Cooperative Exosite-dependent Cleavage of Synaptobrevin by Tetanus Toxin Light Chain*

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The light chain (L chain) of tetanus neurotoxin (TeNT) has been shown to have been endowed with zinc endopeptidase activity, selectively directed toward the Gln76–Phe77 bond of synaptobrevin, a vesicle-associated membrane protein (VAMP) critically involved in neuroexocytosis. In previous reports, truncations at the NH2 and COOH terminus of synaptobrevin have shown that the sequence 39–88 of synaptobrevin is the minimum substrate of TeNT, suggesting either the requirement of a well defined three-dimensional structure of synaptobrevin or a role in the mechanism of substrate hydrolysis for residues distal from the cleavage site. In this study, the addition of NH2- and COOH-terminal peptides of synaptobrevin, S 27–55 (S1) and S 82–93 (S2), to the synaptobrevin fragment S 56–81 allowed the cleavage of this latter peptide by TeNT to occur. This appears to result from an activation process mediated by the simultaneous binding of S1 and S2 with complementary sites present on TeNT as shown by surface plasmon resonance experiments and the determination of kinetic constants. All these results favor an exosite-controlled hydrolysis of synaptobrevin by TeNT, probably involving a conformational change of the toxin. This could account for the high degree of substrate specificity of TeNT and, probably, botulinum neurotoxins.

Tetanus toxin (TeNT)1 and the seven serotypes of botulinum neurotoxins (BoNT/A, /B, /C, /D, /E, /F, and /G), which are produced by several anaerobic bacilli of the genus Clostridium, cause the paralytic syndromes of tetanus and botulism by blocking neurotransmitter release at central and peripheral synapses, respectively (1, 2). They are formed by two disulfide-linked polypeptides, the heavy (H) chain (100 kDa) being responsible for specific binding to neurons and cell penetration, and the light (L) chain (50 kDa) for neuroexocytosis blockade, the reduction of the disulfide bond being required for this process (1, 2). The L chain of these neurotoxins contains the typical His-Glu-Xaa-Xaa-His sequence of zinc endopeptidases (3–5). Accordingly, each toxin has been shown to contain one atom of zinc (6–8), except BoNT/C for which two zinc atoms have been found (9). These L chains, but not their apo-forms, cleave specifically three proteins of the neuroexocytosis apparatus: synaptobrevin, which is hydrolyzed by TeNT, BoNT/B, /D, /F, and /G, SNAP 25, which is cleaved by BoNT/A, /C1, and /E, and syntaxin by BoNT/C1 (10–12). Moreover, as suspected from previous studies on zinc metalloendopeptidases (13), site-directed mutagenesis of the recombinant light chains of TeNT and BoNT/A showed that the two histidines of the motif His-Glu-Xaa-Xaa-His are zinc ligands and the glutamate plays a critical role in the catalytic process (14–17).

Among the group of zinc metalloendopeptidases, a particularity of clostridial neurotoxins is a very high substrate selectivity, contrasting with the wide specificity of zinc proteases belonging to the thermolysin and metzincin families (13). Indeed, except for BoNT/C1 (11, 12), the clostridial neurotoxins appear to cleave a single protein substrate at a single peptide bond (10). The target of five clostridial neurotoxins, synaptobrevin, also known as VAMP (for vesicle-associated membrane protein), is a protein highly conserved in eucaryotes (18–20), which play a central role in neuroexocytosis (21–27). Human synaptobrevin isoform II contains 116 residues with a short COOH-terminal tail anchoring the molecule into the membrane of small synaptic vesicles. Its cytosolic region is composed of a NH2-terminal proline-rich domain of 24 residues followed by a hydrophilic domain of 72 amino acids (Fig. 1). TeNT cleaves synaptobrevin selectively at one single peptide bond Gln76–Phe77 (28). Successive truncations of synaptobrevin at its NH2 and COOH terminus have shown that the removal of both the proline-rich NH2-terminal 1–25 domain and the 93–116 transmembrane anchor does not reduce the rate of hydrolysis by TeNT (29, 30) (Fig. 1). Conversely, truncations of the 27–49 acidic domain (30–32) as well as truncations of the 82–93 basic domain (32) results in a dramatic decrease (100 times) in the rate of cleavage (Fig. 1), the minimum substrate of TeNT being the 50-residue peptide S 39–88 (32). Furthermore, several copies of a common acidic motif are present in the sequence of the three targets of clostridial neurotoxins (33). Antibodies raised against this motif cross-react with synaptobrevin, SNAP-25, and syntaxin and inhibit the cleavage of synaptobrevin by either TeNT or BoNT/B or /G (34). Two copies of this acidic motif are present in the sequence of synaptobrevin and correspond to the 38–47 and 62–71 regions designated V1 and V2, respectively. Substitutions of the acidic residues of V1 by serines in synaptobrevin abolish almost completely its cleavage by TeNT (34, 35). Altogether these findings suggested that domains of synaptobrevin far from the cleavage site could play an important role in the hydrolysis of this peptide either by inducing a cleavable conformation of synaptobrevin at the Gln76–Phe77 bond or by promoting a proteolytic conformation of the TeNT L chain. With the aim of answering these questions,
and S 82–93 are designated S1 and S2, respectively. The cleavage site Gln76–Phe77 of TeNT is indicated by the Sprefix is an abbreviation for human synaptobrevin II, followed by the first and the last residue number in the original sequence. Peptides S27–55 and S82–93 are designated S1 and S2, respectively. These results suggested that sequences distal from the cleaved bond Gln76–Phe77 are involved in the enzyme substrate interaction.

various peptides corresponding to the synaptobrevin (S) acidic domain S1 (for S 27–55) and basic domain S2 (for S 82–93) (Fig. 1) have been prepared by solid-phase synthesis, and their influence on the cleavage of different synthetic synaptobrevin fragments by purified TeNT L chain was studied. We show here that the cleavage of synaptobrevin fragment S 50–93 or S 32–81, too short to be efficiently cleaved by TeNT, occurred following addition of 1 mM S1 or 1 mM S2, respectively, while the same concentrations of S1 or S2 do not affect the high cleavage rate of the complete substrate S 32–93. Moreover, the addition of both 1 mM S1 and 1 mM S2 allowed a cleavage of the short S 56–81 fragment by TeNT L chain to be observed. Surface plasmon resonance experiments demonstrated that the binding of TeNT L chain to S1 is dependent on the presence of S2. Altogether these results show clearly that synaptobrevin cleavage is dependent on the interactions of both well defined NH2- and COOH-terminal domains S1 and S2, with complementary exosites present in TeNT. The influence of S1 and S2 on the kinetic constants of the enzymatic reaction favors an allosteric mechanism (36–38), probably through a conformational change of the toxin.

MATERIALS AND METHODS

Chemicals—Hepes and all other buffer components were purchased from Sigma. High performance liquid chromatography (HPLC) grade solvents (acetonitrile and methanol) were obtained from SDS (Pey sins, France). Solvents, reagents, and protected amino acids for peptide synthesis were obtained from Perkin-Elmer (Roissy, France). Solvents (acetonitrile and methanol) were obtained from SDS (Peypin, France). All the chemicals for HPLC were purchased from J. T. Baker (Philipsburg, NJ). HPLC grade acetic acid was purchased from Merck (Darmstadt, Germany). Ion electrospray mass spectrometry was obtained from Micromass (Manchester, UK). All the chemicals for chemical synthesis were purchased from Sigma.

Synthesis and Purification of Peptides—Assembly of the protected peptide chains was carried out using the stepwise solid phase method of Merrifield (39) on an Applied Biosystems (ABI) 431A automated peptide synthesizer with ABI small scale Fmoc (40) (9-fluorenlymethyl) chemistry. The peptides (S 27–55, S 82–93) were prepared as described in Soleilhac et al. (29) with the exception of the fluorescent substrate [Pya][S] 39–88 whose synthesis has been previously described (32). Peptides were cleaved from the resin, deprotected, diethyl ether-purified, and washed in accordance with ABI guidelines. All the peptides were purified by reverse phase (RP) HPLC on a Vydac C4 column (250 × 10 mm) using acetonitrile (CH3CN) gradients in 0.1% trifluoroacetic acid. Ion electrospray mass spectrometry allowed the molecular weight of synaptobrevin peptides and metabolites to be verified. The HPLC-purified peptides were lyophilized and stored at −20 °C. Peptide concentration was determined by absorbance measurements at 280 nm, on the basis of the extinction coefficient calculated from sequence data (40).

TeNT L Chain Purification—TeNT was kindly provided by Pasteur-Mérieux (France). The double chain was reduced by using dithiothreitol and TeNT L chain was separated from the H chain and purified by ion exchange chromatography as described previously in Soleilhac et al. (32). The TeNT L chain was stored in 20 mM Hepes, pH 7.4, at −20 °C in siliconized Eppendorf tubes. No change in peptidase activity was observed after 6 months.

RP-HPLC Analyses of TeNT-mediated Endoproteolysis of Synaptobrevin Peptides—For substrate peptides S 32–93, S 32–81, S 50–93, and S 56–81, the enzymatic assays were carried out in a typical volume of 100 µl in siliconized Eppendorf tubes. In this case, 100 µM substrate (1 mM for S 56–81) was incubated with 1 µg of TeNT L chain in the presence of 1 mM S1 or/and 1 mM S2 in 20 mM Hepes, pH 7.4, at 37 °C. The cleavage was stopped by adding 50 µl of 0.2 M HCl, and this solution was analyzed by HPLC on a Capcell Pa column (300 Å, 7 µm, 250 × 4.6 mm) under isocratic conditions (24% B for the metabolite S 77–93 and 8% B for S 77–81. Eluent A was trifluoroacetic acid 0.05% in H2O; eluent B was CH3CN 90%, trifluoroacetic acid 0.035% in H2O; ultraviolet detection λ = 214 nm). After elution of the cleavage product, the substrate and the activating peptide were removed by washing the chip with 100% B. The amount of cleavage product released was determined by HPLC from a standard curve obtained by injection of known concentrations of synthetic cleavage products (S 77–93 and S 77–81).

Surface Plasmon Resonance Analysis—Real-time surface plasmon resonance sensorgrams were recorded on a Pharmacia Biosensor BIACore 2000 apparatus. Experiments were run with a CM5 sensor chip (Pharmacia Biosensor, Uppsala, Sweden) on which 0.2 pmol/mm2 peptide S1 (relative response, 688 RU) was covalently bound to the dextran core 2000 apparatus. Experiments were run with a CM5 sensor chip (Pharmacia Biosensor, Uppsala, Sweden) on which 0.2 pmol/mm2 peptide S1 (relative response, 688 RU) was covalently bound to the dextran matrix via free amino groups by the N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide-N-hydroxy-succinimide activation method followed by a deactivation by ethanolamine. Another channel was activated by the same method and deactivated in the same way to serve as a control. The maximal response allowed for a 1/1 stoichiometry binding of TeNT to S1 was calculated to be 9800 RU given the molecular mass of TeNT L chain (50 kDa). Binding experiments were performed at 25 °C with a flow rate of 10 µl/min in 20 mM Hepes, pH 7.4. After each binding experiment, the sensor chip was regenerated by a 6 mM guanidinium chloride pulse of 30 s.

Fluorescence Measurements of TeNT-mediated Cleavage of [Pya][S] 39–88 in Presence of S1 and S2—The enzymatic assay has been described in detail in Soleilhac et al. (32). Briefly, 20 µM substrate [Pya][S] 39–88 (50 amino acids) and different concentrations of S1 and S2 peptides were incubated in siliconized Eppendorf tubes in a total volume of 100 µl of 20 mM Hepes, pH 7.4, containing 250 ng of TeNT L chain (50 nm) for 20 min at 37 °C in the dark. The reaction was stopped by the addition of 900 µl of 72% MeOH, 0.1% trifluoroacetic acid in H2O.

FIG. 1. Primary sequence of fragments of human synaptobrevin II used as substrate or modulator of TeNT enzymatic activity. The S prefix is an abbreviation for human synaptobrevin II, followed by the first and the last residue number in the original sequence. Peptides S 27–55 and S 82–93 are designated S1 and S2, respectively. The cleavage site Gln76–Phe77 of TeNT is indicated by the arrow. TMR indicates the transmembrane region and IR, the intravesicular region. The presence or the absence of cleavage of the different substrates is indicated by + or − signs, respectively.

| Proline rich head | Hydrophilic core | TMR | IR |
|------------------|-----------------|-----|----|
| S1               | TNNR                  |     |    |
| S2               | A                          |     |    |
Determination of the Influence of S1 and S2 on the Kinetic Constants of the Enzymatic Reaction

To determine whether S1 or S2 could promote the cleavage of synaptobrevin fragments S50–93, S32–93, and S56–81 by TeNT L chain, the latter peptides were incubated in the presence of 1 mM S1 or S2 before addition of TeNT L chain. After 20 min at 37 °C, the reaction mixtures were analyzed by RP-HPLC in order to quantify the level of cleavage products generated. No effect on the cleavage rate of the optimal substrate S32–93 was observed after addition of the same concentrations of S1 (Fig. 2, C and D) or S2 (Fig. 3A). In contrast, the cleavage rate of S50–93 in the presence of 1 mM S1 increased 34-fold (52 pmol min⁻¹ μg⁻¹) reaching a level comparable to that of S32–93 (102 pmol min⁻¹ μg⁻¹) (Fig. 2, A and B) but was not enhanced by the addition of 1 mM S2 (Fig. 3B). An even greater potentiating effect was observed following addition of 1 mM S2 to S32–81 (Fig. 3C). This effect is difficult to quantify because, in the absence of S2, the cleavage of S32–81 was undetectable; however, taking 0.1 pmol min⁻¹ μg⁻¹ as the limit of cleavage product detection, it can be assumed to be greater than 150-fold. The addition of 1 mM S1 to this peptide did not improve its cleavage (Fig. 3C). In view of these results the influence of the addition of 1 mM S1 and/or 1 mM S2 was tested on the synaptobrevin fragment S56–81, lacking both S1 (S27–55) and S2 (S43–56) domains. In this case, as expected, the simultaneous presence of the two peptides S1 and S2 allowed a significant TeNT enzyme activity (12 pmol min⁻¹ μg⁻¹) (Fig. 3D) to be detected. Interestingly, no effects could be obtained under the same conditions with a peptide sequence shorter than S1, S37–47 (data not shown), which corresponds to the acidic motif conserved in SNAP-25 and syntaxin (33).

The Presence of S2 Is Required to Observe TeNT L Chain Binding to S3-A convenient method to observe interactions between small soluble peptides and proteins is to use the surface plasmon resonance technique (41). When a 6 μM solution of TeNT L chain was injected into a flow cell containing immobilized S1, no binding was observed (Fig. 4). Contrary to S1, a mixture of 6 μM TeNT L chain with 50 μM S3 showed a specific binding of about 4000 RU (Fig. 4). In a control experiment, injection of 50 μM peptide S3 alone was shown to produce only a small response of 330 RU (Fig. 4). The S3-promoted interaction between TeNT and S3 was dose-dependently reversed by preincubating the TeNT-S3 mixture with a large excess of S3. With 1 mM S3, no detectable binding of TeNT was observed (Fig. 4, inset).

Determination of the Influence of S1 and S2 on the Kinetic Constants of the Enzymatic Activity of TeNT L Chain—The minimum fluorescent substrate [Pya]S39–88, which contains the non-natural fluorescent amino acid pyrenylalanine (Pya) in position 88 (32) was chosen to study the influence of S1 and S2 on the kinetic constants of the enzymatic reaction. Increasing concentrations of peptides S1 and S2 induced a dose-dependent increase in [Pya]S39–88 cleavage, with maximal substrate degradation obtained at 100 μM either S1 or S2 (170 and 600%, respectively) (Fig. 5), which was therefore the concentration used to measure the influence of the peptides on the kinetic constants of the enzymatic reaction (Table I). The addition of peptide S2 produced a shift of the apparent Michaelis constant (Km) value of the substrate from 576 to 51 μM, while the maximal velocity (Vmax) was only slightly affected (166 instead of 111 pmol min⁻¹ μg⁻¹). As expected from a partial overlap of the sequences of S1 and S2, the [Pya]S39–88 substrate, the potentiating effect induced by the peptide S1 was lower, the Km shifting from 576 to 479 μM and the Vmax shifting from 111 to 169 pmol min⁻¹ μg⁻¹ (Table I). In all these studies, control experiments by RP-HPLC indicated that the enhanced fluorescence corresponded to an increase in the formation of the cleavage product [Pya]S77–88 (data not shown). No other cleavage was observed.

The purpose of this study was to investigate the mechanism involved in the highly specific and selective cleavage of synap-
tobrevin at the Gln76–Phe77 bond (28). Previously reported data have shown that the acidic NH$_2$-terminal sequence 27–55 and the basic COOH-terminal sequence 82–93 of synaptobrevin are essential for an efficient cleavage of this peptide (29–32). Different possibilities could account for these data. (i) The hydrolysis of synaptobrevin could require a well defined tertiary structure of the peptide substrate induced by residues far from the cleavage site. (ii) Additional interactions with TeNT could be necessary to generate the Michaelis complex. (iii) Interactions of well defined sequences of the substrate with specific exosites present on tetanustoxin could be necessary to promote synaptobrevin cleavage, as it is the case for allosteric enzymes.

In order to answer these questions, synthetic peptides S$_1$ and S$_2$ corresponding, respectively, to the acidic and basic motif of synaptobrevin were tested for their ability to enhance the cleavage rate of the complementary synaptobrevin sequences by TeNT. Interestingly, the cleavage of the synaptobrevin peptide S 50–93, which lacks almost completely the S$_1$ sequence and which is very poorly cleaved by TeNT, was greatly enhanced (34-fold) by the addition of 1 mM S$_1$. Similarly, the cleavage of the synaptobrevin peptide S 32–81, in which the full sequence of S$_2$ is lacking and which is not cleaved by TeNT, was triggered (150-fold) by the addition of 1 mM S$_2$. The deci-
sive demonstration of the role of the S1 and S2 motifs came from the cleavage of S 56–81, a synaptobrevin peptide lacking both S1 and S2 domains, which was induced by the simultaneous addition of 1 mM S1 and 1 mM S2.

In all these experiments the cleavage rate measured for the mixtures of peptides was 2–7 times lower than that measured with the optimum substrate S 32–93 (Fig. 3). This was not unexpected, since the affinity for TeNT of the isolated fragments and their ability to fit the complementary sites within the protein cannot be as high as when the binding motifs are included in the substrate sequence.

These results do not support the hypothesis of a role for the NH2- and COOH-terminal peptides S1 and S2 in inducing a TeNT-cleavable conformation of synaptobrevin. Indeed, the formation of a unique tertiary structure, comparable to that of S 27–93, by mixing the three short peptides S 27–55 (S), S 56–81, and S 82–93 (S2) is highly improbable. This is supported by the lack of a well defined tertiary structure for synaptobrevin in solution as previously observed by 1H NMR (29). A mechanism which could account for the present results is the promotion of an active conformation of TeNT induced by the binding of the S1 and S2 domains of synaptobrevin to exosites present on the toxin surface, as in the case of allostERIC enzymes (36–38). Surface plasmon resonance analysis clearly showed the binding of the S1 domain of synaptobrevin to TeNT. This interaction occurs only in the presence of the S2 fragment and is not due to a direct interaction between S1 and S2 as shown by 1H NMR analysis (data not shown). This finding strongly suggests a cooperative interaction of both exosites. Conversely, when S2 was immobilized on the sensor chip, the ionic interaction of the highly basic S2 fragment with the multiple negative charges of the dextran matrix probably hampered the correct orientation of the S2 side chains, accounting for the absence of binding observed when either TeNT or a TeNT-S1 mixture was injected through a flow cell coated with S2 (data not shown). However, this interaction has been clearly demonstrated by the 10-fold decrease in the KM value of the fluorescent substrate following addition of the lacking S2 fragment (Table 1).

Table I

| Peptide | KM (mM) | Vmax (pmole min^-1 mg^-1) |
|---------|---------|--------------------------|
| [Pya^{88}]S 39–88 | 576 ± 11 | 111 ± 10 |
| [Pya^{88}]S 39–88 + S1 | 479 ± 12 | 169 ± 11 |
| [Pya^{88}]S 39–88 + S2 | 51 ± 11 | 166 ± 10 |

Aside from the selectivity of other clostridial neurotoxins.

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**FIG. 6. Model of cooperative exosite-dependent cleavage of synaptobrevin by TeNT.** A represents the S2 sequence of synaptobrevin, B the S1 sequence, and C the S1/S2 sequence. TeNT L chain might exist mainly under nonproteolytic conformation(s), which can bind the S2 sequence of synaptobrevin. The S2-ToNT complex undergoes a transformation (STEP 1), which allows TeNT to bind S1. This induces the final conformational transition (STEP 2), and subsequent formation of the Michaelis complex.

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VAMP Cleavage by TeNT