Botulinum neurotoxin types A and E require the SNARE motif in SNAP-25 for proteolysis

Philip Washbourne[a], Rossella Pellizzari[a], Giulia Baldini[b], Michael C. Wilson[c], Cesare Montecucco[a,*]

[a]*Centro C.N.R. Biomembrane and Dipartimento di Scienze Biomediche, Università di Padova, Via Colombo, 3, 35100 Padua, Italy*
[b]*Dept. of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, New York, USA*
[c]*Dept. of Neurosciences, University of New Mexico, School of Medicine, Albuquerque, NM, USA*

Received 25 September 1997; revised version received 16 October 1997

Abstract Botulinum neurotoxins type A and E (BoNT/A and BoNT/E) are metalloproteases with a unique specificity for SNAP-25 (synaptosome-associated protein of 25 kDa), an essential protein component of the neuroexocytotic machinery. It has been suggested that this specificity is directed through the recognition of a nine residue sequence, termed SNARE motif, that is common to the other two SNARE proteins: VAMP (vesicle-associated membrane protein) and syntaxin, the only known substrates of the other six clostridial neurotoxins. Here we analyse the involvement of the four copies of the SNARE motif present in SNAP-25 in its interaction with BoNT/A and BoNT/E by following the kinetics of proteolysis of SNAP-25 mutants deleted of SNARE motifs. We show that a single copy of the motif is sufficient for BoNT/A and BoNT/E to recognise SNAP-25. While the copy of the motif proximal to the cleavage site is clearly involved in recognition, in its absence, other more distant copies of the motif are able to support proteolysis. Also, a non-neuronal isoform of SNAP-25, Syndet, is shown to be sensitive to BoNT/E, but not BoNT/A, whilst the SNAP-25 isoforms from *Torpedo marmorata* and *Drosophila melanogaster* were demonstrated not to be substrates of these metalloproteases.

Key words: SNAP-25; Syndet; SNARE motif; Botulism; Clostridium; Neurotoxin; Zinc endopeptidase

1. Introduction

Botulinum neurotoxins cause the flaccid paralysis typical of botulism by blocking acetylcholine release at the neuromuscular junction. They are di-chain zinc endopeptidases secreted by strains of *Clostridium botulinum*, *C. butyricum* and *C. barati*. The neurotoxins reach their intracellular targets by translocating the light chain (50 kDa) into the cytosol after having been endocytosed via interaction of the heavy chain (100 kDa) with a high affinity receptor complex [1,2]. Serotypes B, D, F and G proteolyse VAMP/synaptobrevin [3–5], serotypes A and E specifically cleave SNAP-25 [6–8] and type C acts on syntaxin and SNAP-25 [9–11]. Syntaxin and SNAP-25, associated with the cytoplasmic face of the plasma membrane of presynaptic nerve terminals, and VAMP, inserted into the synaptic vesicle membrane, have been postulated to act as synaptic vesicle docking receptors (t- and v-SNAREs), and have been implicated in the steps leading to membrane fusion for release of neurotransmitter into the synaptic cleft [12]. SNAP-25, however, seems not only involved in neuroexocytosis, but is also required for axonal growth and synaptogenesis [10,13,14]. These potentially distinct roles of SNAP-25 in development and mature synaptic function may be mediated through the regulated expression of SNAP-25 isoforms encoded by alternative splicing [15]. The three SNAREs, SNAP-25, syntaxin and VAMP are the only eukaryotic proteins to possess a distinct and conserved motif [16]. This short motif which is present twice in VAMP (V1 and V2), twice in syntaxin (X1 and X2) and at four positions in SNAP-25 (S1 to S4) (Fig. 1) is predicted to form an amphipathic α-helix, providing a surface of negative charge on one-third of the surface and a group of hydrophobic residues on the adjacent third of the cylinder [16]. Recent studies suggest that these SNARE motifs are required by the clostridial neurotoxins to interact specifically with their substrates allowing them to then cleave at discrete sites elsewhere in the polypeptide chain. Mutagenesis studies on the two copies of the SNARE motif of VAMP have demonstrated that each of the five VAMP specific clostridial neurotoxins recognise their substrate in distinct ways: TeNT, BoNT/F and BoNT/D interact predominantly with the amino-terminal copy of the motif, whereas BoNT/B and BoNT/G recognize the carboxyl-terminal one [16–19]. The nonapeptide motif is highly conserved not only in SNAREs from mammals but also from *Drosophila melanogaster*, *Torpedo marmorata* [20], *Carassius aureus* [21] and *Arabidopsis thaliana* (X. Gansel, personal communication) (Fig. 1). This suggests that they are probably involved in an essential part of SNARE function.

BoNT/A is the best available therapeutic agent of a variety of human diseases that benefit from a functional inhibition of cholinergic terminals [22,23]. Hence, there is a particular importance in understanding the molecular basis of the action of BoNT/A. Here, we report on mutagenesis studies of SNAP-25 which demonstrate that the most C-terminal motif, S4, is a prerequisite for the specific proteolysis by BoNT/A and BoNT/E. In the absence of S4, the distal motifs, S1, S2 and S3, can substitute in the substrate recognition step, although this leads to reduced efficiency in proteolytic activity. We have extended these studies to show that the non-neuronal SNAP-25 homologue, Syndet, is susceptible to BoNT/E, but not BoNT/A, and that SNAP-25 from *T. marmorata* and *D. melanogaster* are not effective substrates for these neurotoxins.

*Corresponding author. Fax: +39 (49) 8276049.
E-mail: cesare@civ.bio.unipd.it

Abbreviations: BoNT, botulinum neurotoxin; DTT, dithiothreitol; GST, glutathione S-methyl transferase; SNAP-25, synaptosome-associated protein of 25 kDa; SNAREs, soluble NSF accessory protein receptors; TeNT, tetanus toxin; VAMP, vesicle-associated membrane protein

PII S0014-5793(97)01328-8

2. Materials and methods

2.1. Proteins and chemicals

BoNT/A and BoNT/E were obtained from WAKO (Germany) and further purified via immobilized metal-ion affinity chromatography to remove traces of contaminant proteases [24]. Anti-C-terminal SNAP-25 antibodies were prepared as detailed previously [25]. Primary antibodies were detected by immunostaining with an anti-rabbit conjugated with alkaline phosphatase (Sigma) using nitrotetrazolium blue and bromo-chloro-indolylphosphate (Sigma).

2.2. Bacterial strains, plasmid construction and SNAP-25 deletions and mutagenesis

The expression plasmids containing the SNAP-25b isoform coding region were generated from a DNA fragment amplified by PCR from a mouse SNAP-25 cDNA clone characterised previously [25]. The DNA fragment comprises the complete sequence of the SNAP-25 coding region flanked by *Bam*HI and *Eco*RI restriction sites at the 5′ and 3′ ends respectively and was inserted into the pEMBL18$^+$ plasmid. Uracil containing ssDNA was produced within BW313, an *E. coli* dut- ung-strain, for generation of SNAP-25 mutant ΔS4 by site directed mutagenesis. The in vitro reaction was performed as described by Kunkel et al. [26]. The following oligonucleotide was used: 5′-GATGCCCGGGAAAATGGCATCA-TCGGAAAC-3′. The sequence of the mutated gene was checked by dideoxy sequencing using fmol DNA Sequencing System (Promega). N-terminal deletions were created by PCR using oligonucleotide forward primers corresponding to the sense strand of SNAP-25b, incorporating a *Bam*HI site at the 5′ end. The reverse primer was the same as for wild-type SNAP-25b. Wild-type SNAP-25b, mutants and deletions were subcloned into *Bam*HI and *Eco*RI sites of pGEX-4T3 vector (Pharmacia) and transformed into the AB1899 strain of *E. coli*. SNAP-25 and Syndet from mouse, *Drosophila* and *Torpedo* SNAP-25 were also subcloned by PCR, introducing the HA1 epitope tag at the 3′ end and inserted into pGEX 4T3 and expressed in AB1899.

2.3. Protein expression and purification

Mouse SNAP-25, mouse Syndet, *Drosophila* SNAP-25, *Torpedo* SNAP-25 and mouse SNAP-25 mutants were expressed as GST-fusion proteins and were purified by affinity chromatography on GSH-sepharose matrix (Pharmacia) as before [17].

2.4. Assay of proteolytic activity

After treatment with DTT 10 mM for 30 min at 37°C, BoNT/A or BoNT/E (50 nM or 150 nM final concentration) were added to each of the GST-fusion SNAP-25s (0.5 μg/μl final concentration) in phosphate buffer, 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4 and incubated at 37°C for variable periods of time. The concentrations of the two proteases were selected after preliminary experiments to provide rates of cleavage capable of evidentiating differences among SNAP-25 mutants. Samples were analysed by Western blotting onto nitrocellulose after electrophoresis in a 13% polyacrylamide SDS gel and were probed with an anti-SNAP-25 C-terminal antibody or with an anti-HA1 tag monoclonal (Boehringer Mannheim).

3. Results and discussion

3.1. Minimal length for proteolysis of SNAP-25 by BoNT/A and BoNT/E includes SNARE motif S4

A characteristic feature of the three targets of the clostridial neurotoxins: SNAP-25, VAMP and syntaxin, is the common presence of a nine residue sequence, termed the SNARE motif [16]. Experimental evidence indicates that one copy of the SNARE motif present in VAMP is implicated in the binding followed by proteolysis by the five VAMP specific clostridial neurotoxins [17–19]. Fig. 1 illustrates the location and sequence of the four copies of the SNARE motif present in the SNAP-25 molecule, together with the cleavage sites of BoNT/A and BoNT/E. Beginning from the amino-terminus, the four copies of the motif are termed S1, S2, S3 and S4.

In order to determine which of the four copies of the SNARE motif present in neuronal SNAP-25 is important for the recognition by the SNAP-25 specific clostridial neurotoxins, we constructed a series of N-terminal deletions of mouse SNAP-25b expressed as recombinant GST-fusion proteins and measured their rate of proteolytic cleavage. Fig. 2 shows the various chimeric GST-SNAP-25 constructs that have been prepared (top panel), expressed in *E. coli* and purified by affinity chromatography on Sepharose-GSH columns, together with their kinetics of cleavage by BoNT/A (left panel) and BoNT/E (right panel). Progressive deletions beginning from the distal amino-terminal copy of the motif do not alter the rate of cleavage of the substrate both by BoNT/A and by BoNT/E. Moreover the deleted SNAP-25 substrates are cleaved as long as a single copy of the SNARE motif (S4) is present. However, removal of a further 14 residues containing the S4 motif from Δ1-140 to produce Δ1-154 totally abolishes proteolytic activity. Hence, the minimal segment of the SNAP-25 molecule required for the proteolytic cleavage by both BoNT/A and BoNT/E has to include S4, and this explains the outcome of a previous study on the minimal length of SNAP-25 segments still cleaved by BoNT/A [8].

3.2. Interchangeability of recognition motifs

We considered that the resistance of the 154–206 C-terminal

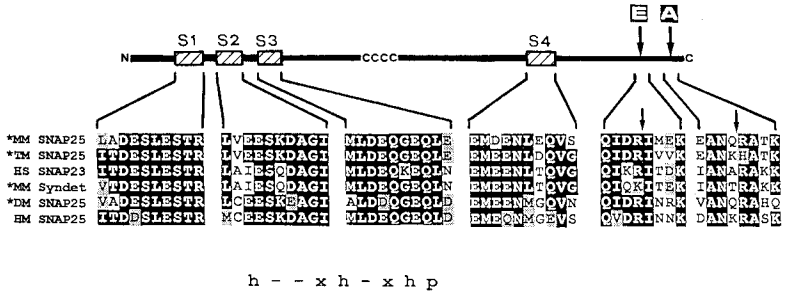

Fig. 1. Schematic structure of SNAP-25 with cleavage sites of neurotoxins and positions of SNARE motifs. The amino acid sequences corresponding to the four copies of the SNARE motif and the cleavage sites in diverse SNAP-25 homologues are presented. Below is the consensus sequence of the SNARE motif. The sequences were aligned using CLUSTALW. White on black letters are identical amino acids, black on grey are conserved and black on white are non-conserved. Arrows indicate cleavage sites of BoNT/A (A) and BoNT/E (E). MM SNAP-25, *Mus musculus* (GenBank ID M22012); TM, *Torpedo marmorata* SNAP-25 (Swiss-Prot ID P36976); HS, *Homo sapiens* SNAP-23 (GenBank ID U55936); MM Syndet, *Mus musculus* Syndet (GenBank ID U73143); DM, *Drosophila melanogaster* SNAP-25 (Swiss-Prot ID P36975); HM, *Hirudo medicinalis* SNAP-25 (GenBank ID U85806). h, hydrophobic residues; −, negatively charged residues, x, any residue; p, polar residue. An asterisk designates isoforms studied in this paper.

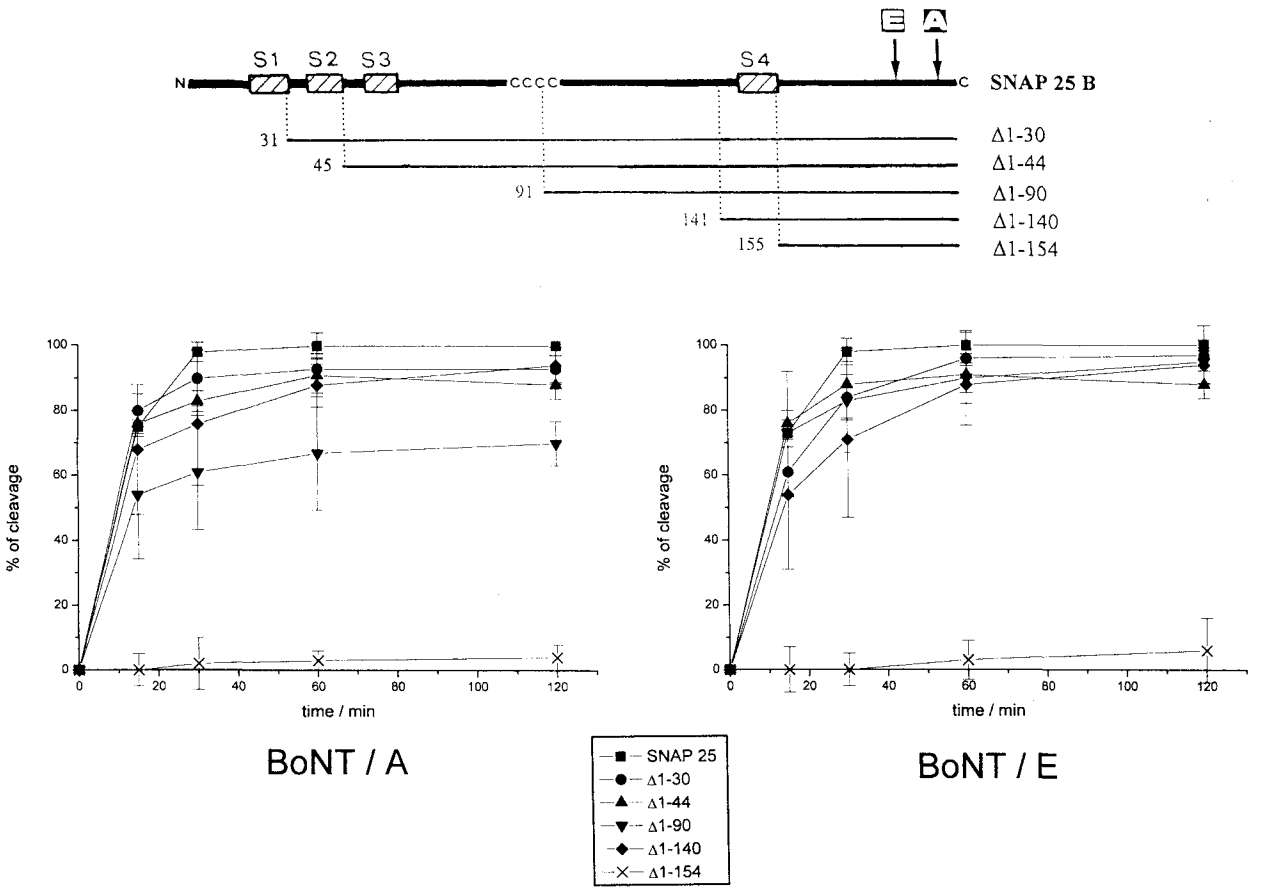

Fig. 2. Proteolysis by BoNT/A and BoNT/E of N-terminal deletion mutants of SNAP-25. The upper diagram shows the deletion mutants that have been fused to GST and assayed for their susceptibility to BoNT/A and BoNT/E. All constructs were incubated with BoNT/A 50 nM (left panel) and BoNT/E 50 nM (right panel), samples were taken after 0, 15, 30, 60 and 120 min, submitted to electrophoresis and Western blot. The alkaline phosphatase stained blots were quantified by densitometric scanning. Data are averages of three independent experiments and bars are S.D. values.

SNAP-25 fragment to cleavage by either BoNT/A or BoNT/E might be due to a conformational change caused by deletion of the amino-terminal portion of the protein through to S4 or by steric hindrance imposed by GST on this shortened polypeptide. To examine this in detail a construct was generated in which S4 was specifically deleted from full length SNAP-25. As shown in Fig. 3, deletion of S4 had little effect on the rate of proteolysis by either BoNT/A or BoNT/E. Consequently, N-terminal deletions were generated using the ΔS4 mutant as template in order to reveal other regions which could play a role in substrate recognition. As shown, the rate of cleavage by BoNT/A is reduced progressively for each SNARE motif removed, but cleavage is completely abolished when all remaining SNARE motifs were deleted. BoNT/E shows a smaller dependence of the rate of proteolysis on sequential removal of the N-terminal SNARE motifs, but it is entirely inactive on a SNAP-25 mutant not containing any copy of the motif. These results suggest that it is unlikely that the inability of BoNT/A or BoNT/E to cleave the minimal 154–206 fragment was due to GST steric hindrance as a polypeptide lacking the proximal S4 motif, but as long as 116 amino acids (Δ1-90ΔS4) was resistant to neurotoxin proteolysis. In addition, the results imply that the N-terminal SNARE motifs S1, S2 and S3 can substitute to different extents for the absence of S4.

3.3. *Syndet is cleaved by BoNT/E only, whilst SNAP-25 from Drosophila and Torpedo are susceptible to neither BoNT/A nor BoNT/E*

Recently, SNAP-25 from other species and non-neuronal isoforms have been identified and their sequences reported [20,21,27,28]. To gain further information on the extent of the specificity of BoNT/A and BoNT/E recognition and proteolytic activity, several SNAP-25 isoforms were assayed as substrates for these toxins. They were cloned behind GST and a HA1 epitope tag was fused at the C-terminal end. In this way, cleavage could be monitored by Western blotting with an anti-HA1 monoclonal antibody. Fig. 4 shows that the addition of a HA1 epitope to the C-terminus of the mouse SNAP-25 GST-fusion protein does not alter its kinetics of cleavage by either BoNT/A or BoNT/E. Whilst the two non-mammalian isoforms proved not to be susceptible to BoNT/A or BoNT/E (data not shown), Syndet is effectively cleaved by BoNT/E, but not by BoNT/A, at about a quarter of the rate of the GST-mouse SNAP-25-HA1 polypeptide (Fig. 4).

SNAP-25, VAMP and syntaxin are the only known substrates of the eight clostridial neurotoxins which effectively block synaptic transmission. The unique proteolytic activity exerted by the clostridial neurotoxins on the three SNAREs

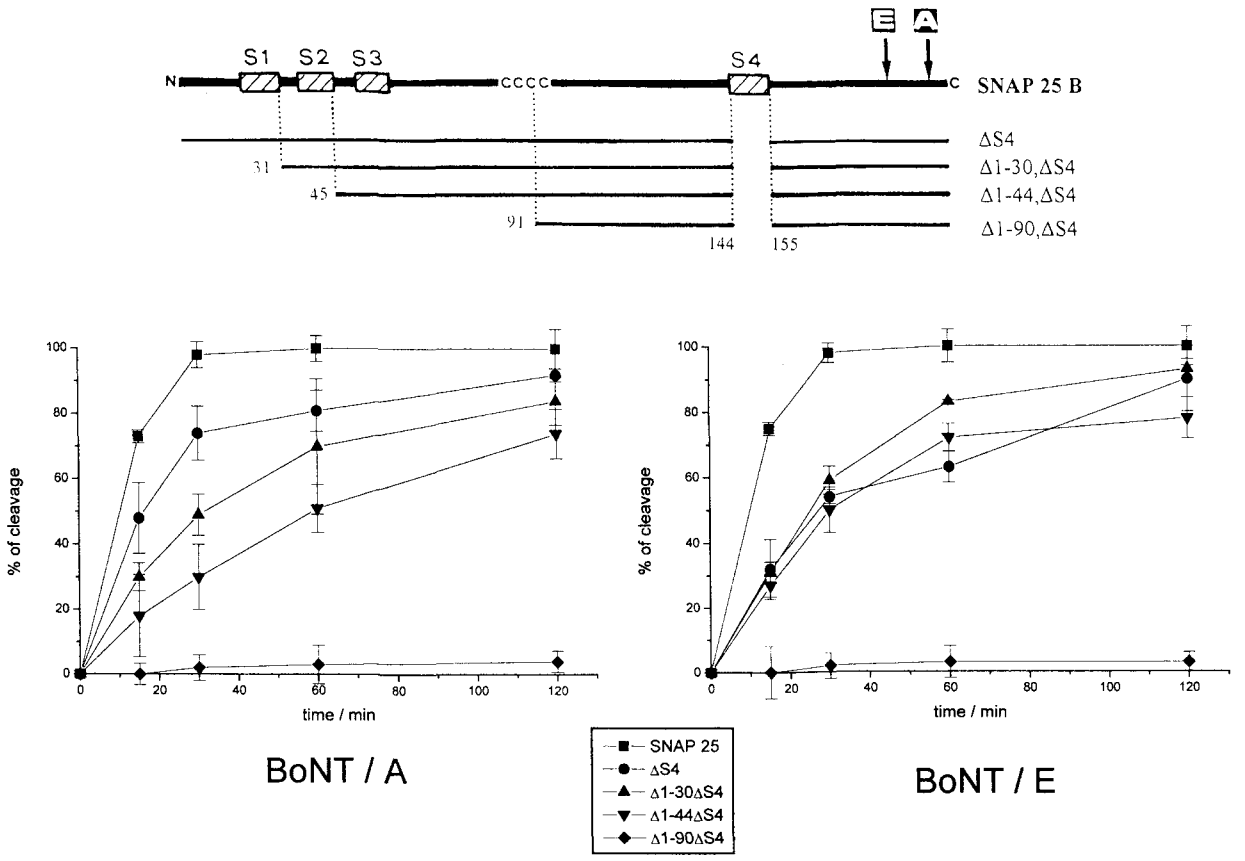

Fig. 3. Neurotoxin proteolysis of deletion mutants that progressively remove the three N-terminal SNARE motifs in the absence of S4. The upper diagram shows deletion mutants of SNAP-25, but which include a loop out deletion of amino acids 145 to 154 (ΔS4). All constructs were incubated with BoNT/A 50 nM (left panel) and BoNT/E 50 nM (right panel), samples were taken after 0, 15, 30, 60 and 120 min, submitted to electrophoresis and Western blot. The alkaline phosphatase stained blots were quantified by densitometric scanning. Data are averages of three independent experiments and bars are S.D. values.

can not be explained by recognition of regions encompassing only the cleavage sites, as short peptides are not hydrolysed [8,29–32]. Moreover, the same peptide bonds cleaved by BoNT/A and BoNT/E are present in other regions of SNAP-25 (Gln15-Arg16, Arg59-Ile60, Arg191-Ile192) and yet these peptide bonds are not hydrolysed. In VAMP concrete evidence has been provided for the requirement of the motif V1 by TeNT and V2 by BoNT/B and BoNT/G [17,18] and for BoNT/D and BoNT/F [19].

We have shown here that the SNARE motif is also necessary for proteolysis of SNAP-25 by BoNT/A and BoNT/E: both neurotoxins require the most C-terminal motif, S4, for efficient cleavage. However, in its absence, the three N-terminal copies of the motif (S1, S2, S3) can substitute for S4 as a recognition motif, though to different extents for the two toxins. It is unusual to find proteases which interact with their substrates at more than 50 amino acids from the cleavage site, and this result provides some information on the structure of SNAP-25 in solution. It indicates that SNAP-25 is a flexible molecule that can 'snap' into a series of different conformations, with the amino-terminal SNARE motif being in a similar position with respect to the cleavage site as is S4. Such flexibility of SNAP-25 may be at the basis of its capability of interacting with different proteins at synaptic terminals: syntaxin and VAMP 2, synaptotagmin and voltage gated Ca^{2+}-channels [33].

The lack of proteolysis of the non-mammalian isoforms of SNAP-25 by BoNT/A and BoNT/E is probably due to non-conservative mutations around the cleavage sites. The resistance of torpedo SNAP-25 to both toxins can be accounted for

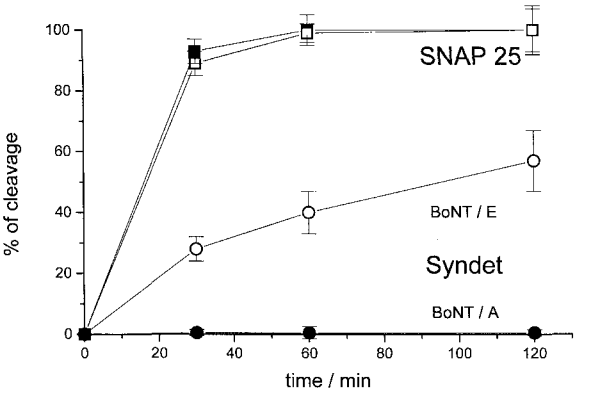

Fig. 4. Proteolysis of the non-neuronal SNAP-25 isoform, Syndet, by BoNT/E. SNAP-25 and Syndet cDNAs were fused with GST and HA1 tag was added to the 5′ end. The diagram shows the extent of proteolysis of the purified recombinant SNAP-25 (squares) and Syndet (circles) by BoNT/A (closed) and BoNT/E (open) at 50 nM after 0, 30, 60 and 120 min. The samples were submitted to electrophoresis and Western blot. The blots were decorated with anti-HA1 antibodies and quantified by densitometric scanning. Data are averages of three independent experiments and bars are S.D. values.

by the substitution of a glutamine to lysine at the cleavage site of BoNT/A and an Asp to Val substitution at P_3' of the BoNT/E cleavage site. Similar considerations can be made on the lack of proteolysis of *D. melanogaster* SNAP-25 by both proteases. BoNT/A has been reported to cause a block in acetylcholine release in the torpedo electric organ [34]. This result suggests that there may be another SNAP-25 isoform to be identified in cartilaginous fishes or that this isoform may only be cleaved in vivo. Recently, Syndet, a novel isoform of SNAP-25 highly expressed in non-neuronal cells has been characterised [28]. Because of the growing use of clostridial neurotoxins in cell biology studies where the role of a given SNARE is probed by following the effect of their functional abolition by neurotoxin proteolysis [35], it was of some interest to assay the effect of the two neurotoxins on Syndet. Its lack of cleavage by BoNT/A is most likely to be attributed to the threonine for glutamine substitution at the cleavage site (Fig. 1). On the other hand, its cleavage by BoNT/E indicates that the lysine for arginine substitution at the P_1 position of the cleavage site of BoNT/E, with respect to SNAP-25, is at least partially conservative. The relative susceptibilities of the diverse SNAP-25 homologues suggest that these neurotoxic metalloproteases not only use distant recognition motifs, but have also stringent requirements around their cleavage site.

Acknowledgements: We would like to thank D. Larhammar for *Torpedo* and *Drosophila* SNAP-25 clones, S. Censini for the synthesis of several oligonucleotides and N. Bortoletto for technical support. This study was supported by Telethon grant 763 and by MURST 60%.

References

[1] Simpson, L.L. (1986) Annu. Rev. Pharmacol. Toxicol. 26, 427–453.
[2] Montecucco, C. and Schiavo, G. (1995) Quart. Rev. Biophys. 28, 423–472.
[3] Schiavo, G., Shone, C.C., Rossetto, O., Alexander, F.C. and Montecucco, C. (1993) J. Biol. Chem. 268, 11516–11519.
[4] Schiavo, G., Malizio, C., Trimble, W.S., Polverino de Laureto, P., Milan, G., Sugiyama, H., Johnson, E.A. and Montecucco, C. (1994) J. Biol. Chem. 269, 20213–20216.
[5] Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., Roques, B., Fykse, E.M., Südhof, T.C., Jahn, R. and Niemann, H. (1994) J. Biol. Chem. 269, 12764–12772.
[6] Blasi, J., Chapman, E.R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Sudhof, T.C., Niemann, H. and Jahn, R. (1993) Nature 365, 160–163.
[7] Schiavo, G., Santucci, A., Dasgupta, B.R., Mehta, P.P., Jontes, J., Benfenati, F., Wilson, M.C. and Montecucco, C. (1993) FEBS Lett. 335, 99–103.
[8] Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Sudhof, T.C., Jahn, R. and Niemann, H. (1994) J. Biol. Chem. 269, 1617–1620.
[9] Foran, P., Lawrence, G.W., Shone, C.C., Foster, K.A. and Dolly, J.O. (1996) Biochemistry 35, 2630–2636.
[10] Osen Sand, A., Staple, J.K., Naldi, E., Schiavo, G., Rossetto, O., Petitpierre, S., Malgaroli, A., Montecucco, C. and Catsicas, S. (1996) J. Comp. Neurol. 367, 222–234.
[11] Williamson, L.C., Halpern, J.L., Montecucco, C., Brown, J.E. and Neale, E.A. (1996) J. Biol. Chem. 271, 7694–7699.
[12] Rothman, J.E. and Wieland, F.T. (1996) Science 272, 227–234.
[13] Osen Sand, A., Catsicas, M., Staple, J.K., Jones, K.A., Ayala, G., Knowles, J., Grenningloh, G. and Catsicas, S. (1993) Nature 364, 445–448.
[14] Lakin, N.D., Morris, P.J., Theil, T., Sato, T.N., Moroy, T., Wilson, M.C. and Latchman, D.S. (1995) J. Biol. Chem. 270, 15858–15863.
[15] Bark, I.C., Hahn, K.M., Ryabinin, A.E. and Wilson, M.C. (1995) Proc. Natl. Acad. Sci. USA 92, 1510–1514.
[16] Rossetto, O., Schiavo, G., Montecucco, C., Poulain, B., Deloye, F., Lozzi, L. and Shone, C.C. (1994) Nature 372, 415–416.
[17] Pellizzari, R., Rossetto, O., Lozzi, L., Giovedi, S., Johnson, E., Shone, C.C. and Montecucco, C. (1996) J. Biol. Chem. 271, 20353–20358.
[18] Wictome, M., Rossetto, O., Montecucco, C. and Shone, C.C. (1996) FEBS Lett. 386, 133–136.
[19] Pellizzari, R., Mason, S., Shone, C.C. and Montecucco, C. (1997) FEBS Lett. 409, 339–342.
[20] Risinger, C., Blomqvist, A.G., Lundell, I., Lambertsson, A., Nassel, D., Pieribone, V.A., Brodin, L. and Larhammar, D. (1993) J. Biol. Chem. 268, 24408–24414.
[21] Risinger, C. and Larhammar, D. (1993) Proc. Natl. Acad. Sci. USA 90, 10598–10606.
[22] Jankovic, J., Hallett, M., Eds. (1994) Therapy with Botulinum Toxin, Marcel Dekker, Inc., New York.
[23] Montecucco, C., Schiavo, G., Tugnoli, V. and de Grandis, D. (1996) Mol. Med. Today 2, 418–424.
[24] Rossetto, O., Schiavo, G., Polverino de Laureto, P., Fabbiani, S. and Montecucco, C. (1992) Biochem. J. 285, 9–12.
[25] Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E. and Wilson, M.C. (1989) J. Cell Biol. 109, 3039–3052.
[26] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[27] Ravichandran, V., Chawla, A. and Roche, P.A. (1996) J. Biol. Chem. 271, 13300–13303.
[28] Wang, G.Y., Witkin, J.W., Hao, G.M., Bankaitis, V.A., Scherer, P.E. and Baldini, G. (1997) J. Cell Sci. 4, 505–513.
[29] Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de laureto, P., DasGupta, B.R. and Montecucco, C. (1992) Nature 359, 832–835.
[30] Shone, C.C., Quinn, C.P., Wait, R., Hallis, B., Fooks, S.G. and Hambleton, P. (1993) Eur. J. Biochem. 217, 965–971.
[31] Shone, C.C. and Roberts, A.K. (1994) Eur. J. Biochem. 225, 263–270.
[32] Foran, P., Shone, C.C. and Dolly, J.O. (1994) Biochemistry 31, 15365–15374.
[33] Hanson, P.I., Heuser, J.E. and Jahn, R. (1997) Curr. Opin. Neurobiol. 7, 310–315.
[34] Rabasseda, X., Blasi, J., Marsal, J., Dunant, Y., Casanova, A., Bizzini, B., Schiavo, G. and Montecucco, C. (1988) Toxicon 26, 329–336.
[35] Rappuoli, R. and Montecucco, C. (Eds.) (1997) Guidebook to Protein Toxins and Their Use in Cell Biology, Oxford University Press, Oxford.