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Allosteric-type control of synaptobrevin cleavage by tetanus toxin light chain

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SUMMARY

The light chain of tetanus neurotoxin (TeNT L chain) has been shown to be endowed with zinc endopeptidase activity, selectively directed towards the Gln⁷⁶-Phe⁷⁷ bond of synaptobrevin, a vesicle-associated membrane protein 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. 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.

INTRODUCTION

Tetanus toxin (TeNT) 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]. They are formed by two disulfide-linked polypeptides, the heavy chain H (100 kDa) being responsible for specific binding to neurons and cell penetration, and the light chain

L (50 kDa) for neuroexocytosis blockade, the reduction of the disulfide bond being required for this process [1]. The L chain of these neurotoxins contains the typical His-Glu-Xaa-Xaa-His sequence of zinc endopeptidases [2]. Accordingly, each toxin has been shown to contain one atom of zinc [3]. 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 [4].

Among the group of zinc metallopeptidases, a particularity of clostridial neurotoxins is a very high

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HUMAN SYNAPTOBREVIN II

Proline rich head		Hydrophilic core			TMR	IR
1	MSATAATAPPAAPAGEGGPPAPPNLT	39	QQAQVDEVVDIMRVNVDKVLERDQKLS	76-77	88	116
				↓		
		32	LQQAQVDEVVDIMRVNVDKVLERDQKLS	76-77	93	
						Cleavage by TeNT
						+++
			50	VDKVLERDQKLS	93	
						-
		32	LQQAQVDEVVDIMRVNVDKVLERDQKLS	81		
						-
	27	37	47	55	82 + + + +	+ 93
	TSNRRLQQAQVDEVVDIMRVNVDKVLE				AKLKRKYWKNL	
						S ₁
						S ₂

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 as 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 absence of cleavage of the different substrates is indicated by the presence of + or -, respectively. These results suggest that sequences distal from the cleaved bond Gln⁷⁶-Phe⁷⁷ are involved in the enzyme-substrate interaction.

substrate selectivity, contrasting with the wide specificity of zinc proteases belonging to the thermolysin and metzincin families [5]. Indeed, the clostridial neurotoxins appear to cleave a single protein substrate at a single peptide bond [4]. The target of five clostridial neurotoxins, synaptobrevin, also known as VAMP (for vesicle-associated membrane protein), is a protein highly conserved in eucaryotes [6] which plays a central role in neuroexocytosis [7]. TeNT cleaves synaptobrevin selectively at one single peptide bond Gln⁷⁶-Phe⁷⁷ [8]. 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 [9,10] (Fig. 1). Conversely, truncations of the 27–49 acidic domain [10–12] as well as truncations of the 82–93 basic domain [12] result 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 [12]. 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, 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 the purified TeNT L chain was studied.

MATERIALS AND METHODS

Chemicals

Hepes and all other buffer components were purchased from Sigma (St. Louis, MO, U.S.A.). High-performance liquid chromatography (HPLC) grade solvents (acetonitrile and methanol) were obtained from SDS (Peypin, France). Solvents, reagents and protected amino acids for peptide synthesis were obtained from Perkin Elmer (Roissy, France). All the chemicals for surface plasmon resonance (SPR) experiments were provided by Pharmacia Biosensor (Uppsala, Sweden).

Synthesis and purification of peptides

Assembly of the protected peptide chains was carried out on an Applied Biosystems (ABI) 431A automated peptide synthesizer with ABI small-scale Fmoc chemistry. The peptides (S 27–55, S 82–93, S

32–93, S 32–81, S 50–93, S 56–81, S 77–93 and S 77–81) were synthesized as outlined by Cornille et al. [9].

TeNT L chain purification

TeNT was kindly provided by Pasteur-Mérieux (Marcy l'étoile, France). The double chain was reduced by using dithiothreitol (DTT) and the TeNT L chain was separated from the H chain and purified by ion exchange chromatography as previously described by Soleilhac et al. [12]. The TeNT L chain was stored in 20 mM Hepes (pH 7.4) at -20°C in siliconized eppendorfs. 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 eppendorfs. In this case, 100 μM of substrate (1 mM for S 56–81) was incubated with 1 μg TeNT L chain in the presence of 1 mM S_1 or/and 1 mM S_2 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 C_8 column (300 \AA , 7 μM , 250 \times 4.6 mm) under isocratic conditions (24% B for the metabolite S 77–93 and 8% B for S 77–81; eluent A: TFA 0.05% in H_2O ; eluent B: CH_3CN 90%, TFA 0.038% in H_2O , ultraviolet detection $\lambda = 214$ nm).

SPR analysis

Real-time SPR 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 mm^{-2} of peptide S_1 (relative response 688 RU (resonance units)) was covalently bound to the dextran matrix via free amino groups by the EDC-NHS 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 control. The maximal response allowed for a 1/1 stoichiometry binding of TeNT to S_1 was calculated to be 9800 RU given the molecular weight of

TeNT L chain (50 kDa). Binding experiments were performed at 25°C with a flow rate of 10 $\mu\text{l}/\text{min}^{-1}$ in 20 mM Hepes (pH 7.4). After each binding experiment, the sensor chip was regenerated by a 6 M guanidinium chloride pulse of 30 s.

RESULTS

Promoting effects of peptides S_1 or/and S_2 on the cleavage of fragments S 50–93, S 32–81 and S 56–81 by TeNT L chain

In order to see if S_1 or S_2 could promote the cleavage of synaptobrevin fragments S 50–93, S 32–81 and S 56–81, which are poorly or not at all cleaved by TeNT L chain, the latter peptides were incubated in the presence of 1 mM S_1 or S_2 before the 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 S 32–93 was observed after the addition of the same concentrations of S_1 or S_2 (Fig. 2A). In contrast, the cleavage rate of S 50–93 in the presence of 1 mM S_1 increased 34-fold (52 pmol $\text{min}^{-1} \mu\text{g}^{-1}$), reaching a level comparable to that of S 32–93 (102 pmol $\text{min}^{-1} \mu\text{g}^{-1}$), but was not enhanced by the addition of 1 mM S_2 (Fig. 2B). An even greater potentiating effect was observed following the addition of 1 mM S_2 to S 32–81 (Fig. 2C). This effect is difficult to quantify because, in the absence of S_2 , the cleavage of S 32–81 was undetectable; however, taking 0.1 pmol $\text{min}^{-1} \mu\text{g}^{-1}$ as the limit of cleavage product detection, it can be assumed to be greater than 150-fold. The addition of 1 mM S_1 to this peptide did not improve its cleavage (Fig. 2C). In view of these results, the influence of the addition of 1 mM S_1 or/and 1 mM S_2 was tested on the synaptobrevin fragment S 56–81, lacking both S_1 (S 27–55) and S_2 (S 82–93) domains. In this case, as expected, the simultaneous presence of the two peptides S_1 and S_2 allowed a significant TeNT enzyme activity (12 pmol $\text{min}^{-1} \mu\text{g}^{-1}$) (Fig. 2D) to be observed.

The presence of S_2 is required to observe TeNT L chain binding to S_1

A convenient method to observe interactions between small soluble peptides and proteins is to use

Hydrolysis of Synaptobrevin fragments by TeNT				Cleavage rate ($\mu\text{mole}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$)
		TeNT ^y Cleavage		
		32	93	
		LQQTQAQVDEVDIMRVNVDKVLERDQKLSLDDRADALQAGASQFETSAAKLKPKRYWVKNL		
A				102 ± 5
		27	55	
		NLTSNRRLQQTQAQVDEVDIMRVNVDKLE + S 32-93		105 ± 5
			+ 82 S2 93	
		S 32-93 + AKLKPKRYWVKNL		103 ± 5
		50	93	
		VDKVLERDQKLSLDDRADALQAGASQFETSAAKLKPKRYWVKNL		1.5 ± 0.5
B		27	55	
		NLTSNRRLQQTQAQVDEVDIMRVNVDKLE + S 50-93		52 ± 2
		S 50-93 + 82 S2 93		1.5 ± 0.5
		AKLKPKRYWVKNL		
		32	81	
		LQQTQAQVDEVDIMRVNVDKVLERDQKLSLDDRADALQAGASQFETSA		< 0.1
C				
		S 32-81 + 82 S2 93		15 ± 0.5
		AKLKPKRYWVKNL		
		27	55	
	NLTSNRRLQQTQAQVDEVDIMRVNVDKLE + S 32-81		< 0.1	
		56	81	
		HDKLSELDORADALQAGASQFETSA		< 0.1
D		27	55	
		NLTSNRRLQQTQAQVDEVDIMRVNVDKLE + S 56-81		< 0.1
		S 56-81 + 82 S2 93		< 0.1
		AKLKPKRYWVKNL		
		+ 82 S2 93		12 ± 0.5
		AKLKPKRYWVKNL		

Fig. 2. Influence of the addition of S₁ or/and S₂ on the enzymatic cleavage of different synaptobrevin peptides by TeNT L chain. Peptide substrate (10⁻⁴ M) (10⁻³ M in the case of S 56-81) was incubated in the presence or absence of 1 mM S₁ or/and 1 mM S₂ in a total volume of 100 μ l of 20 mM hepes (pH 7.4) with 1 μ g of TeNT L chain at 37 °C. The reaction was stopped by the addition of 50 μ l of HCl (0.2 N). The released cleavage products S 77-93 or S 77-81 were quantified by RP-HPLC as described in the Materials and Methods section and in Fig. 2. The cleavage rate is expressed as \pm SEM of three independent experiments performed in duplicate.

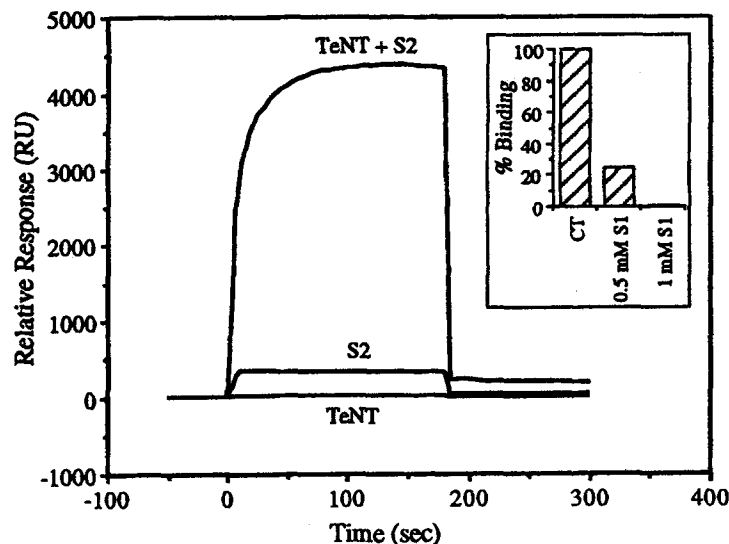


Fig. 3. Binding of TeNT L chain to S₁ in the presence of S₂ observed by SPR (BIAcore). Resonance units (RU) are plotted as a function of time (sensorgrams) for representative experiments. Thirty microliter of 6 μ M TeNT L chain in 20 mM Hepes (pH 7.4) (bottom trace), or 30 μ l of 50 μ M S₂ in 20 mM Hepes (pH 7.4) (middle trace), or 30 μ l of 6 μ M TeNT L chain + 50 μ M S₂ in 20 mM Hepes (pH 7.4) (top trace) were passed into a flow cell coated with S₁ at a flow rate of 10 μ l/min⁻¹. The 0 abscissa represents the beginning of injection. The inset represents the abolition of TeNT binding observed by coinjection of TeNT + S₂ with increasing concentrations of S₁. The relative responses expressed in percentage of binding were calculated at the equilibrium ($t = 150$ s after injection).

the SPR technique. When a 6 μM solution of TeNT L chain was injected into a flow cell containing immobilized S_1 , no binding was observed (Fig. 3). In contrast, injection of a mixture of 6 μM of TeNT L chain with 50 μM S_2 showed a specific binding of about 4000 RU (Fig. 3). In a control experiment, injection of 50 μM of peptide S_2 alone was shown to produce only a small response of 330 RU (Fig. 3). The S_2 -promoted interaction between TeNT and S_1 was dose-dependently reversed by preincubating the TeNT- S_2 mixture with a large excess of S_1 . With 1 mM S_1 , no detectable binding of TeNT was observed (Fig. 3, inset).

DISCUSSION AND CONCLUSIONS

The purpose of this study was to investigate the mechanism involved in the highly specific and selective cleavage of synaptobrevin at the Gln⁷⁶-Phe⁷⁷ bond [8]. Previously reported data have shown that the acidic NH₂-terminal sequence 27–55 and the basic COOH-terminal sequence 82–93 of synaptobrevin are essential for an efficient cleavage of this peptide [9–12]. 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 decisive demonstration of the role of the S_1 and S_2 motifs came from the cleavage of S 56–81, a synaptobrevin peptide lacking both S_1 and S_2 domains, which was induced by the simultaneous addition of 1 mM S_1 and 1 mM S_2 .

Furthermore, SPR analysis clearly showed the binding of the S_1 domain of synaptobrevin to TeNT. This interaction occurs only in the presence of the S_2 fragment and is not due to a direct interaction between S_1 and S_2 as shown by ¹H NMR analysis (data

not shown). This finding strongly suggests a cooperative interaction of both exosites.

Altogether, these findings suggest that peptides S_1 and S_2 favor the cleavage of synaptobrevin fragments by interaction with corresponding exosites present at the toxin surface, and are in good agreement with an allosteric model of enzyme functioning [13]. According to this hypothesis, the predominant conformational state of TeNT L chain in solution could correspond to non-proteolytic form(s) in equilibrium with a very low percentage of the proteolytic form. The higher affinity of the S_1 and S_2 domains for the latter form would increase its population, their interaction with the corresponding exosites on TeNT triggering the proteolytic cleavage of the VAMP. Nevertheless, further physicochemical experiments will be necessary to physically observe the proposed change of TeNT conformation. As far as we know, a cooperative mechanism has never been described for zinc proteases, and for proteases in general such a mechanism is only known for thrombin, a trypsin-like serine protease, which makes use of exosites for recognition of its substrate [14]. TeNT L chain selectivity is dependent on this type of mechanism.

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