differing among VAMP-2 and TI-VAMP were individually replaced in VAMP-2 with the corresponding amino acids of TI-VAMP. Some of those were in addition replaced with alanine. All identical residues were substituted by alanine, and Ala-37, which corresponds to TI-VAMP Ala-131 was replaced with leucine. VAMP-2 mutants were radiolabeled by in vitro transcription/translation and incubated for 1 h in the presence of 0.2 nM LC/F, 0.2 nM LC/D, 20 nM LC/B, or 10 nM LC/T. Samples were analyzed by Tris/Tricine-PAGE using 15% gels. Columns represent percentages of cleavage versus the wild-type VAMP-2-(1–96). Data represent means ± S.D. of at least four independent experiments. The dotted lines specify thresholds of 10, 33, and 66% reduction of cleavability, and the color code applied to the columns is as follows: green, no or less than 10% reduction of cleavability; yellow, more than 10% reduction of cleavability; pink, more than 33% reduction of cleavability; red, more than 66% reduction of cleavability. Open column overlays specify the results of parallel mutations to alanine in those positions. Dark blue horizontal bars below individual charts denote putative exosites, light blue bars show the cleavage sites, and dark blue dots show interspersed anchor points (see “Discussion”).
Reduction of LC/D cleavage by 33% was caused by mutations at nine amino acid residues, of which three reduced cleavage by 66%. The nine critical amino acid positions are distributed over the segment Val-39 to Leu-60. Considering the conservation of Val-39, Asp-40, and Glu-41 in TI-VAMP, these data perfectly matched the determination of the segment 42–64 (cf. TI-VH23) to permit optimal substrate cleavage. As for LC/F, mutation of few positions, Val-43, Gln-58, and Ser-61, apparently caused a reduction in cleavability through steric or electrostatic interference, as indicated by analysis of alanine mutations at these positions. Conversely, the positive charge at position 59 is crucial for substrate cleavage, since the mutation to alanine evoked a much stronger negative effect compared with the arginine mutation (Fig. 2, open bars).

Mutation of residues in the segment Val-39 to Met-46 increased K_m, indicating that this region is solely crucial for the formation of the Michaelis complex (Fig. 3, middle). Mutation of Met-46 increased K_m 6-fold, which was in agreement with its strongest effect on the cleavage rate (Fig. 2).

Next, cleavability of the various VAMP-2 mutants by LC/T was analyzed. Eleven mutations decreased cleavage to less than 66% of wild-type VAMP-2, five of them to less than 33%. Amino acid substitutions affecting cleavage by TeNT were found in two clusters. One cluster of amino acids locates around the scissile bond (residues 69–80), and the second comprises residues 41–45. Again, mutation of a residue located at a site remote from the scissile peptide bond had an effect only on K_m (V43K), whereas mutation of cleavage site residues A74S, Q76T, and E78K diminished exclusively k_cat (Fig. 3, right). Three more residues whose mutation diminished cleavage by 12–27% were identified even further upstream (residues 31–35). Hence, TeNT requires a substrate segment of at least 51 amino acids for optimal cleavage, whereas the required parts of VAMP-2 by BoNT/F and D comprise 31 and 26 amino acids, respectively. The essential part for BoNT/B, which shares the scissile bond with TeNT, had earlier been shown by mapping experiments to be even shorter and to comprise the substrate segment Ser-61 to Ser-80 (20). Interestingly, the systematic mutagenesis for TeNT showed that merely four residues in the region between the cleavage site and the remote cluster (amino acids 41–45) marginally reduced cleavability (Fig. 2). In this respect, TeNT resembles BoNT/A (23, 26).

Replacement of 10 TI-VAMP Amino Acids with the Corresponding Ones of VAMP-2 Creates a Readily Cleavable Substrate for BoNT/D—In order to scrutinize results of the systematic mutagenesis analysis, we set out to create a TI-VAMP variant containing as few mutations as possible that would be readily cleavable by BoNT/D. VAMP-2 residues crucial for cleavage were assembled successively in corresponding positions in TI-VAMP. As a starting point, we recognized that...
Substrate Recognition Mode of BoNT/D, BoNT/F, and TeNT

replacement of Glu-152 and Glu-155 with glutamine and serine, respectively, resulted in a TI-VAMP variant (designated TI-D-2) that was cleaved, albeit merely at high LC/D concentrations (Fig. 4). Import of further VAMP-2 residues stepwise increased the sensitivity of TI-VAMP to LC/D. A TI-VAMP variant that exhibited a cleavability similar to that of the genuine substrate VAMP-2 ultimately possessed 10 mutations (TI-D-10; Fig. 4) if the above mentioned inhibitory effect of the longin domain was neglected. At assay conditions, 32.6 ± 2.6% of TI-D10 was cleaved, which was comparable with 36.5 ± 5.8% for the hybrid TI-VH85 (Fig. 4). This finding confirmed the results of the preceding single amino acid replacement experiments in VAMP-2. Interestingly, to obtain a BoNT/B-sensitive TI-VAMP, an exchange of a similar number of amino acids (11 amino acids) was required (20). It remains to be shown whether TI-D-5, TI-D-8, or TI-D-10 could be useful for studies of the cellular function of TI-VAMP.

DISCUSSION

Here, we report comprehensive information about substrate amino acids that are essential for VAMP cleavage by BoNT/B, BoNT/D, and TeNT and their role in the catalytic process. The data show that similar to the SNAP-25 proteolyzing BoNT/A and BoNT/E, VAMP-specific CNTs initiate substrate interaction, employing an exosite located N-terminal of the scissile bond. However, the lengths of these exosites as well as their distance from the scissile bond vary from one to the other CNT.

The recently solved structure for SNAP-25 bound to a proteolytically inactive mutant of BoNT/A L chain combined with biochemical data for mutated SNAP-25 and wild-type L chain illustrated that this L chain initially contacts its substrate involving an exosite remote from the scissile peptide bond. The exosite comprises about 12 residues of SNAP-25 (positions 156–167). SNAP-25 then wraps around LC/A in a U-turn-like fashion; thereby, some further interactions along the substrate-toxin interface are established. The scissile peptide bond Gln-197–Arg-198 next becomes exposed to the active site residues of LC/A. This is probably ensured by a certain order of binding steps of several substrate cleavage site residues to L chain pockets around the active site (21, 23, 26, 28).

The present study provides evidence for a conserved recognition strategy of VAMP cleaving CNTs. First, exosite-like segments in VAMP-2 were identified for BoNT/D, BoNT/F, and TeNT and have recently been identified for BoNT/B as well (20). These clusters of residues are clearly distinguishable from the respective cleavage site and may be delineated to amino acids 31–41 (for LC/F), 39–46 (for LC/D), 63–67 for (LC/B), and 41–45 (for TeNT; Fig. 2), although except for LC/F, they are shorter than that of BoNT/A. However, amino acids at those sites fulfilled the criterion for exosite residues in that their mutation exclusively affected \( k_m \) values, thus indicating that they are only involved in the formation of the Michaelis complex. Second, at least for TeNT, the putative exosite would be separated from the cleavage site (if defined by P4-P4') by 27 amino acids, thus comparable with 25 residues for the BoNT/A exosite. The distance of the exosites for BoNT/F, D, and B are shorter, being 13, 9, and 5 residues, respectively, the last resembling in this aspect the configuration for BoNT/E (27). Third, as discovered for the SNAP-25-LC/A interface, the present study also identifies single residues in the region linking respective exosites and cleavage sites that could function as further anchor points.

In the final step prior to peptide bond cleavage, the scissile bond has to be properly aligned at the active site of the catalytic L chain. This is probably mediated via a yet undefined order of cleavage site residue interactions. Results of the present study together with earlier findings demonstrate that the importance of individual cleavage site positions significantly differs from one to another CNT. According to our data, BoNT/D for instance involves the substrate positions P3, P1, and P1'. However, the P1' amino acid interaction at the toxin S1' pocket is uniformly indispensable. This may mean that the P1'-S1' interaction sets the ultimate alignment before peptide bond cleavage can occur. Crystal structures of each CNT L chain are available, but except for presumable S1' pockets in the L chains (data not shown), we were unable to identify binding pockets for the other important VAMP cleavage site residues in the L chains. It will be interesting to find out if VAMP aligns in a similar fashion to SNAP-25 at the L chain active site.

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Substrate Recognition Mode of BoNT/D, BoNT/F, and TeNT

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