Charakterisierung der Substratspezifität clostridieller Neurotoxine

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Zusammenfassung

Zu den clostridiellen Neurotoxinen (CNT) gehören die sieben unterschiedlichen Serotypen der Botulinus Neurotoxine (BoNT, bezeichnet mit den Buchstaben A-G) und Tetanus Neurotoxin (TeNT). Die Toxine werden als einzelne Proteine mit einer Molekülmasse von ungefähr 150 kDa synthetisiert und nach der Synthese durch bakterielle Proteasen modifiziert. Das aktive Toxin besteht aus der schwere Kette mit einer Molekülmasse von 100 kDa, welche für die hochspezifische Wirtszellbindung und die Internalisierung zuständig ist, sowie der leichten Kette (50 kDa). Die leichte Kette ist eine Zink-Protease, welche spezifisch Mitglieder der SNARE Proteinfamilie hydrolysiert. Dies führt zu einer Inhibierung der Neurotransmitterausschüttung. Dabei spalten BoNT/A und BoNT/E SNAP-25, während BoNT/B, D, F, G und TeNT VAMP-2 spalten. Eine Sonderrolle nimmt BoNT/C ein. Es spaltet sowohl SNAP-25, als auch Syntaxin 1a.

In dieser Arbeit wurde die Substratspezifität von BoNT/B, D, F und TeNT erstmalig ausführlich charakterisiert. Dabei diente der Unterschied zwischen VAMP-2 und dem CNT insensitiven SNARE Protein TI-VAMP als Ausgangsbasis. Die Untersuchungen der Substratspezifität von BoNT/C basierten auf einem bereits veröffentlichten Co-Kristall zwischen BoNT/A und SNAP-25.

Durch umfangreiche Mutationsanalysen konnte neben der Spaltstelle ein zweiter Hauptinteraktionsbereich zwischen Toxin und Substrat identifiziert werden. Diese primäre Bindungsregion umfasst dabei vier oder mehr Aminosäuren, wobei Mutationen dieser Reste in kinetischen Analysen ausschließlich einen Effekt auf die Substratbindung zeigen. Die Größe dieser Bindungsregion und deren Abstand zur eigentlichen Spaltregion variiert zwischen den einzelnen Serotypen.

Die beobachteten negativen Effekte der Mutationen in der Spaltstelle (P5 bis P5’ Position) können alle auf eine Reduktion der Wechselzahl zurückgeführt werden. Austausche in der P1’ Position zeigen bei allen untersuchten Serotypen einen drastischen Effekt. Dies ist auf die hohe Spezifität der korrespondierenden S1’ Taschen in den leichten Ketten bezüglich Größe und Ladung zurückzuführen. Die weiteren Reste in der Spaltstelle dienen vermutlich der korrekten Ausrichtung des Substrates und zeigen Serotyp spezifische Effekte.

Die in dieser Arbeit gewonnenen Daten liefern weitere wichtige Hinweise für die Konstruktion geeigneter Inhibitoren. Die konstruierten BoNT/B und BoNT/D sensitiven TI-VAMP Mutanten bilden eventuell hilfreiche Werkzeuge für weitere funktionelle Studien.

Stichworte: Clostridielle Neurotoxine, VAMP, SNAP-25, Substraterkennung

Abstract

The group of clostridial neurotoxins (CNT) consists of the seven different serotypes of botulinum neurotoxin (BoNT, named A-G) and tetanus neurotoxin (TeNT). The toxins are produced as single chain molecules of 150 kDa and subsequently proteolytically activated. The active toxin consists of a 100 kDa heavy chain (HC), which is responsible for specific host cell binding and internalisation and the 50 kDa light chain (LC). The light chain is a zinc protease which hydrolyses specific members of the SNARE protein family. This leads to an inhibition of neurotransmitter release. BoNT/A and BoNT/E cleave SNAP-25, while BoNT/B, D, F, G and TeNT hydrolyse VAMP-2. BoNT/C displays a special role. It cleaves both, SNAP-25 and Syntaxin 1a.

Within this study the substrate specificity of BoNT/B, D, F and TeNT was extensively characterised for the first time. Thereby the difference between VAMP-2 and the CNT insensitive SNARE protein TI-VAMP in the areas of toxin-substrate interaction served as a starting basis. The examination of the substrate specificity of BoNT/C was based on a previously released co-crystal of SNAP-25 bound to BoNT/A.

By extensive mutation analysis it was possible to identify a second main interaction area besides the cleavage site in the toxin-substrate interaction for all examined serotypes. This primary binding site consists of four or more residues and kinetic analysis showed that mutations of these residues only have an effect on substrate binding. These binding sites exhibit different sizes and distances to the scissile peptide bonds for each neurotoxin.

The observed negative effects of mutations in the cleavage site (P5 to P5’ position) could all be referred to a reduction of the turnover number. Exchange in the P1’ position showed a drastic negative effect in all assayed serotypes, because of the high specificity of the corresponding S1’ light chain pockets regarding size and charge. The other cleavage site residues are likely needed for the correct adjustment of the substrate and show serotype specific effects.

The data regarding the substrate specificity of BoNT/B, C, D, F and TeNT obtained from this work provide important information for proper inhibitory design. The constructed BoNT/D and BoNT/B sensitive TI-VAMP mutants could be useful tools for further functional studies.

Keywords: clostridial neurotoxins, VAMP, SNAP-25, substrate recognition

Inhaltsverzeichnis

Abkürzungsverzeichnis

µ	Mikro
Å	Angström
A, Ala	Alanin
Abb	Abbildung
AMP	Ampicillin
AS	Aminosäure(n)
ATP	Adenosintriphosphat
BoNTA-G	Botulinum Neurotoxin Serotyp A-G
Bp	Basenpaare
bzw.	beziehungsweise
C	Kohlenstoff
C, Cys	Cystein
ca.	circa
CNT	Clostridielle Neurotoxine
CO_2	Kohlendioxid
C-terminal	carboxyterminal
D, Asp	Aspartat
d.h.	das heißt
E, Glu	Glutamat
E. coli	*Escherichia coli*
f	Femto
F, Phe	Phenylalanin
FDA	*Food and Drug Administration*
g	Gramm
G, Gly	Glycin
griech.	griechisch
GSH	reduziertes Glutathion
h	Stunde(n)
H, His	Histidin
HC	schwere Kette
H_C	carboxyterminale Hälfte der schweren Kette (HC), Bindungsfragment
HEPES	4-(2-Hydroxyethyl)-1-piperazinethansulfonsäure
H_N	aminoterminale Hälfte der schweren Kette (HC), Translokationsdomäne
I, Ile	Isoleucin
IPTG	Isopropyl-1-thio-ß-D-galactopyranosid
k	Kilo
K, Lys	Lysin
K_{cat}	Wechselzahl
K_M	Michaeliskonstante
k.A.	keine Angabe möglich
L, Leu	Leucin
LD_{50}	mittlere letale Dosis
Da	Dalton

l	Liter
lat	lateinisch
LC	leichte Kette
LMW	*Low Molecular Weight Marker* (Molekülmassenstandard)
Lsg	Lösung
m	Milli, Meter
M, Met	Methionin
Max	Maximum
MHH	Medizinische Hochschule Hannover
min	Minute(n)
mol	Mol
N	Stickstoff
n	nano
N, Asn	Asparagin
Ni-NTA	Nickeltrinitriloessigsäure
N-terminal	aminoterminal
OD_{600}	Optische Dichte bei 600 nm
P, Pro	Prolin
PAGE	Polyacrylamid-Gelelektrophorese
PCR	Polymerase-Kettenreaktion
pH	negativer dekadischer Logarithmus der Wasserstoffionenkonzentration
PMSF	Phenylmethylsulfonylfluorid
Q, Gln	Glutamin
R, Arg	Arginin
RT	Raumtemperatur
s	Sekunde
S, Ser	Serin
SD	*standard deviation* (Standardabweichung)
SDS	Natriumdodecylsulfat (*sodium dodecyl sulfate*)
SNAP-25	*Synaptosome-associated Protein of 25 kDa*
SNARE	*soluble NSF attachment protein receptor*
SV	*synaptic vesicle protein*
T, Thr	Threonin
Tab	Tabelle
TEMED	N, N, N´, N´-Tetramethylethylendiamin
TeNT	Tetanus Neurotoxin
TI-VAMP	*tetanus-insensitive vesicle associated membrane protein*
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol
V, Val	Valin
v/v	Volumen pro Volumen
VAMP	vesicle associated membrane protein
W, Trp	Tryptophan
w/v	Gewicht pro Volumen (*weight per volume*)
wt	Wildtyp (*wild type*)
Y, Tyr	Tyrosin
z.B.	zum Beispiel
Zn	Zink

1. Einleitung

Viele pathogene Bakterien produzieren Proteintoxine, welche erst im Zellinneren wirken. Diese Toxine binden an die Zelloberfläche, werden in vesikuläre Kompartimente aufgenommen und transportieren ihre katalytische Untereinheit in das Zytosol, wo bestimmte Proteinsubstrate enzymatisch modifiziert werden. Aufgrund ihres ähnlichen Aufbaus werden diese Toxine mehrheitlich den sogenannten AB-Toxinen zugeordnet. Die Bindung an die Zelloberfläche und die Translokation der katalytischen Untereinheit werden dabei von der B-Untereinheit übernommen, während die A-Untereinheit die katalytisch aktive Domäne ist. Zu den AB-Toxinen gehört auch die Gruppe der clostridiellen Neurotoxine (CNT), Tetanus Neurotoxin (TeNT) und die sieben serologisch unterscheidbaren Botulinus Neurotoxine (BoNT/A-G). Diese stellen mit einer Maus LD_{50} zwischen 0,1 und 1ng/kg Körpergewicht (Schiavo *et al.,* 2000) die giftigsten bekannten natürlichen Substanzen dar und sind verantwortlich für die beiden Krankheiten Tetanus bzw. Botulismus.

1.1. Botulismus und Tetanus

Im Jahre 1817 beschrieb der deutsche Mediziner Justinus Kerner zum ersten Mal bei einem Patienten den Zustand der generalisierten Muskelerschlaffung, nachdem dieser verdorbene Wurst verzehrt hatte. Er nannte diese Vergiftung Botulismus (*botulus*, lat. = Wurst).
Botulismus wird durch die Aufnahme von verdorbener Nahrung (Lebensmittelvergiftung), durch eine Besiedlung des Intestinaltraktes (hauptsächlich bei Kleinkindern = Kleinkind-Botulismus) oder einer Wunde (Wund-Botulismus) durch verschiedene Stämme von *Clostridium botulinum, Clostridium butyricum* und *Clostridium barati* hervorgerufen. Die bis heute bekannten sieben verschiedenen Serotypen der Botulinus Neurotoxine wurden aus verschiedenen Quellen isoliert und chronologisch von A bis G benannt (Tab. 1). BoNT/C und D treten in der Natur nur bei wilden Tieren bzw. bei Nutztieren auf (Popoff, 1995). Vor kurzem konnte gezeigt werden, dass BoNT/C in Bezug auf Dosis und Dauer der Wirkung, einen ähnlichen Effekt auf Menschen hat, wie BoNT/A und B (Eleopra *et al.,* 2004). Der 1969 in einer Erdprobe aus Südamerika gefundene Serotyp G (Gimenez und Ciccarella, 1970) konnte bis heute nicht als Quelle für natürlichen Botulismus identifiziert werden.
Die obligat anaeroben Bakterien produzieren Säure- und Protease-resistente Vorgänger-Neurotoxine. Diese hochmolekularen Proteinkomplexe können den Gastrointestinaltrakt passieren und zerfallen nach Erreichen des Dünndarms in ihre Komponenten. Dort gelangt

das reine Neurotoxin in den Blutkreislauf und erreicht schließlich seinen Wirkungsort: die präsynaptische Membran motoneuraler Nervenendigungen (Maksymowych und Simpson, 1998).

Tab.1 Auflistung des Enddeckungsjahres und der Quelle der sieben verschiedenen BoNT Serotypen.

Serotyp	Quelle	Jahr
A	Mensch	1897
B	Mensch	1904
C	Vogel	1922
D	Rind	1929
E	Mensch	1934
F	Mensch	1960
G	Boden	1970

Ein Fall von Tetanus (*tetanos*, griech. = sich kontrahieren) wurde zum ersten Mal vor über 2500 Jahren von Hippokrates beschrieben. Eines der Hauptsymptome ist die hyperkontrahierte Skelettmuskulatur. Die Krankheit endet häufig mit dem Tod durch Atem- oder Herzstillstand (Bleck, 1989). Hervorgerufen wird Tetanus durch eine Wundinfektion mit *Clostridium tetani*. Dieses gram-positive, anaerobe Bakterium, welches 1891 von Kitasato entdeckt wurde, kommt weltweit nahezu in allen Böden vor. Das vom Bakterium gebildete TeNT gerät in den Blutkreislauf und erreicht schließlich die motoneuronale Endplatte. Dort wird es aufgenommen und im Gegensatz zum BoNT retrograd zu inhibitorischen Neuronen des Rückenmarks transportiert, wo es die Ausschüttung von Glycin verhindert. Durch die hohe Immunisierungsrate stellt Tetanus in den Industrieländern keine Gefahr mehr dar (2 Fälle pro 10 Millionen Einwohnern pro Jahr). Anders in Entwicklungsländern, wo längst nicht so viele Menschen gegen Tetanus geimpft sind. 1998 gab es laut Weltgesundheitsbehörde (WHO) in diesen Gebieten ca. 410.000 Todesfälle. Der überwiegende Teil dieser Todesfälle entfällt in Entwicklungsländern auf neugeborene Kinder, bei denen es infolge unsteriler Abnabelung nach Hausgeburten zur Einbringung von Tetanussporen in die Nabelwunde des Neugeborenen und damit zur Infektion kommt.

1.2. Klinischer Einsatz clostridieller Neurotoxine

Aufgrund ihrer inhibitorischen Wirkung auf die Neurotransmitterausschüttung werden BoNT in der Therapie verschiedener Krankheiten eingesetzt, welche durch eine Hyperaktivität der cholinergen Nervenendigungen hervorgerufen werden. In den 1970er Jahren konnte der Amerikaner Alan Scott durch gezielte Injektion hochverdünnter BoNT/A-Dosen bei Primaten beobachten, wie überstimulierte Muskeln über mehrere Monate entspannt blieben. Dabei traten bei den Tieren keinerlei Nebeneffekte auf (Johnson, 1999). Wenig später setzte er BoNT/A bei der Therapie verschiedener Augenerkrankungen ein. Im Jahre 1989 wurde BoNT/A von der US-amerikanischen Arzneimittelzulassungsbehörde (FDA) unter dem Namen Oculinum für die Behandlung von Augenerkrankungen und hemifazialer Spasmen zugelassen. Heutzutage wird BoNT/A für medizinische Zwecke von verschiedenen Firmen unter unterschiedlichen Namen hergestellt. Dabei wird das Neurotoxin zusammen mit den Vorgänger-Komplexproteinen aufgereinigt. Die Anwendungsgebiete von BoNT/A sind in den letzten Jahren rapide angewachsen (über 100 verschiedene klinische Einsatzgebiete). Dazu zählen auch die Behandlung von Erkrankung des autonomen Nervensystems und der Schmerztherapie. Zusätzlich zu BoNT/A wird in letzter Zeit auch der native BoNT/B-Komplex eingesetzt.

Aufgrund der hohen Impfrate gegen Tetanus kann TeNT nicht als natives Toxin in der Medizin eingesetzt werden. In Forschungsarbeiten wird das H_C-Fragment allerdings als möglicher Träger eingesetzt, der gezielt Wirkstoffe in das zentrale Nervensystem transportieren kann (Schiavo *et al.,* 2000).

1.3. Aufbau clostridieller Neurotoxine

Clostridielle Neurotoxine werden als einkettige Polypeptide von ungefähr 150 kDa synthetisiert und anschließend von bakteriellen Proteasen in zwei Ketten gespalten, welche allerdings noch über eine Disulfidbrücke verbunden bleiben (Niemann, 1991; Montecucco und Schiavo, 1994) (Abb.2). Die tertiäre Struktur konnte in den letzten Jahren durch kristallographische Arbeiten geklärt werden (Lacy *et al.,* 1998; Swaminathan *et al.,* 2000).

Die leichte Kette (ca. 50 kDa) bildet die katalytische Untereinheit des Toxins. Sie enthält ein konserviertes HExxH-Motiv (Niemann *et al.,* 1994), welches man auch bei Zink-

Endopeptidasen findet und wodurch die clostridiellen Neurotoxine zur Familie der zink-abhängigen Metalloproteasen gezählt werden.

Die schwere Kette besteht aus zwei funktionelle Domänen, jede ca. 50 kDa schwer. Der C-terminale Bereich (H_C) ist die Bindungs-Domäne und für die Bindung an neuronale Zellen verantwortlich. Den N-terminale Teil (H_N) bezeichnet man als Translokations-Domäne, welche sehr wahrscheinlich bei der Translokation der leichten Kette in das Zytosol involviert ist.

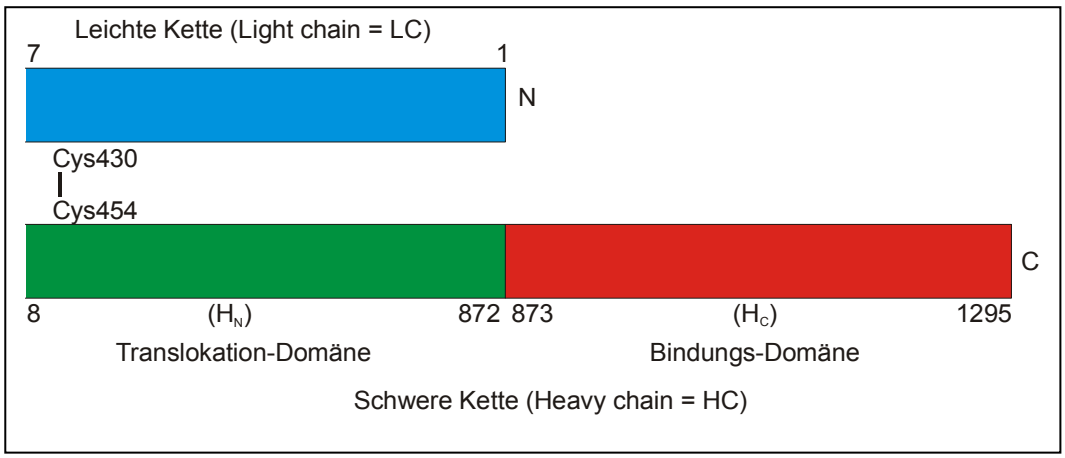

Abb.2 Schematischer Aufbau eines clostridiellen Neurotoxins (BoNT/A). Leichte und schwere Kette sind über eine einzelne Disulfidbrücke (Cys430-Cys454) miteinander verbunden. Die leichte Kette (blau) vermittelt die Endopeptidase-Aktivität, während die schwere Kette für die Bindung (roter Anteil) und die Translokation (grüner Anteil) zuständig ist.

1.4. Funktionsweise clostridieller Neurotoxine

Das H_C-Fragment bindet zuerst spezifisch an bestimmte Ganglioside auf der Zelloberfläche neuronaler Zellen, wodurch eine Anhäufung von Toxin-Molekülen auf der Zelle stattfindet. Ein zweiter Rezeptor, ein Protein, vermittelt schließlich die Aufnahme des Toxins über rezeptorvermittelte Endozytose (Kap. 1.4.1. und 1.4.2.). Eine pH-induzierte Konformationsänderung ermöglicht es der H_N-Domäne vermutlich in die Membran einzudringen und eine Pore zu bilden, durch welche die leichte Kette in das Zytosol gelangen kann (Kap. 1.4.3.). Dort spaltet die leichte Kette ihr entsprechendes Substrat, wodurch eine Inhibition der Acetylcholinfreisetzung hervorgerufen wird. Dies führt zu einer Erschlaffung der Muskulatur (Abb.3). Im Gegensatz zu BoNT, wird TeNT innerhalb der Zelle retrograd zu inhibitorischen Neuronen weitertransportiert, wo es die Ausschüttung von hemmenden Neurotransmittern unterbricht (Kap. 1.4.4.). Dies wiederum führt zu den bekannten Tetanus-

Symptomen, nämlich einer dauerhaften Muskelspannung. Den Unterschied zwischen Botulismus und Tetanus liegt also nicht im Wirkungsmechanismus, sondern im Wirkungsort.

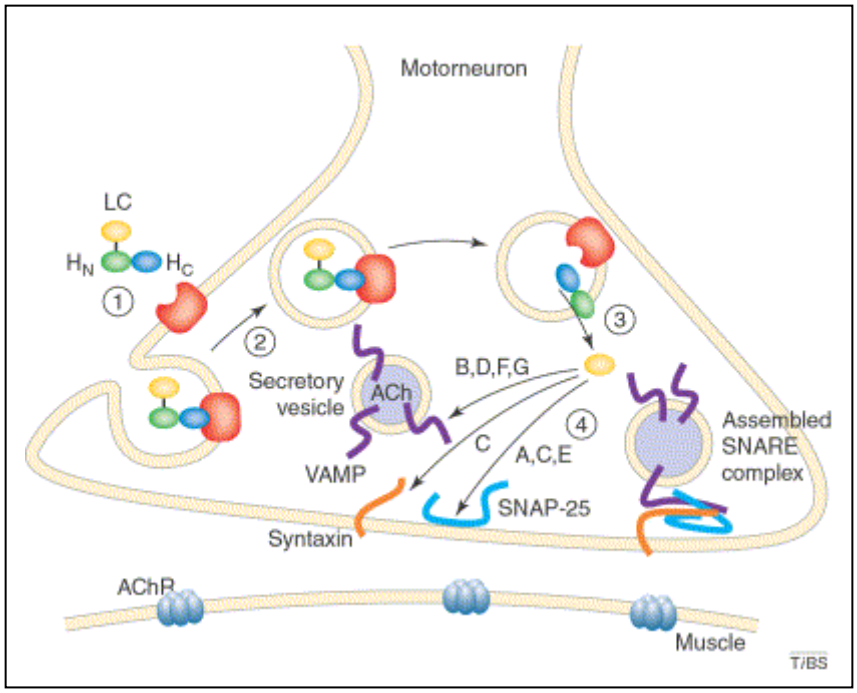

Abb.3 Schematisierte Darstellung der Wirkungsweise von BoNT. Punkt 1: Rezeptorvermittelte Bindung an neuronale Zellen. 2: Internalisierung in endosomale Strukturen. 3: Translokation der leichten Kette. 4: Proteolyse der Substratproteine. (Turton *et al.,* 2002)

1.4.1. Bindung

Die Bindung der CNT an die Membran neuronaler Zellen wird durch das H_C-Fragment vermittelt (Lalli *et al.,* 1999) und läuft in zwei Teilschritten ab.

Es konnte gezeigt werden, dass CNT zunächst an komplexe Ganglioside binden, insbesondere G_{D1b}, G_{T1b} und G_{Q1b} (Schiavo *et al.,* 2000). Knockout-Mäuse, welche keine komplexen Ganglioside bilden können, sind nahezu resistent gegen TeNT und BoNT/A, B und E (Kitamura *et al.,* 1999; Bullens *et al.,* 2002). Die Affinität der Bindung von CNT an Ganglioside, welche im hohen nM Bereich liegt, ist wesentlich geringer als die Affinität mit der sie an neuronales Gewebe binden (K_D = 1,2 nM; Halpern and Neale, 1995). Als mögliche Erklärung dieser Unterschiede wurde bereits früh ein Doppelrezeptormodell postuliert. Weil die Bindung von TeNT und BoNT/A an Rattensynaptosomen proteasesensitiv ist (Dolly *et al.,* 1982), ging man früh von einem Protein als zweitem Rezeptor aus. In der Tat konnten kürzlich Synaptische Vesikel (SV) Proteine als Proteinrezeptoren identifiziert werden.

BoNT/B und G interagieren mit Synaptotagmin I und II. Dabei besitzt BoNT/B eine höhere Affinität zu Synaptotagmin II, während BoNT/G besser an Synaptotagmin I bindet (Dong *et al.,* 2003; Nishiki *et al.,* 1996; Rummel *et al.,* 2004). Erst vor kurzem konnte SV2 als Proteinrezeptor für BoNT/A identifiziert werden (Mahrhold *et al.,* 2006, Dong *et al.,* 2006).

1.4.2. Internalisierung

Nach der Bindung an ihre entsprechenden Rezeptoren gelangen die CNT nicht direkt ins Zytosol, sondern werden in vesikuläre Strukturen endozytiert (Dolly *et al.* 1982). Diese Aufnahme findet spontan statt, kann allerdings durch Stimulation der Nervenzelle verstärkt werden (Schiavo *et al.,* 2000) Aufgrund der Tatsache, dass synaptische Vesikelproteine als Rezeptoren für CNT dienen, kann man davon ausgehen, dass die Toxine in eben jenen Vesikeln aufgenommen werden (Baldwin and Barbieri, 2007) Obwohl der zweite Rezeptor für TeNT noch nicht gefunden wurde, konnte zumindest gezeigt werden, dass es in Synaptischen Vesikeln aufgenommen wird. (Matteoli *et al.* 1996)

1.4.3. Translokation

Um das Zytosol zu erreichen muss die leichte Kette die hydrophobe Barriere der Vesikelmembran passieren. Es ist mittlerweile eine anerkannte These, dass dafür das Lumen des Vesikels angesäuert werden muss. Dies wurde für alle Neurotoxine nachgewiesen (Simpsons *et al.,* 1994). Durch die Reduktion des pH-Werts kommt es im CNT zu einer Konformationsänderung von einer wasserlöslichen „neutralen“ Form zu einer „sauren“ Form mit oberflächlichen hydrophoben Segmenten, wodurch es den Toxinen möglich ist in die Lipiddoppelschicht der Membran einzudringen. Dort können sie Ionenkanäle bilden (Boquet und Duflot, 1982). BoNT/C z.B. bildet in Lipidmembranen ähnliche Kanäle wie das Diphtherietoxin (Donovan und Middlebrook, 1986; Koriazova und Montal, 2003). Die Porengröße der gebildeten Ionenkanäle ist sehr gering (Sheridan, 1998), so dass sich die Frage stellt, ob die leichte Kette das Zytosol über diesen Weg erreichen kann. Allerdings konnte gezeigt werden, dass die leichte Kette bei niedrigem pH eine „molten globule“-Struktur einnimmt und so in der Lage sein dürfte, den gebildeten Kanal zu passieren. Diese pH-induzierten Strukturänderungen sind komplett reversibel (Li und Singh, 2000).
Der Translokationsprozess ist der bislang am wenigsten verstandene Schritt in der Funktionsweise clostridieller Neurotoxine.

1.4.4. Proteolyse

Die leichte Kette bildet die katalytische Untereinheit des Neurotoxins. Sie besitzt ein konserviertes HExxH-Motiv, welches typisch für Zinkproteasen, wie z.b. Thermolysin ist. Dabei koordinieren die beiden Histidine (BoNT/A: His222 und His226) zusammen mit einem weiter stromabwärts liegenden Glutamat das zentrale Zinkatom und das Glutamat (BoNT/A: Glu223) das Wassermolekül im aktiven Zentrum (Lacy *et al.,* 1998).
CNT unterbrechen die Neurotransmitterübertragung indem sie hochspezifisch SNARE (soluble NSF attachment protein receptor) Proteine spalten. BoNT/A und E spalten SNAP-25 (synaptosomal-associated protein of 25 kDa); BoNT/B, D, F, G und TeNT hydrolysieren VAMP (vesicle-associated membrane protein – oder auch Synaptobrevin genannt); BoNT/C spaltet als einziges zwei verschiede SNARE-Proteine, nämlich SNAP-25 und Syntaxin (Humeau *et al.,* 2000, Schiavo *et al.,* 2000). (Kap. 1.5.)

1.5. Die Substrate der clostridiellen Neurotoxine

Anfang der 1990er Jahre konnten die Arbeitsgruppen von H. Niemann bzw. C. Montecucco zeigen, das die SNARE-Proteine Syntaxin1, SNAP-25 und VAMP die Substrate der clostridiellen Neurotoxine darstellen (Niemann *et al.,* 1994; Schiavo *et al.,* 1994; Humeau *et al.,* 2000). SNARE-Proteine sind mit ihren verschiedenen Isoformen (Abb. 4) an allen wichtigen Endo- bzw. Exozytosevorgängen innerhalb der Zelle beteiligt, indem sie den für die Membranfusion notwendigen SNARE-Komplex bilden (Kap. 1.5.4.).
Die Sensitivität gegenüber den CNT war entscheidend um die Funktion der SNARE-Proteine näher zu untersuchen. Der erste Nachweis für die Rolle der SNARE-Proteine bei der Exozytose wurde z.B. durch die Tatsache gewonnen, dass die Tetanus-induzierte Blockade der Sekretion durch die Wirkung des Toxins auf VAMP2 hervorgerufen wurde (Link *et al.,* 1992; Schiavo *et al.,* 1992). So stellen die CNT ein praktisches molekulares Werkzeug für die Untersuchung der Endo- bzw. Exozytosevorgängen dar.
Allerdings gibt es auch diverse SNARE-Proteine, welche Neurotoxin-Insensitiv sind. So wird TI-VAMP (tetanus-insensitive vesicle associated membrane protein) oder auch Endobrevin (VAMP8) von keinem CNT gespalten (Martinez-Arca *et al.,* 2000), während z.B. VAMP1 aus der Ratte im Gegensatz zur humanen und murinen Isoform insensitiv gegenüber BoNT/B und TeNT ist.

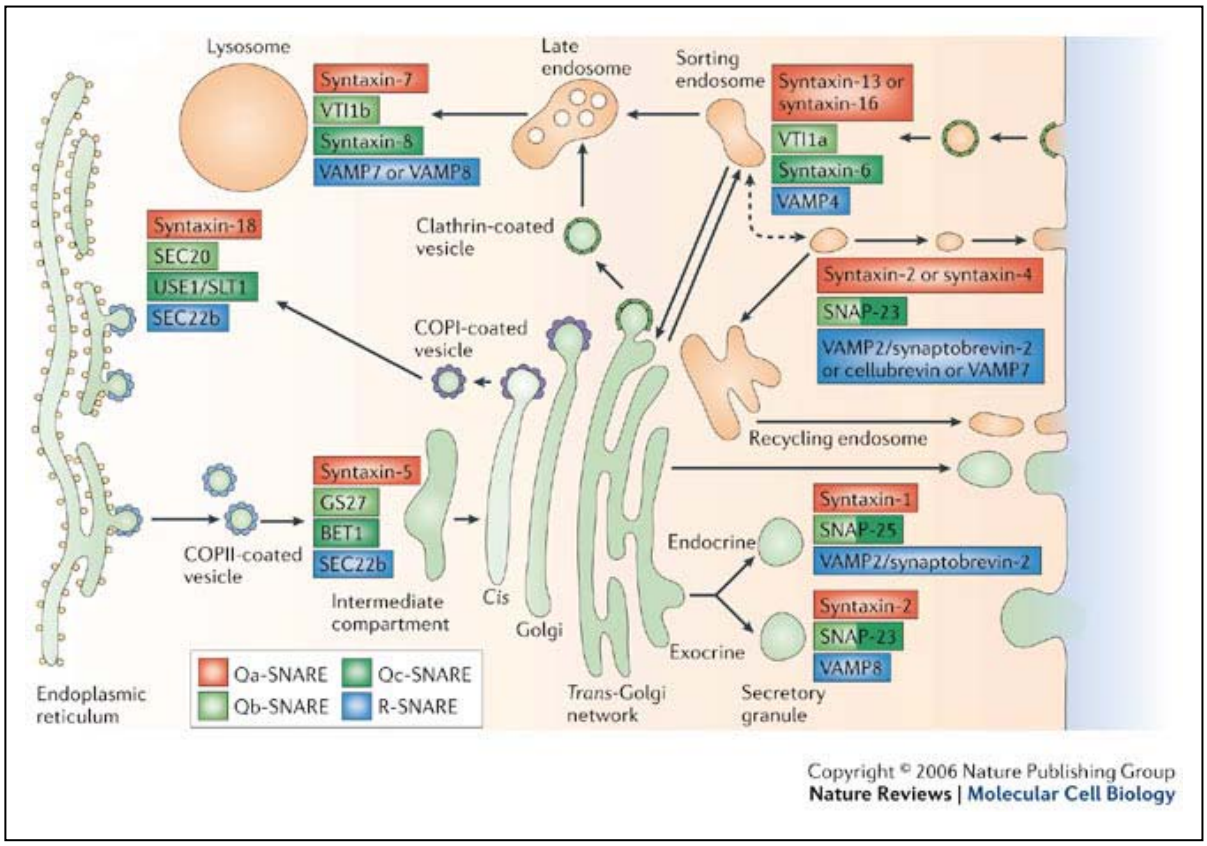

Abb.4 Intrazelluläre Lokalisierung diverser SNARE-Proteine. Die verschiedenen Isoformen der SNARE-Proteine sind distinkten Zellkompartimenten zugeordnet. Rot: Syntaxin Familie, Grün: SNAP-25 Familie, Blau: VAMP Familie. In dieser Abbildung ist auch die Zuordnung nach Qa-, Qb-, Qc- und R-SNARE angegeben. (Jahn und Scheller, 2006)

1.5.1. Syntaxin

Syntaxin ist ein Membranprotein mit einer Größe von 35 kDa, welches über eine C-terminale Transmembrandomäne in der Plasmamembran verankert ist. Der N-terminale Bereich (Aminosäuren 1-120) besteht aus drei langen α-helikalen Strukturen, welche wahrscheinlich für Protein-Protein-Wechselwirkungen zuständig sind. Die Aminosäuren 180-262 bilden das SNARE-Motiv welches für die Interaktion mit den anderen SNARE Proteinen VAMP und SNAP-25 wichtig ist und über das die Bildung des SNARE-Komplexes vermittelt wird (Hayashi *et al.,* 1994). In Säugetieren findet man mehr als 20 Syntaxin Isoformen und einige Homologe in der Hefe und in Pflanzen (Jahn und Scheller, 2006). Dabei sind Syntaxin 1a, 1b, 2, und 3 BoNT/C sensitiv, während Syntaxin 4 nicht gespalten wird (Humeau *et al.,* 2000).

1.5.2. SNAP-25

SNAP-25 (25 kDa synaptosomal-associated protein) besitzt keine Transmembrandomäne, kann sich aber über zentralgelegene palmitoylierte Cysteine in der Plasmamembran verankern (Lane und Liu, 1997). Die beiden Aminosäureabschnitte 1-83 und 120-206 (SNARE-Motive) sind an der SNARE-Komplexbildung beteiligt (Sutton *et al.,* 1998). Es exstieren zwei verschiedene Isoformen von SNAP-25, welche durch alternatives Spleißen entstehen und zu 96% konserviert sind (Puffer *et al.,* 2001). Eine weitere Isoform ist SNAP-23, welches allerdings nicht in neuronalen Zellen vorkommt (Ravichandran *et al.,* 1996). Die Sensitivität gegenüber Botulinus Neurotoxinen variiert in den verschiedenen Spezies. Humanes SNAP-23 wird von keinem Toxin gespalten, während SNAP-23 aus Maus und Huhn sensitiv gegenüber BoNT/A und E ist (Humeau *et al.,* 2000).

1.5.3. VAMP

VAMP (oder auch Synaptobrevin) ist ein 13 kDa großes synaptisches Vesikelprotein mit vier verschiedenen funktionale Domänen (Baumert *et al.,* 1989). Der 33 Aminosäure lange N-terminale Bereich ist Prolinreich und Isoformspezifisch. Die Aminosäuren 33-96 (SNARE-Motiv) sind hochkonserviert und für die Bildung des SNARE-Komplexes zuständig. C-terminal findet man die Transmembrandomäne und einen unterschiedlich langen intravesikulären Bereich. Anhand des N-terminalen Abschnitts unterteilt man die Proteine in kurze Synaptobrevine (*brevin*, lat. = kurz) und in Longine mit einer 120-140 AS langen Longin Domäne (Filippini *et al.,* 2001). Diese Domäne hat einen großen regulatorischen Einfluss auf die SNARE-Komplexbildung (Martinez-Arca *et al.,* 2000).

1.5.4. Der synaptische SNARE-Komplex

Das molekulare Model der SNARE-vermittelten Membranfusion wurde im Laufe der Jahre immer weiter gefestigt. Nach diesem Modell vermitteln SNARE-Proteine in gegenüberliegenden Membranen die Fusion durch Nutzung der freien Energie, die bei der Bildung eines parallelen „Vier-Helix-Bündels“ entsteht. Gebildet wird dieses Bündel jeweils aus einem VAMP, einem Syntaxin und zwei SNAP-25 SNARE-Motiven (Abb. 5A). Das

Zentrum bilden dabei 16 aufeinander gestapelte Ebenen von interagierenden Seitenketten (Antonin *et al.,* 2002) (Abb. 5B). Die interagierenden Seitenketten der einzelnen Ebenen sind zum größten Teil hydrophob Einzige Ausnahme ist die zentrale „Null"-Ebene. Hier liegen drei hochkonservierte Glutamine (Q) und ein hochkonserviertes Arginin (R) (Abb. 5C). Nach diesen Resten werden die SNARE-Proteine in Qa-, Qb-, Qc- und R-SNARE Proteine unterteilt (Fasshauer *et al.,* 1998).

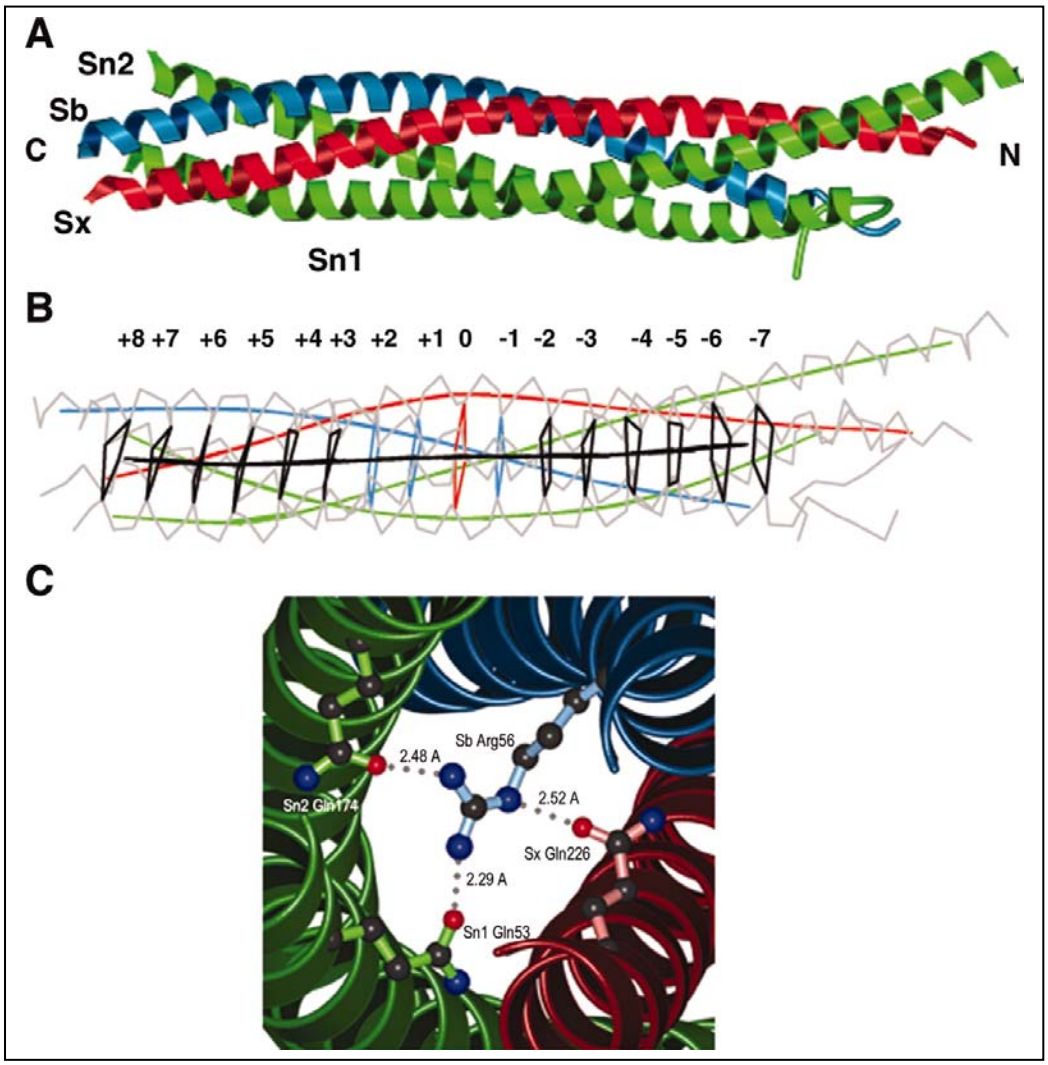

Abb.5 Der synaptische SNARE-Komplex. A) Kristallstruktur des SNARE-Kernkomplexes, bestehend aus Syntaxin (rot), VAMP (blau) und SNAP-25 (grün). B) Schematische Darstellung der 16 Ebenen des Kernkomplexes. Die „Null"-Ebene ist in Rot dargestellt. C) Detailansicht der „Null"-Ebene mit dem zentralen Arginin des VAMP (blau) und den drei Glutaminen der anderen SNARE-Proteine. (Sutton *et al.,* 1998)

Man postuliert, dass die bei der Bildung des ternären komplexes freigesetzte Energie dafür genutzt wird, die elektrostatische Barriere zwischen der Vesikel- und der präsynaptischen

Membran zu überwinden und damit entscheidend zur Vermischung der Membranlipide zwischen beiden Membranen beizutragen.

1.5.5. Substratspezifität der leichten Ketten

Die leichten Ketten von BoNT und TeNT sind die selektivsten aller bekannten Proteasen (Oost *et al.,* 2003). Sie spalten in ihren jeweiligen Substraten nur eine spezielle Peptidbindung (Schiavo *et al.,* 2000). Die Aminosäuren im Bereich um die Spaltstelle werden nach Schechter und Berger von P_5 – P_5' durchnummeriert. Dabei entspricht P_1' der ersten Aminosäure nach der Spaltstelle. Die korrespondierenden Taschen in der Protease werden mit S_5 – S_5' benannt.

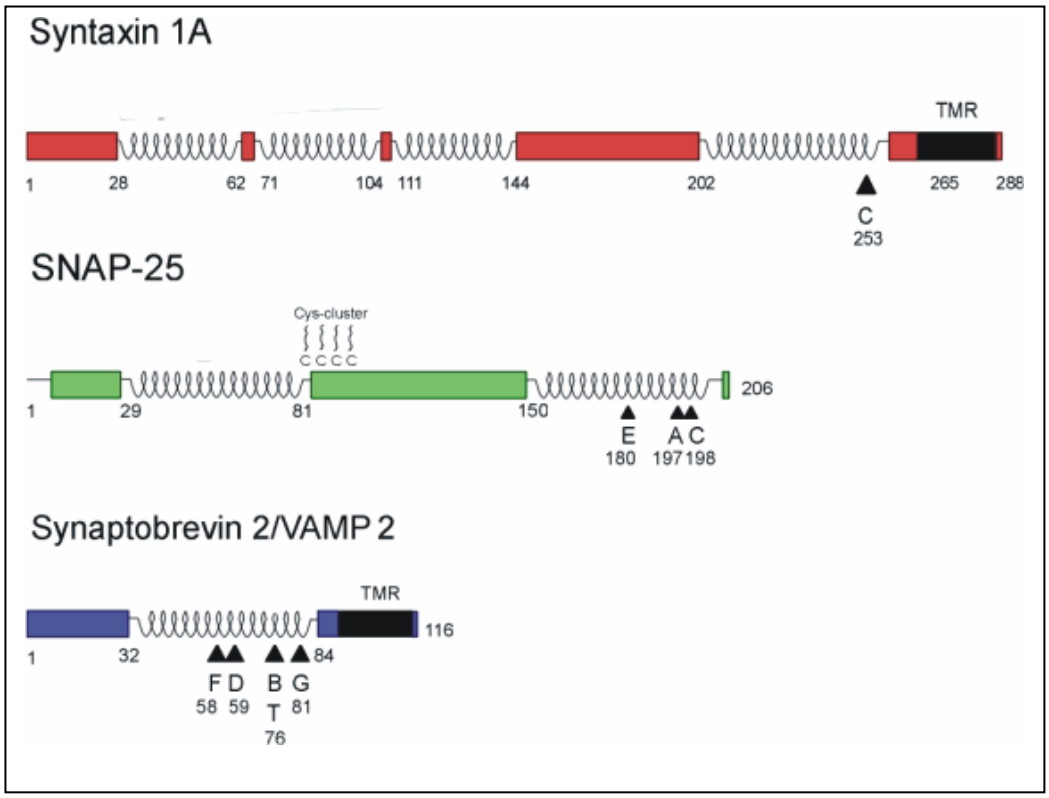

Abb.6 Die Substrate der clostridiellen Neurotoxine. Die Pfeile markieren die Spaltstellen der verschiedenen Neurotoxine.

Für die korrekte Erkennung und Spaltung werden Aminosäureabschnitte im Substrat benötigt, welche sowohl die eigentliche Spaltstelle, als auch Sequenzen weit davon entfernt beinhalten (Vaidyanathan *et al.,* 1999; Yamasaki *et al.,* 1994; Schmidt *et al.,* 1997; Foran *et al.,* 1994;

Cornille *et al.,* 1997; Schmidt *et al.,* 1995; Washbourne *et al.,* 1997). Bereits einzelne Punkmutationen in diesen Bereichen können die Spalteffizienz teilweise stark verringern (Rossetto *et al.,* 1994; Pellizzari *et al.,* 1996). Eine kürzlich vorgestellte Co-Kristallstruktur der leichten Kette von BoNT/A mit seinem Substrat SNAP-25 (Abb. 7) ermöglicht neue Einblicke in die Enzym/Substart-Interaktion (Breidenbach und Brunger, 2004).

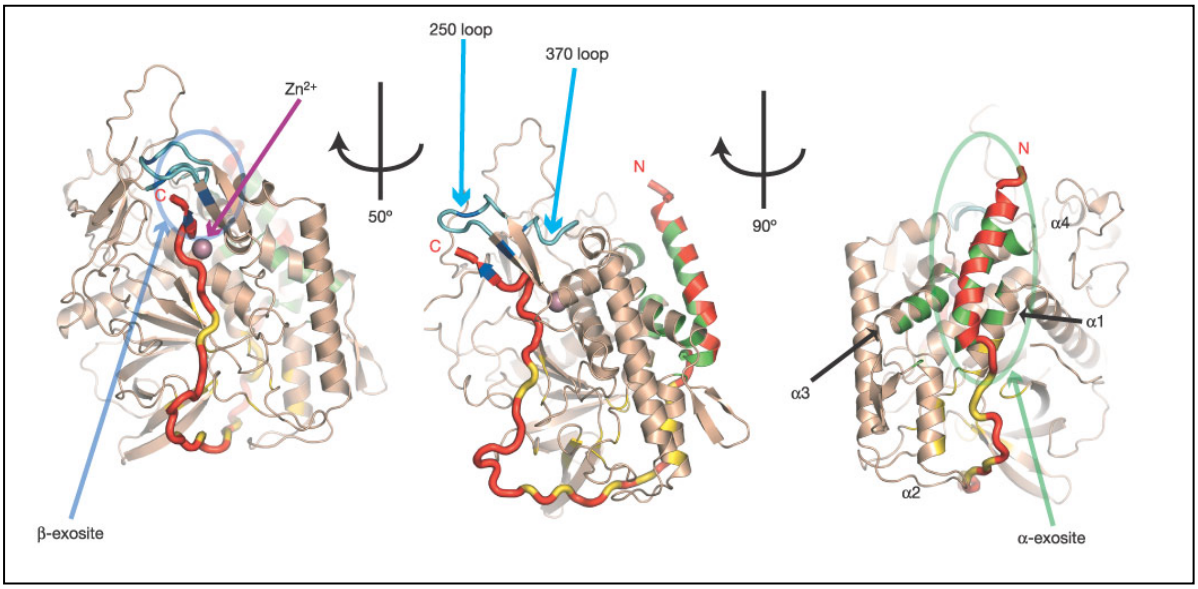

Abb.7 Interaktion zwischen SNAP-25 und LC/A. Es sind drei verschiedene Ansichten des Komplexes zu sehen, jeweils um die vertikale Achse gedreht. SNAP-25 (rot) bindet über verschiedene Ankerpunkte (gelb markiert) und einen kleinen a-helikalen Bereich (α-exosite) mit der leichten Kette von BoNT/A (braun).

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

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

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**Corresponding author*

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

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

Introduction

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

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

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

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

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

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

Results

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

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

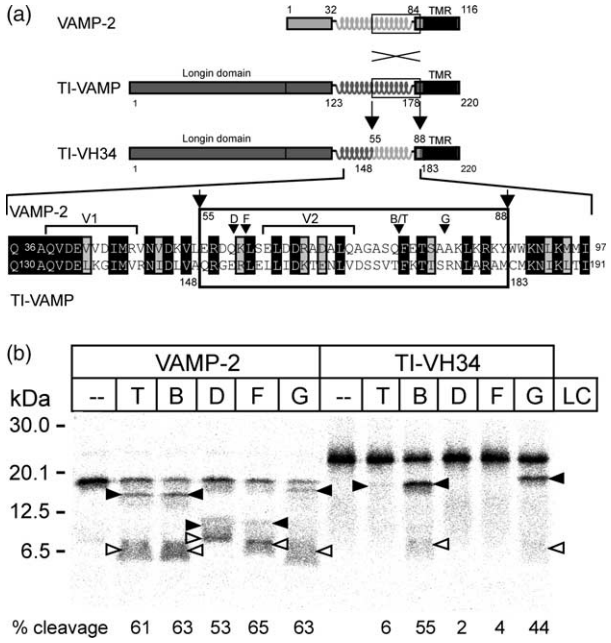

Figure 1. Effects of CNTs on TI-VH34. (a) Schematic representation of TI-VAMP (grey), the BoNT/B substrate VAMP-2 (light grey), and the VAMP hybrid, TI-VH34. The coiled-coil domains mediating the interaction with partner SNAREs are displayed. Numbers below or above specify amino acid border positions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

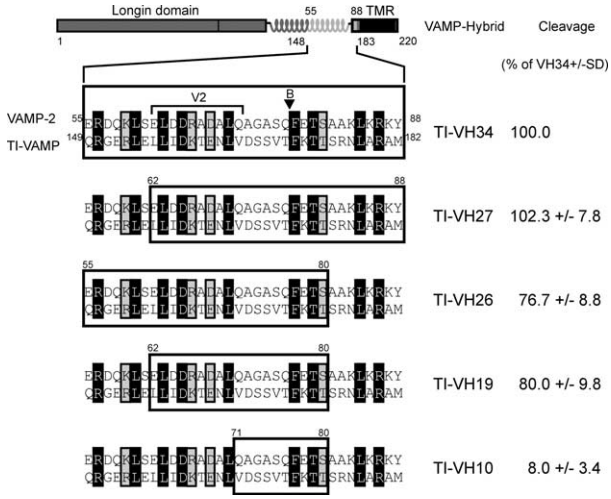

Figure 2. Effects of BoNT/B on various VAMP hybrids. Left panel: Alignment of the 34 amino acid residues encompassing the region of VAMP-2 supposed to mediate the interaction with BoNT/B with the corresponding region of TI-VAMP. VAMP-2-derived segments of the various generated hybrids are boxed. Identical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

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

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

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

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

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

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

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

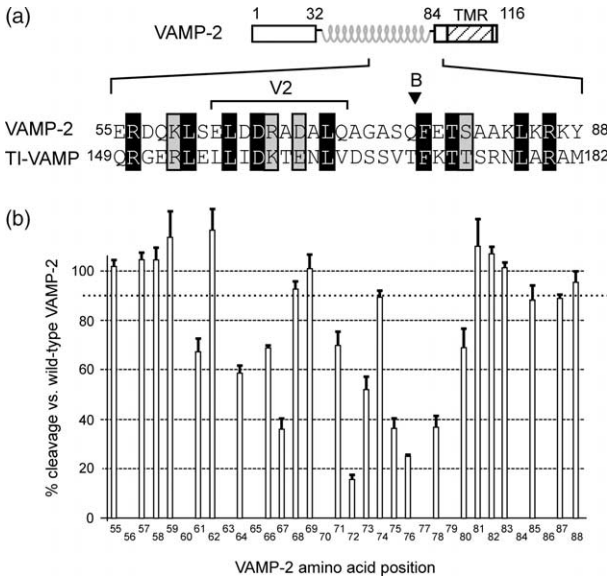

Figure 3. Cleavage analysis of various VAMP-2 point mutants. (a) Schematic representation of VAMP-2 and alignment of the amino acid region supposed to bind to BoNT/B with the corresponding region of TI-VAMP. Identical residues are indicated by white letters on a black background. Conserved residues are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

residues, whose mutation reduced the cleavage rate of VAMP-2, in TI-VAMP (TI-VH10-11) raised the sensitivity even further. Anyway, the assembly of aspartate, alanine, and lysine in positions 158, 161, and 179, respectively, in TI-VH-10 created a hybrid VAMP that possesses merely 11 amino acid residues originating from VAMP-2, but exhibits full sensitivity to BoNT/B (Figure 4). Therefore this hybrid, TI-VH10-10, was referred to as BBs-TI-VAMP for BoNT/B-sensitive TI-VAMP and further analysed. BBs-TI-VAMP could represent a useful tool for further studies on the cellular function of TI-VAMP.

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

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

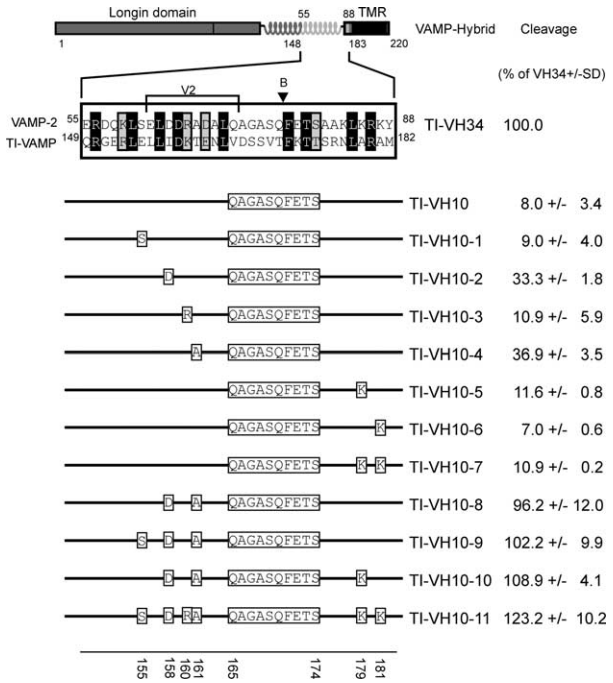

Figure 4. Generation of BoNT/B-sensitive TI-VAMP variants. Left panel: Alignment of the 34 amino acid residues encompassing the region of VAMP-2 supposed to mediate the interaction with BoNT/B with the corresponding region of TI-VAMP. Identical residues are indicated by white letters on a black background.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

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

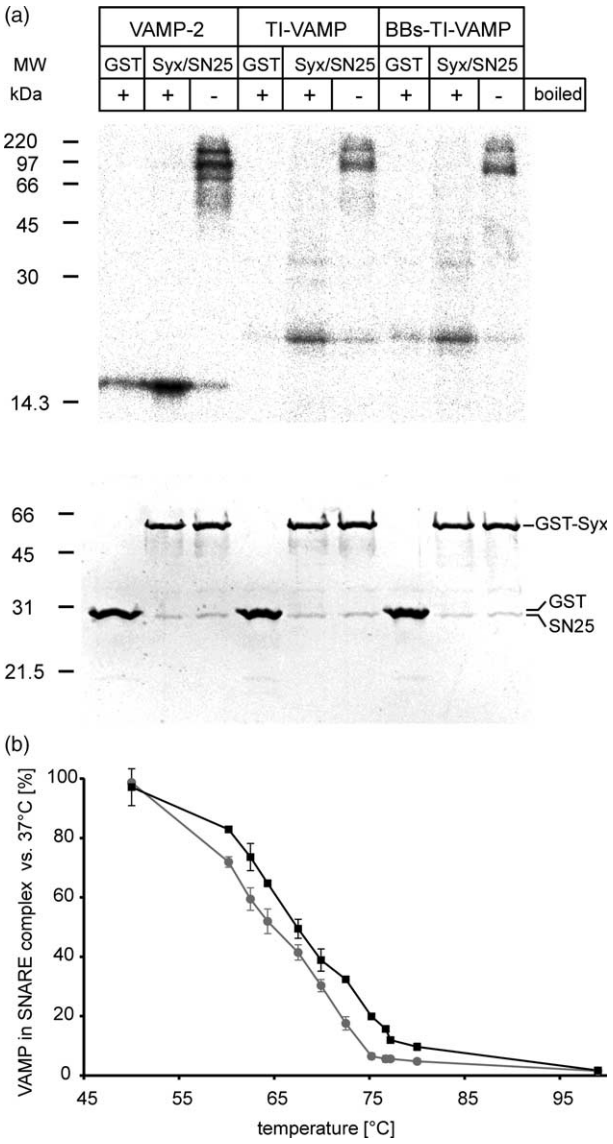

Figure 5. Analysis of the capacity of BBs-TI-VAMP to form SNARE complexes *in vitro*. (a) GST and binary GST-Syx-1A/SNAP-25His6 (SN25) complexes pre-assembled on GT-Sepharose beads were incubated overnight with radiolabelled VAMP-2, TI-VAMP, or BBs-TI-VAMP. Bead-bound protein was analysed by SDS-PAGE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

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

Discussion

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

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

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

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

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

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

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

Materials and Methods

Plasmid constructions

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

Expression and purification of recombinant CNT L chains and SNARE proteins

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

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

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

In vitro transcription and translation

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

Cleavage assays

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

SNARE complex assembly and stability assays

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

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

Molecular modelling

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

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Supplementary Data

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

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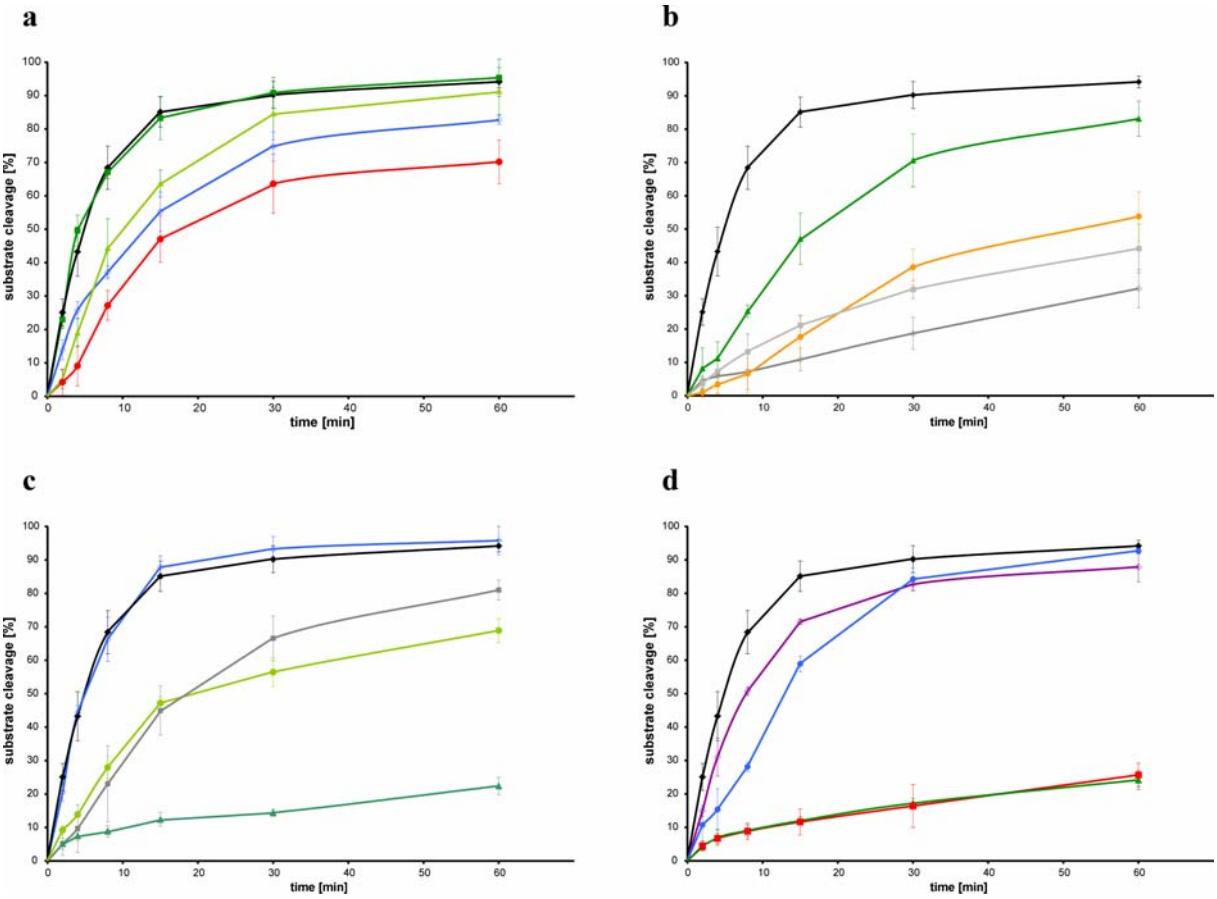

Supplementary Fig. 1. Cleavage analysis of various VAMP-2 point mutants at micromolar substrate concentration. VAMP-2 mutants, produced in *E. coli* and purified by means of their N-terminal hexahistidine tag, were incubated for 1 hour in the presence of 10 nM BoNT/B L chain in toxin assay buffer. At times specified aliquots were withdrawn and mixed with double-concentrated sample buffer. Samples were analysed by Tris/Tricine-PAGE using 15% gels. Quantification of VAMP-2 and its cleavage products was done by means of an LAS-3000 imaging system and Coomassie blue stained gels. Data represent means ± SD of three independent experiments. **(a)** VAMP-2 wild-type (black diamonds); VAMP-2-Gln58Ile (green squares); VAMP-2-Ser61Glu (light green triangles); VAMP-2-Asp64Ile (red circles); VAMP-2-Arg66Lys (blue diamonds) **(b)** VAMP-2 wild-type (black diamonds); VAMP-2-Ala67Thr (light grey squares);VAMP-2-Ala72Asp (grey diamonds); VAMP-2-Gly73Ser (orange circles) **(c)** VAMP-2 wild-type (black diamonds); VAMP-2-Ala74Ser (grey squares);VAMP-2-Ser75Val (dark green triangles); VAMP-2-Ser80Thr (light green circles); VAMP-2-Lys83Asn (blue diamonds) **(d)** VAMP-2 wild-type (black diamonds); VAMP-2-Gln76Thr (green triangles);VAMP-2-Glu78Lys (red squares); VAMP-2-Lys87Ala (blue circles); VAMP-2-Tyr88Met (purple diamonds).

2.2. Structural and biochemical studies of botulinum neurotoxin serotype C1 light chain protease: implications for dual substrate specificity

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Structural and Biochemical Studies of Botulinum Neurotoxin Serotype C1 Light Chain Protease: Implications for Dual Substrate Specificity

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Abstract:

Clostridial neurotoxins are the causative agents of the neuroparalytic disease botulism and tetanus. They block neurotransmitter release through specific proteolysis of one of the three soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs) SNAP-25, syntaxin, and synaptobrevin, which constitute part of the synaptic vesicle fusion machinery. The catalytic component of the clostridial neurotoxins is their light chain (LC), a Zn^{2+} endopeptidase. There are seven structurally and functionally related botulinum neurotoxins (BoNTs), termed serotype A to G, and tetanus neurotoxin (TeNT). Each of them exhibits unique specificity for their target SNAREs and peptide bond(s) they cleave. The mechanisms of action for substrate recognition and target cleavage are largely unknown. Here, we report structural and biochemical studies of BoNT/C1-LC, which is unique among BoNTs in that it exhibits dual specificity toward both syntaxin and SNAP-25. A distinct pocket (S1') near the active site likely achieves the correct register for the cleavage site by only allowing Ala as the P1' residue for both SNAP-25 and syntaxin. Mutations of this SNAP-25 residue dramatically reduce enzymatic activity. The remote α−exosite that was previously identified in the complex of BoNT/A-LC and SNAP-25 is structurally conserved in BoNT/C1. However, mutagenesis experiments show that the α−exosite of BoNT/C1 plays a less stringent role in substrate discrimination in comparison to that of BoNT/A, which could account for its dual substrate specificity.

2.3. Substrate Recognition Mechanism of VAMP/Synaptobrevin Cleaving Clostridial Neurotoxins

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Substrate Recognition Mechanism of VAMP/Synaptobrevin Cleaving Clostridial Neurotoxins

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Abbreviations: BoNTs, botulinum neurotoxins; CNT, Clostridial neurotoxins; LC, light chain; SNAREs, soluble N-ethylmaleimide-sensitive-factor attachment protein receptor;; TeNT, tetanus neurotoxin; TI-VAMP, Tetanus toxin insensitive VAMP; VAMP, vesicle associated membrane protein (also designated synaptobrevin).

ABSTRACT

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. Whereas the mode of SNAP-25 cleavage by BoNT/A, 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/D, F, and TeNT by means of saturation mutagenesis of VAMP/synaptobrevin. For each of the toxins four or more residues clustered at an N-terminal site remote to 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, the region comprising four residues of either side of the scissile bond, do not play a role in the catalytic process. Mutation of residues in the proximity of the scissile bond exclusively affects the turnover number of the neurotoxins, however, the importance of individual positions at the cleavage sites varied for each toxin. The data evidence that similar to the SNAP-25 proteolyzing BoNT/A and BoNT/E, VAMP/synaptobrevin specific CNTs also initiate substrate interaction employing an exosite located N-terminal of the scissile peptide bond.

Clostridial neurotoxins (CNTs), i.e. tetanus neurotoxin (TeNT) and seven serotypes of botulinum neurotoxins (BoNTs A to G) interfere with neurotransmitter release. BoNTs act in extremely low doses in motorneurons at the neuromuscular junction and thereby cause muscle paralysis. TeNT operates in inhibitory interneurons of the spinal cord that down regulate the activity of motorneurons and thus causes the opposite effect of BoNTs. CNTs are synthesized as single chain protein toxins by bacteria of the genus *Clostridium* and become later on proteolytically activated. They consist of a catalytic domain (designated L chain), a translocation domain that transfers the L chain subsequent to receptor mediated endocytosis across the membrane of the endosomal compartment, and a cell binding subunit that mediates the selective binding to nerve cells. Upon delivery to the cytosol, the L chain is released from the rest of the molecule by reduction of the disulphide bridge by which it is tethered to the translocation domain. The L chains finally attack their intracellular substrates by acting as zinc endoproteases. They exhibit individual substrate specificity. BoNT/C hydrolyses various syntaxins. BoNT/A, C, and E cleave certain members of the synaptosomal-associated protein of 25 kDa (SNAP-25) family, and all other CNTs proteolyze certain vesicle associated membrane proteins (VAMPs)/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 (syx 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 and SNAP-23 together with which TI-VAMP carries 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 characterization 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.

MATERIALS AND METHODS

Plasmid constructions

The cytosolic portion (amino acids 1-96) encoding segment of the wild-type rat VAMP-2 gene was inserted into the pET15b vector (Merck Biosciences GmbH, Schwalbach Ts., Germany). The open reading frame for human TI-VAMP was subcloned in pSP72 (Promega, Mannheim, Germany). Point mutations in pET15b-VAMP-2 and pSP72-TI-VAMP were introduced by oligonucleotide primer based PCR mutagenesis using Pwo-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) or the GeneTailor site-directed mutagenesis system (Invitrogen Corporation, Carlsbad, USA). Hybrid VAMP encoding plasmids (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.

Expression and purification of recombinant VAMP-2 and CNT L chains

The *E. 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 Ni^{2+}-nitrilotriacetic acid-agarose beads according to the manufacturer´s instructions. Fractions containing recombinant proteins were dialysed against toxin assay buffer (150 mM K^+ glutamate, 10 mM HEPES-KOH, pH 7.2), frozen in liquid nitrogen, and kept at -70°C.

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.

In vitro transcription and translation

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

Cleavage assays

Cleavage assays contained 1 µL of the transcription/translation mixture of [^{35}S]-methionine-labelled VAMP-2 or TI-VAMP variants and purified L chain and were incubated for 60 min at 37°C in a total volume of 10 µL of toxin assay buffer. 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-Laboratories GmbH, Munich, Germany). Subsequently, gels were dried and radiolabelled proteins were visualised employing a BAS-1500 phosphoimager (Fuji Photo Film, Co., Ltd., Tokyo, Japan). Quantification of radiolabelled 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 to 100 µM. Each of the various substrate concentrations was endowed by the addition of 1 µl of radio-labeled His_6VAMP-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, Japan). 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. K_m and V_{max} values were derived from Lineweaver-Burk plots using the GraphPad Prism 4.03 program (GraphPad Software Inc, San Diego, USA).

Molecular modelling

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

RESULTS AND DISCUSSION

VAMP recognition by BoNT/F depends on a more extended substrate segment compared to BoNT/D

In order to define essential substrate segments for BoNT/D and BoNT/F, we generated a series of hybrids that consisted of various parts of the genuine VAMP-2 substrate which replaced corresponding areas of the full-length non-cleavable TI-VAMP (TI-VAMP hybrids TI-VH; Fig. 1). *In vitro* translated hybrid substrates were incubated for 1 hour with LCD or LCF (both used at 0.2 nM final concentration). The extent of cleavage was determined by phosphor imaging and expressed as percentage of cleavage of VAMP-2(1-96). Optimal cleavage for LCD was still observed with a VAMP hybrid that comprised a 23 amino acid segment (Val-42 to Asp-64) of VAMP-2 (TI-VH23), whereas TI-VH44 (VAMP-2 region Ala-21 to Asp-64) still yielded optimal cleavage for LCF. N- or C-terminal elongation of these VAMP-2 portions did not enhance the activity of LCD or LCF, although maximum values of 48.5% or 22.2% relative to VAMP-2(1-96) were only achieved for LCD and LCF, 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, amino acids 1-93, e.g. in TI-VH23 indeed strongly improved cleavage by LCD (83.8 ± 2.5%). In agreement with this data, attachment of the longin domain to VAMP-2 (TI-VAMP-2(32-116)) decreased cleavage to 50.8 ± 3.8%. The presence of the longin domain similarly affected cleavage by LCF. It cleaved 42.6 ± 2.7% of TI-VH44 lacking residues 1-93 within one hour 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 LCF. Therefore, cleavage of full-length VAMP-2 versus VAMP-2(1-96) was studied. As expected, both variants were proteolyzed with comparable efficiency by LCD (94.0 ± 4.3% of VAMP-2(1-96)), but proteolysis of full-length VAMP-2 by LCF 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 LCF. Besides a negative effect for the longin domain, the cleavage analysis of various TI-VH proteins suggested that LCD involves 23 substrate residues, whereas LCF requires a far longer VAMP-2 segment, and that residues located C-terminal to the cleavage site, i.e. more than five residues downstream of the scissile peptide bond, do not appear to be important for substrate recognition and cleavage by LCD and LCF. This finding questions an earlier observation according to which Asp-64, Asp-65, and Asp-68 were involved in the interaction with LCF (*17*). Results obtained for point mutations in positions 64 and 68 as well as that a VAMP peptide comprising Leu-32 to Asp-65 is effectively cleaved clearly support our mapping data (Fig. 2; see below) (*15*).

Identification of substrate residues involved in formation of the Michaelis complex and the hydrolytic process

Next, saturation mutagenesis of VAMP-2(1-96) at positions 27 to 88 was performed to identify every individual VAMP-2 residue that contributes to the interaction with CNTs. Residues being not conserved among TI-VAMP and VAMP-2 were mutated to those present in the corresponding position of TI-VAMP. Conserved residues were replaced with alanine. The only conserved alanine (Ala-37) was converted to leucine. Mutated VAMP-2 proteins were initially produced as radiolabelled molecules by *in vitro* transcription/translation and incubated for one hour with LCD, LCF, LCT, and LCB. The percentage of cleavage versus VAMP-2(1-96) was calculated. Amino acids, whose substitution resulted in an at least 33% diminished cleavage rate, were considered critical for substrate cleavage. According

to this presetting, 14 amino acids within the segment Thr-27 to Tyr-88 proved to be important for LCF. Seven mutations even reduced the cleavability by over 66% (Fig. 2). In agreement with the results of the previous mapping experiments, significant effects were not observed for mutations downstream of Leu-60. To find out, if critical amino acids contribute to substrate binding or the catalytic process, kinetic parameters were determined. Mutation of residues remote to the scissile peptide bond, i.e. mutations Q33D, V39A, and V43, drastically increased K_M, but had no effect on k_{cat}. Vice versa, replacement of residues around the scissile bond, D57G, Q58E, K59R, and L60A solely diminished k_{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_M and k_{cat} values (Tab. 1). Together, residues in the direct neighborhood to the scissile bond are involved in the catalytic process, whereas those remote to the cleavage site are exclusively responsible for substrate binding. The data for mutants of the cleavage site are in full compliance with those of earlier work conducted with synthetic VAMP-2 peptides (*15*).

Reduction of LCD cleavage by >33% was caused by mutations at nine amino acids, three of which reduced cleavage by >66%. The nine critical residues 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 (cp. TI-VH23) to govern optimal substrate cleavage. As for LCF, this analysis confirmed that residues downstream of the cleavage site are also not important for the interaction of LCD (Fig. 2). Determination of the kinetic parameters showed that like for LCF, amino acid substitutions in the proximity to the scissile bond affected k_{cat}, but left K_M unaltered. Mutation of residues in the segment Val-39 to Met-46 increased K_M, indicating that they are solely crucial for the formation of the Michaelis complex (Tab. 1). 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 LCT was analyzed. Eleven mutation 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 to 80) and the second comprises residues 41-44. Additional five

residues whose mutation diminished cleavage by 12 to 27% were identified even further upstream (residues 31 to 35). Hence, TeNT requires a substrate segment of at least 51 amino acids, whereas the required parts of VAMP-2 for optimal cleavage by BoNT/F and D comprise 31 and 28 amino acids, respectively. The essential part for BoNT/B, which shares the scissile bond with TeNT, had earlier been shown by mapping experiments to depend on the substrate segment Ser-61 to Ser-80 (*20*). In line with this finding, the saturation mutagenesis of residues in the region upstream of Ser-61 discovered merely four residues that marginally reduced cleavability (Fig. 2). Again, mutation of a residue located at a remote site to the scissile peptide bond had only an effect on K_M (V43K), whereas mutation of cleavage site residues A74S, Q76T, and E78K diminished exclusively k_{cat} (Tab. 1).

Replacement of ten 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 saturation mutagenesis, we set out to create a TI-VAMP variant containing as little as possible mutations 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 replacement of Glu-153 and Glu-155 with glutamine and serine, respectively, resulted in a TI-VAMP variant (designated TI-D2) that was cleaved, albeit merely at high LCD concentrations (Fig. 3). Import of further VAMP-2 residues stepwise increased the sensitivity of TI-VAMP to LCD. A TI-VAMP variant that exhibited a similar cleavability as the genuine substrate VAMP-2 ultimately possessed ten mutations (TI-D-10; Fig. 3), 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 to 36.5 ± 5.8% for the hybrid TI-VAMP-2(32-116) (Fig. 3). 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 (eleven) of amino acids was required (*20*). It remains to be shown, whether TI-D-5, TI-D8, or TI-D-10 could be a useful for studies on the cellular function of TI-VAMP.

VAMP and SNAP-25 proteolyzing CNTs likely pursue a similar substrate recognition strategy

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 to the scissile peptide bond. The exosite comprises about twelve residues of SNAP-25 (position 156 to 167). SNAP-25 then wraps around LCA in a U-turn like fashion: Thereby some further interactions along the substrate-toxin interface are established. The scissile peptide bond Q197-R198 becomes next exposed to the active site residues of LCA. 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 (Fig. 4). Firstly, exosite like segments in VAMP-2 were identified for BoNTs D, 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 31-43 (for LCF), 39-46 (for LCD), 61-67 for (LCB), and 41-45 (for TeNT; Fig. 2), although they are except for LCF smaller 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 they are only involved in the formation of the Michaelis complex. Secondly, at least for TeNT the putative exosite would be separated from the cleavage site (if defined by each four residues on either side of the scissile bond) by 27 amino acids, thus comparable to 25 residues for the BoNT/A exosite. The distance of the exosites for BoNT/F, D and B are shorter, being eleven, eight, and four residues, respectively, the latter resembling in this aspect the configuration in BoNT/E (*27*). Thirdly, as discovered for the SNAP-25-LCA interface, the present studies also identify single residues in the region linking tentative 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 not defined 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 form one to another CNT, According to our data BoNT/D for instance involves the substrate positions P3, P2, P1' and P2' (Fig. 4). 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 at the active site.

CONCLUSIONS

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 sizes of these exosites as well as their distance to the scissile bond vary from one to the other CNT.

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Table 1. Kinetic parameters of VAMP-2 cleavage by BoNT/D, BoNT/F, and TeNT

	BoNT/D-LC						BoNT/F-LC						TeNT-LC					
Mutation	K_M (μM)	SD	K_{cat} (1/sec)	SD	K_{cat}/K_M	No. of exp.	K_M (μM)	SD	K_{cat} (1/sec)	SD	K_{cat}/K_M	No. of exp.	K_M (μM)	SD	K_{cat} (1/sec)	SD	K_{cat}/K_M	No. of exp.
wild-type	33.3	3.3	24.6	3.2	0.74	4	28.7	4.9	22.0	6.0	0.76	4	54.9	4.0	2.0	0.57	0.036	4/5
Q33D	34.2	8.7	22.1	3.2	0.65	4	83.0	4.8	23.6	0.9	0.28	2						
V39A	84.4	12.5	21.5	1.7	0.25	4	119.3	42.2	19.4	1.4	0.16	2						
V43K	85.6	15.2	19.2	3.9	0.22	4	91.4	3.4	19.9	9.1	0.22	2	98.4	1.9	1.8	0.12	0.018	2
M46A	193.7	33.3	21.6	7.8	0.11	2	39.0	6.1	19.3	2.7	0.49	4						
E55Q	46.7	1.1	21.5	5.2	0.46	2	43.0	8.5	18.3	0.7	0.43	3						
D57G	48.6	2.8	17.7	9.5	0.37	3	22.8	1.9	1.1	0.3	0.05	2						
Q58E	41.8	11.3	11.9	2.9	0.28	2	24.8	1.8	6.9	3.4	0.28	4						
K59R	41.5	11.2	15.3	3.4	0.37	4	35.6	18.7	2.9	1.0	0.08	2						
L60A	35.4		8.9		0.25	1	28.0	2.8	7.2	2.9	0.26	3						
S61E	28.6	1.4	6.4	1.1	0.22	2	34.0	0.5	24.8	0.2	0.73	2						
Q71V													79.6	2.1	1.6	0.33	0.020	2
G73S													73.5	8.1	0.8	0.06	0.011	2
A74S													60.3	2.2	0.9	0.20	0.015	2
Q76T													54.6	10.5	0.8	0.01	0.015	2
E78K													55.2	2.5	0.6	0.10	0.012	2

FIGURE CAPTIONS

Fig. 1. Determination of VAMP-2 regions that are essential for cleavage by BoNT/F and BoNT/D using various VAMP hybrids. Left panel: Schematic representation of VAMP-2, TI-VAMP, and various TI-VAMP-VAMP-2 hybrids (TI-VH; numbers specify the number of VAMP-2 residues). F, D, B, and T together with numbers are line item specifications for the scissile bonds of respective BoNTs or TeNT. Numbers above (gray) and below (black) line drawings define border residues of VAMP-2 and TI-VAMP in the various hybrid proteins, respectively. Right panel: hybrid proteins were radiolabelled by *in vitro* transcription/translation and incubated for 1 hour in the presence of 0.2 nM L chain of BoNTs D or F. Samples were analyzed by SDS-PAGE and phosphor imaging. Values represent the percentage of cleavage versus VAMP-2(1-96) ± SD of two to eight independent experiments. Note that several TI-VHs were also analyzed as constructs lacking the longin domain (Δ Longin). n.a., not applicable; n.d., not determined.

Fig. 2. Cleavage analysis of various VAMP-2 point mutants. Upper panel: Schematic representation of VAMP-2 and alignment of the amino acid region that was analyzed by saturation mutagenesis with the corresponding region of TI-VAMP. Identical residues are indicated by white letters on 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”. Lower panels: In order to determine the effect on hydrolysis by CNT L chains each non conserved amino acid of VAMP-2 was individually replaced by the corresponding amino acid of TI-VAMP. Conserved residues were substituted for alanine, and the conserved Ala-37 was replaced with leucine. VAMP-2 mutants were radiolabelled by *in vitro* transcription/translation and incubated for 1 hour in the presence of 0.2 nM LCF, 0.2 nM LCD, 20 nM LCB, or 10 nM LCT. 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 ± SD of at least four independent experiments. Dotted lines specify thresholds of 10%, 33%, and 66% reduction of

cleavability. Dark blue horizontal bars below individual charts denote putative exosites, light blue bars the cleavage sites, and dark blue dots interspersed anchor points (see text).

Fig. 3. Generation of BoNT/D sensitive TI-VAMP variants. Left panel: Alignment of a 31 amino acids segment encompassing the region of VAMP-2 supposed to be essential for proteolysis by BoNT/D with the corresponding region of TI-VAMP. Identical residues are indicated by white letters on black background. Conserved residues are shown in boxes on grey background. "D" denotes the scissile peptide bond for BoNT/D. Numbers below specify mutated TI-VAMP residues. Right panel: Mutated TI-VAMP variants were radiolabelled by *in vitro* transcription/translation and incubated for 1 hour in the presence of 0.3 to 300 nM LCD. The extent of cleavage was determined subsequent to SDS-PAGE (15% gels) and phosphor imaging. Values represent the average percentage of cleavage of two to six independent experiments. SD values did not exceed 30% of the measured values (e.g. TI-D-8: 9 ± 2.6%).

Fig. 4. Model for substrate recognition by VAMP cleaving CNTs. (A) VAMP-2 is anchored via its C-terminal transmembrane domain in synaptic vesicles. **(B)** The catalytic domain of the neurotoxin (BoNT/D serves as example; LCD) initiates the interaction *via* a VAMP exosite (black squares) located N-terminal to the cleavage site implicating four or more amino acid side groups. **(C)** Single further contacts (open circle) along the substrate may then be established in order to bring the cleavage site (four residues on either side of the scissile bond; P4P3P2P1P1'P2'P3'P4') close to the LC active site. **(D)** The scissile bond gets properly positioned by means of accommodating substrate cleavage site residues at corresponding LC pockets around the catalytic centre (these are the P3, P2, P1', and P2' substrate residues in case of LCD). **(E)** The scissile peptide bond gets hydrolyzed, and the cleavage products dissociate from the LC **(F)**.

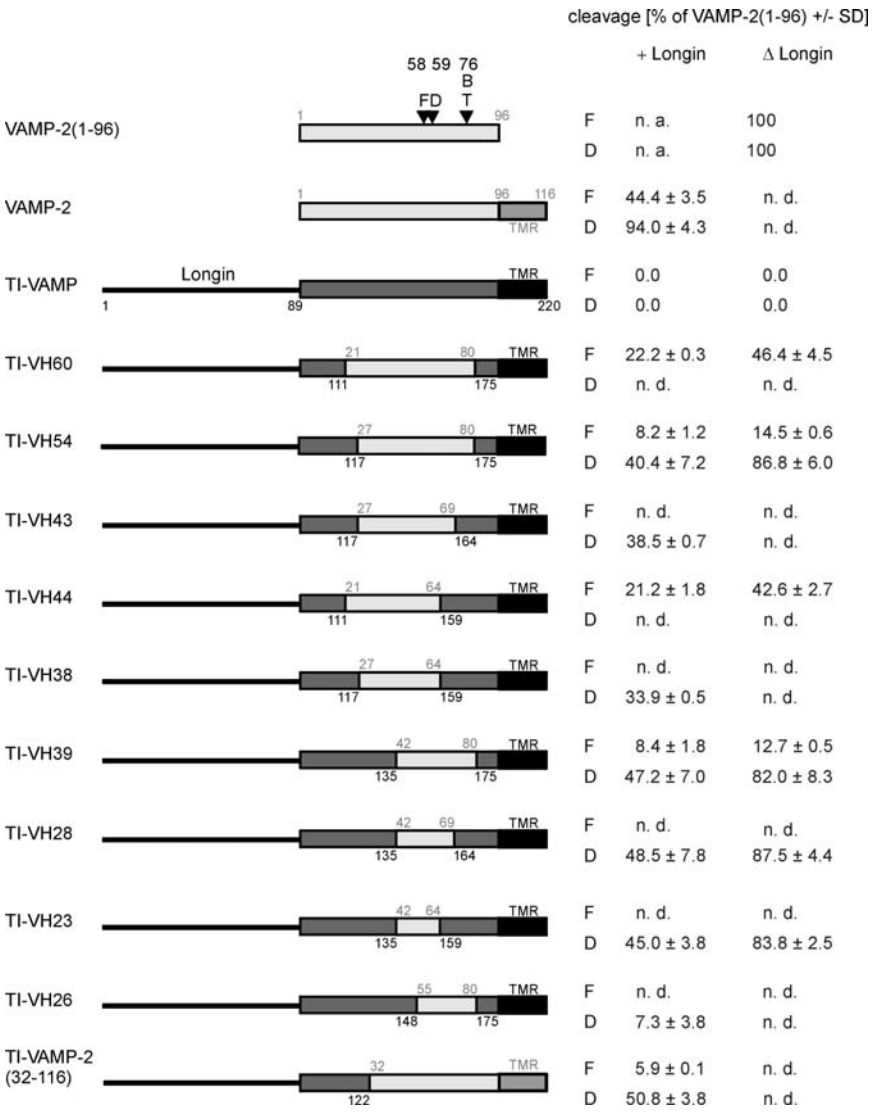
cleavage [% of VAMP-2(1-96) +/- SD]
+ Longin
Δ Longin
58 59 76
FD
B
T
VAMP-2(1-96)
1
96
F n. a. 100
D n. a. 100
VAMP-2
1
96
116
TMR
F 44.4 ± 3.5 n. d.
D 94.0 ± 4.3 n. d.
TI-VAMP
Longin
TMR
1
89
220
F 0.0 0.0
D 0.0 0.0
TI-VH60
21
80
TMR
111
175
F 22.2 ± 0.3 46.4 ± 4.5
D n. d. n. d.
TI-VH54
27
80
TMR
117
175
F 8.2 ± 1.2 14.5 ± 0.6
D 40.4 ± 7.2 86.8 ± 6.0
TI-VH43
27
69
TMR
117
164
F n. d. n. d.
D 38.5 ± 0.7 n. d.
TI-VH44
21
64
TMR
111
159
F 21.2 ± 1.8 42.6 ± 2.7
D n. d. n. d.
TI-VH38
27
64
TMR
117
159
F n. d. n. d.
D 33.9 ± 0.5 n. d.
TI-VH39
42
80
TMR
135
175
F 8.4 ± 1.8 12.7 ± 0.5
D 47.2 ± 7.0 82.0 ± 8.3
TI-VH28
42
69
TMR
135
164
F n. d. n. d.
D 48.5 ± 7.8 87.5 ± 4.4
TI-VH23
42
64
TMR
135
159
F n. d. n. d.
D 45.0 ± 3.8 83.8 ± 2.5
TI-VH26
55
80
TMR
148
175
F n. d. n. d.
D 7.3 ± 3.8 n. d.
TI-VAMP-2
(32-116)
32
TMR
122
F 5.9 ± 0.1 n. d.
D 50.8 ± 3.8 n. d.

Fig. 1.

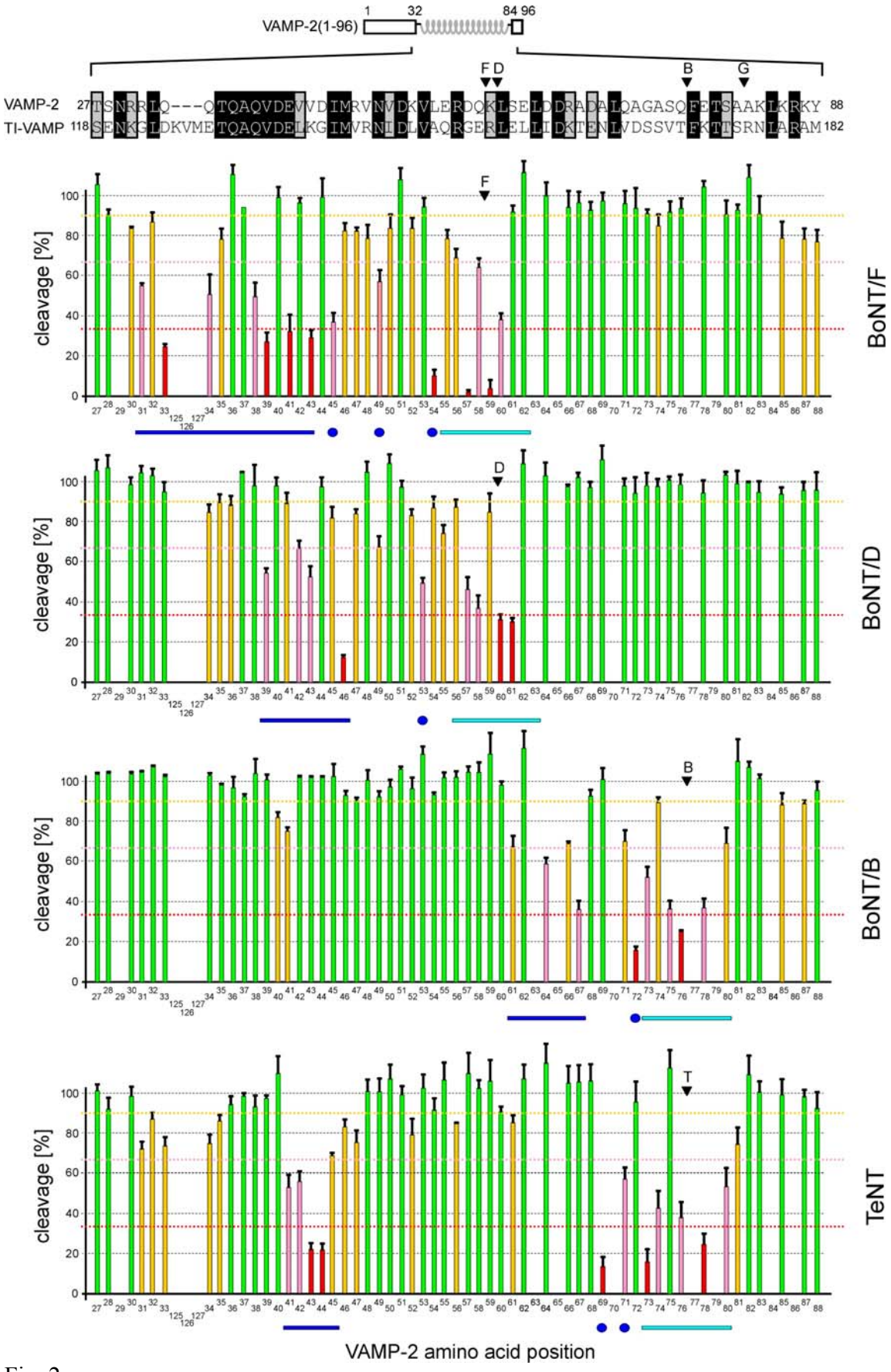
VAMP-2(1-96)
1
32
84 96
F D
B
G
VAMP-2 27 TSNRRLQ---QTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY 88
TI-VAMP 118 SENKGLDKVMETQAQVDELKGIMVRNIDLVAQRGERLELLIDKTENLVDSSVTFKTTSRNLARAM 182
cleavage [%]
BoNT/F
BoNT/D
BoNT/B
TeNT
VAMP-2 amino acid position

Fig. 2.

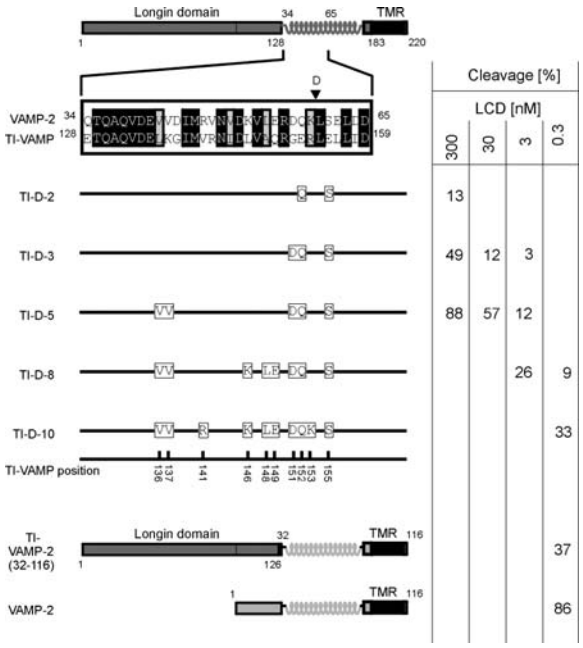
Longin domain
TMR
Cleavage [%]
LCD [nM]
300
30
3
0.3
VAMP-2
TI-VAMP
TI-D-2
TI-D-3
TI-D-5
TI-D-8
TI-D-10
TI-VAMP position
TI-VAMP-2 (32-116)
VAMP-2

Fig. 3.

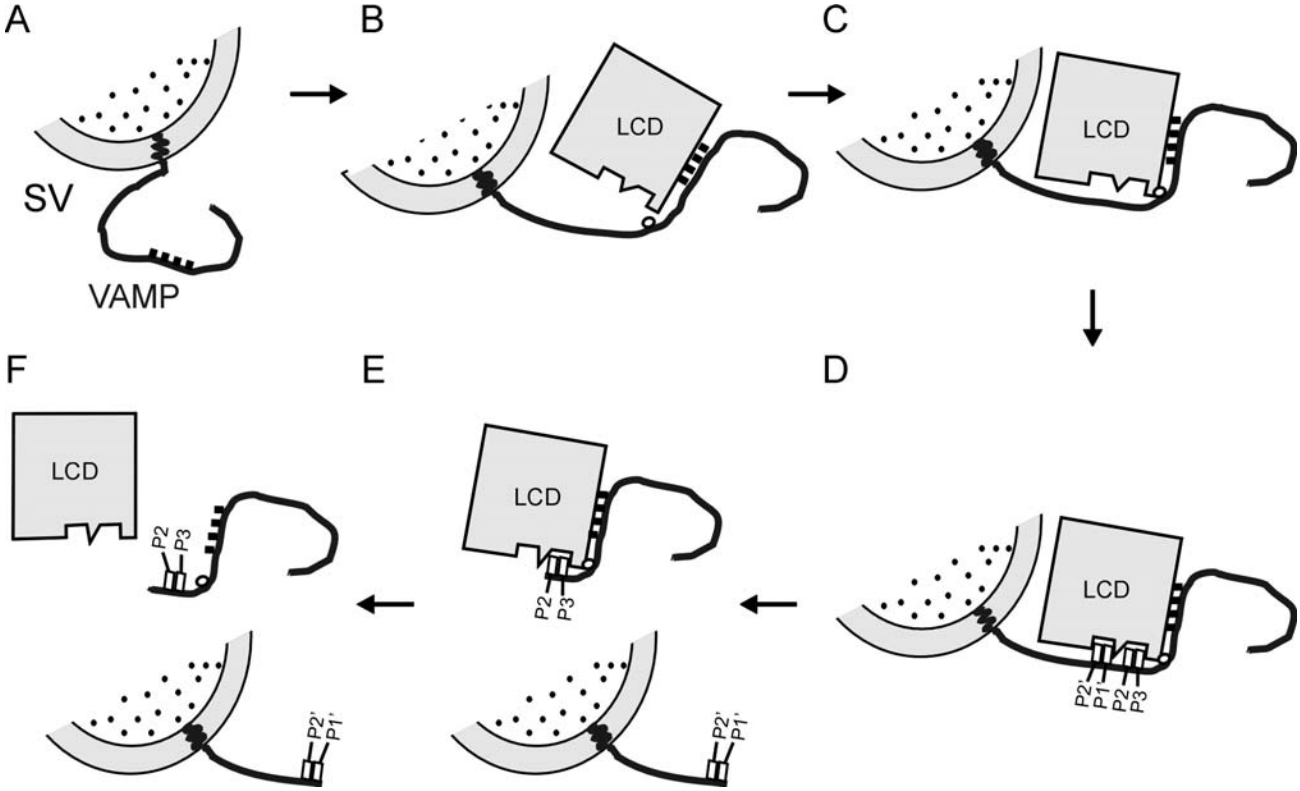
A
B
C
SV
VAMP
LCD
LCD
D
LCD
P2'
P1'
P2
P3
E
LCD
P2
P3
P2'
P1'
F
LCD
P2
P3
P2'
P1'

Fig. 4.

3. Diskussion

In den letzten Jahren wurden die Kristallstrukturen aller leichten Ketten veröffentlicht: BoNT/A (Lacy *et al.,* 1998; Segelke *et al.,* 2004; Breidenbach und Brunger, 2004), BoNT/B (Swaminathan und Eswaramoorthy, 2000), BoNT/C (Jin *et al.,* 2007), BoNT/D (Arndt *et al.,* 2006), BoNT/E (Agarwal *et al.,* 2004), BoNT/F (Agarwal *et al.,* 2005), BoNT/G (Arndt *et al.,* 2005) und TeNT (Breidenbach und Brunger, 2005; Rao *et al.,* 2005). Insgesamt gibt es bis auf Abweichungen in der Oberflächenstruktur kaum Unterschiede (Kap. 2.2., Abb.1B). Das aktive Zentrum ist bei allen leichten Ketten gleich aufgebaut. Es besteht aus dem für Zinkproteasen typischen HExxH Motiv und gleicht damit dem des Thermolysins (Lacy *et al.,* 1998). Umso erstaunlicher ist die Tatsache, dass sich die Aminosäuresequenz einiger Serotypen deutlich voneinander unterscheiden (Tab. 2).

Tab. 2 Sequenzidentität der leichten Ketten in % (MegAlign, ClustalW; DNAStar Inc, USA)

	LC/A	LC/B	LC/C	LC/D	LC/E	LC/F	LC/G	LC/T
LC/A		30,0	30,7	34,1	32,1	32,3	33,3	29,7
	LC/B		31,6	32,9	35,0	37,5	60,4	51,0
		LC/C		46,8	31,2	32,3	34,3	32,4
			LC/D		32,4	34,2	35,8	34,0
				LC/E		55,8	34,7	40,3
					LC/F		38,4	43,9
						LC/G		48,8
							LC/T	

Für die Substraterkennung werden abseits der Spaltstelle zusätzliche lange Bereiche benötigt (α-exosites). Damit nehmen die CNT eine Sonderrolle unter den Metalloproteasen ein, die sonst nur relativ einfache Erkennungssequenzen benötigen.

Für die Vorhersage von möglichen Interaktionspunkten zwischen Substrat und CNT wurden bereits mehrere Computermodelle anhand der jeweiligen kristallographischen Daten erstellt (Arndt *et al.,* 2006; Agarwal *et al.,* 2005; Olson und Armendinger, 2002). Allerdings sind dies rein theoretische Ergebnisse. Wirklich verlässliche Daten liefern nur biochemische Analysen.

In dieser Arbeit konnte der Mechanismus der Substraterkennung von BoNT/B, BoNT/C, BoNT/D, BoNT/F und TeNT näher charakterisiert werden. Die Resultate zeigen, welche Substrataminosäuren zur Bindung und zum katalytischen Mechanismus der Botulinus Neurotoxine B, C, D, F und Tetanus-Neurotoxin wichtig sind. Mit diesem Wissen könnte man nun die leichten Ketten gezielt mutieren und so Neurotoxinvarianten generieren die in der

Lage sind, insensitive SNAREs zu spalten. Außerdem könnte die Spezifität von Inhibitoren weiter verbessert werden.

3.1. Spaltung von VAMP-2

VAMP-2 wird von BoNT/B (Schiavo *et al.,* 1992), BoNT/D (Schiavo *et al.,* 1993a; Yamasaki *et al.,* 1994), BoNT/F (Yamasaki *et al.,* 1994; Schiavo *et al.,* 1993b), BoNT/G (Yamasaki *et al.,* 1994; Schiavo *et al.,* 1994a) und TeNT (Schiavo *et al.,* 1992; Link *et al.,* 1992) gespalten. TI-VAMP wird selbst bei hohen Enzymkonzentrationen (bis zu 10.000x mehr) von keinem der fünf Neurotoxine gespalten, obwohl die beiden Proteine (VAMP-2 und TI-VAMP) zu ca. 40% identisch sind. In dieser Arbeit wurde die molekulare Grundlage für die Resistenz von TI-VAMP geklärt und damit gleichzeitig die Substraterkennung von VAMP-2 näher beleuchtet, indem Austausche zwischen diesen beiden Proteinen durchgeführt wurden.
Es konnte gezeigt werden, dass die Bereiche im Substrat, welche für eine optimale Aktivität der leichten Ketten nötig sind, bei BoNT/B die Aminosäuren Asp64-Ser80 (Kap. 2.1., Fig. 4), bei BoNT/D Val42-Asp64, bei BoNT/F Thr27-Asp42 und bei TeNT Glu41-Ser80 (Kap. 2.3., Fig. 2) umfassen.

3.1.1. Spaltstellen

BoNT/B und TeNT spalten jeweils zwischen Gln76-Phe77. BoNT/D (Lys59-Leu60) und BoNT/F (Gln58-Lys59) spalten an zwei benachbarten Peptidbindungen. Ein Austausch in der P_1' Position hat bei allen Serotypen einen drastischen negativen Effekt auf die Spaltbarkeit (Tab. 3). Dies lässt sich darauf zurückführen, dass die korrespondierenden S_1' Taschen in den leichten Ketten genau auf die Reste der P_1' Position zugeschnitten sind. Bereits eine kleine Änderung der Ladung oder der Größe zeigt bei diesen stringenten Voraussetzungen starke negative Auswirkungen. Die S_1' Taschen von LC/B und LC/T sind fast gleich groß, weil sich bei beiden Serotypen ein Phenylalanin an der P_1' Position befindet. Die S_1' Taschen bei LC/D (P_1' Position: Leucin) und LC/F (P_1' Position: Lysin) sind dagegen wesentlich kleiner (Abb. 8). Bei BoNT/F hat der Austausch in P_1' vom nativen Lysin zum größeren Arginin den stärksten Effekt, hier kann eine Interaktion aus sterischen Gründen nicht mehr stattfinden. Bei

BoNT/D hat die Mutation Leu-Ala zur Folge, dass die hydrophobe Wechselwirkung mit der S_1' Tasche nicht mehr so stark ist (Kap. 2.3., Fig. 2)**.**

Die Effekte der Aminosäureaustausche in den restlichen Positionen der Spaltstelle (P_5 bis P_5') fallen bei den Serotypen unterschiedlich aus. Bei BoNT/F führen alle Mutationen stromaufwärts der P_1' Position zu einer Reduktion der Spaltbarkeit um mindestens 25%, während bei BoNT/D und TeNT nur Mutationen in drei Positionen einen Effekt zeigen. Die Aminosäuren stromabwärts der P_1' Position haben einen geringeren Einfluss auf die Spaltbarkeit (Kap. 2.3., Fig. 2). Hier kommt es nur zu vereinzelten Wechselwirkungen mit den leichten Ketten. Dies unterstützt die Vermutung, dass das Substrat bei allen leichten Ketten in der gleichen Orientierung gebunden wird.

3.1.2. α-exosites

Die Größe der α-exosites und deren Abstand zur Spaltstelle unterscheiden sich stark voneinander (Abb. 8). Bei BoNT/D und BoNT/F ist zumindest der Abstand zur Spaltstelle ungefähr gleich groß (ca. 10 Aminosäuren), während BoNT/B (5 Aminosäuren) und TeNT (20 Aminosäuren) diesbezüglich deutlich abweichen. Einige Reste innerhalb der α-exosite sind scheinbar besonders wichtig für die Interaktion mit der leichten Kette. Dass ein Austausch von Met46 bei Spaltung durch BoNT/D zu einer fast kompletten Inhibierung der Spaltung führt, konnte auch von anderen Arbeitsgruppen nachgewiesen werden (Yamasaki *et al.,* 1994; Pellizzari *et al.,* 1997). Yamasaki und Kollegen konnten ferner zeigen, dass für die Spaltung von VAMP-1 eine 3700-fach höhere Enzymkonzentration benötigt wird, weil hier an Position 46 statt des Methionins ein Isoleucin vorliegt (Yamasaki *et al.,* 1994). Es wird vermutet, dass dieses Methionin eine starke Wechselwirkung mit dem Histidin an Position 132 in BoNT/D eingeht (Arndt *et al.,* 2006). Frühere Untersuchungen an BoNT/B offenbarten, dass das Asp64 eine wichtige Rolle einnimmt (Pellizzari *et al.,* 1996; Wictome *et al.,* 1996; Shone *et al.,* 1993). Auch in dieser Arbeit konnte der drastische Effekt einer Mutation an dieser Position beobachtet werden (Kap. 2.1., Fig. 3). Noch nicht beschrieben wurden die starken Effekte der Austausche von Val43 und Asp44 bei der Spaltung durch TeNT (Kap. 2.3., Fig. 2). Ferner kann der Effekt der Doppelmutante Asp40/Glu41 in der Arbeit von Pellizzari und Kollegen (Pellizzari *et al.,* 1996) nach den Daten dieser Arbeit allein auf die Glu41 Mutation zurückgeführt werden, weil der Einzelaustausch Asp40 zu Alanin keinen negativen Effekt auf die Spaltung hat. Bei der Spaltung durch BoNT/F wurde

durch Verkürzungsmutanten gezeigt, dass der Bereich zwischen Thr27-Gln36 wichtig ist (Yamasaki *et al.,* 1994). In dieser Arbeit konnten die beteiligten Wechselwirkungen in dieser Region zum ersten Mal näher charakterisiert werden. Dabei zeigen die beiden Glutamine an Position 33 und 34 den größten negativen Effekt auf die Spalteffizienz (Kap. 2.3., Fig. 2).
Die kinetischen Experimente mit Mutanten der α-exosite belegen deutlich deren Bedeutung für die korrekte Bindung von VAMP2. Die Reduktion der Spaltbarkeit ist bei allen Mutanten auf eine Erhöhung des K_M Wertes zurückzuführen, während der K_{cat} Wert keine signifikante Veränderung aufweist (Kap. 2.3., Tab. 1).

3.2. Spaltung von SNAP-25 und Syntaxin

Als hilfreiche Ausgangsbasis für die Untersuchungen zur Wechselwirkung von BoNT/C mit SNAP-25 und Syntaxin diente die im Jahr 2004 veröffentlichte Kokristallstruktur von BoNT/A mit SNAP-25 (Breidenbach und Brunger, 2004). Durch die hohe Strukturähnlichkeit der leichten Ketten von BoNT/A und BoNT/C konnten Voraussagen zur Interaktion von BoNT/C gemacht werden.

3.2.1 Spaltstellen

BoNT/C spaltet SNAP-25 zwischen Arg198-Ala199 (Vaidyanathan *et al.* 1999). Mutationen in der P_1 Position (Arg198) in SNAP-25 führen zu einer drastischen Reduktion der Spaltbarkeit. Ein Ladungswechsel vom positiv geladenen Arginin zu einem negativ geladenen Aspartat oder Glutamat zeigt dabei den größten Effekt auf die Spaltbarkeit (Vaidyanathan *et al.,* 1999).
Ersetzt man das Alanin in der P_1' Position (Ala199) von SNAP-25 durch größere Aminosäuren, so erhält man einen drastischen Rückgang der Spaltung. So kann man bei einem Austausch Ala-Arg keine Spaltung mehr nachweisen. Die beobachteten Effekte durch Mutationen in der P_1' Position beeinflussen immer nur die Katalyse, nicht aber die Bindung des Toxins. Dies konnte durch kinetische Experimente nachgewiesen werden (Kap. 2.2., Tab. 2). Die Intoleranz der P_1' Position gegenüber großen Seitenketten kann dadurch erklärt werden, dass die korrespondierende S_1' Tasche in der leichten Kette von BoNT/C sehr klein ist. Dort passen nur Aminosäuren mit kleinen Seitenketten hinein. Die Ala-Gly Mutante

würde zwar sterisch passen, aber dadurch dass Glycin nicht hydrophob ist, fällt hier eine wichtige Wechselwirkung weg. Bei BoNT/A ist die S_1' Tasche sehr viel größer (Breidenbach und Brunger, 2004). BoNT/A spaltet SNAP-25 zwischen Gln197-Arg198, also nur eine Aminosäure weiter N-terminal gegenüber BoNT/C (Binz *et al.* 1994). Das relativ große Arginin an Position 198 passt nur in die größere S_1' Tasche von BoNT/A, nicht aber in die kleine S_1' Tasche von BoNT/C. Dort findet erst das Alanin an Position 199 Platz. Dadurch kann der Versatz der Spaltstelle zwischen BoNT/A und C erklärt werden. Weitere Untersuchungen in diesem Bereich haben gezeigt, dass die SNAP-25 Positionen P_5, P_3, P_1' und P_4' und die entsprechenden Taschen in BoNT/A S5, S3, S_1' und S_4' für die korrekte Justierung des Spaltstelle wichtig sind (Chen *et al.,* 2006; Chen *et al.,* 2007). Bei BoNT/C wurden bis jetzt nur die P_1 Position untersucht. Im Gegensatz zu BoNT/A spielt diese eine wichtige Rolle in der Substraterkennung (Vaidyanathan *et al.,* 1999). Für eine Klärung der genauen Abläufe um die Spaltstelle herum müssten weitere Mutationen in diesem Bereich produziert werden.

Mutationen in der Spaltstelle von Syntaxin1a wurden bisher noch nicht durchgeführt. BoNT/C spaltet Syntaxin1A (Blasi *et al.,* 1993) zwischen Lys253-Ala254 (Schiavo *et al.,* 1994b). Vergleicht man die verschiedenen Syntaxin Isoformen miteinander kann man hieraus Schlüsse auf die Erfordernisse der Spaltstelle ziehen. Im Gegensatz zu allen anderen spaltbaren Isoformen besitzt Syntaxin 4 an der P_1 Position statt eines Lysins ein Isoleucin. Daraus lässt sich ableiten, dass für die Spaltung mit BoNT/C in der P_1 Position des jeweiligen Substrates eine positive Ladung vorhanden sein muss (SNAP-25 = Arg; Syntaxin = Lys).

3.2.2. α-exosites

Für die optimale Aktivität von BoNT/A und C ist ein großer Bereich von SNAP-25 nötig, welcher die Aminosäuren 93-202 beinhaltet (Vaidyanathan *et al.,* 1999). Dies bedeutet, dass nicht nur die Reste flankierend zur Spaltstelle, sondern auch Aminosäuren weiter Stromaufwärts für eine optimale Toxin-Substrat Interaktion zuständig sind. Im BoNT/A-SNAP-25 Cokristall von Breidenbach und Brunger findet man zwischen den SNAP-25 Aminosäuren Gln152 und Met167 einen Bereich starker Wechselwirkung, die α-exosite. Hier findet vermutlich der erste Kontakt zwischen Toxin und Substrat statt (Breidenbach und Brunger, 2004). Eine Deletion dieser Region führt zu einer kompletten Inhibition der Spaltung durch BoNT/A (Vaidyanathan *et al.,* 1999; Kap. 2.2., Tab. 2). Die in dieser Arbeit

zusätzlich erstellten Punktmutationen führen teilweise zu einer starken Reduktion der Spaltung (Kap. 2.2., Tab. 2). Aufgrund von Sequenzvergleichen zwischen BoNT/A und C kann man davon ausgehen, dass die α-exosite auch für die Interaktion zwischen BoNT/C und seinen entsprechenden Substraten wichtig ist. Allerdings konnten bei Experimenten mit BoNT/C keine vergleichbar starken Effekte festgestellt werden. Lediglich Austausche der Aminosäuren Gln152 (vermutliche Wechselwirkung mit BoNT/C Lys365) und Asp166 (vermutliche Wechselwirkung mit BoNT/C Lys346) zeigten eine leichte Reduktion der Spaltungseffizienz bedingt durch schlechtere Bindung (Kap. 2.2., Tab. 2). Die geringeren Effekte der Aminosäureaustausche im Bereich der α-exosite kann man darauf zurückführen, dass BoNT/C mit Syntaxin noch ein zweites Substrat besitzt und eine zu stringente Substratspezifität in diesem Fall die Erkennung beider Substrate unmöglich machen würde. Es ist durchaus denkbar, dass bei der Interaktion von BoNT/C und SNAP-25 auch noch Bereiche wichtig sind, die in dieser Arbeit nicht untersucht wurden. So, ist das optimale Substrat für BoNT/C (SNAP-25: AS 93-202) länger als das für BoNT/A (SNAP-25: AS 146-202) (Vaidyanathan *et al.*, 1999).

Die Verschiebung der Spaltstelle bei SNAP-25 zwischen BoNT/A und C um eine Position wird durch einen flexiblen Bereich (Glu183-Ile192) im Substrat gewährleistet, welcher keinerlei Kontakte zu BoNT/A aufweist (Breidenbach und Brunger, 2004). Wie zu erwarten war, haben Veränderungen in diesem Loop-Bereich keinen Einfluss auf die Spaltung von SNAP-25 durch BoNT/A. Interessanterweise steigern Deletionen in diesem Bereich die Spaltung durch BoNT/C (Kap. 2.2., Tab. 2). Durch die Deletion einzelner Aminosäuren kann es zu neuen Wechselwirkungsmöglichkeiten kommen. Tatsächlich könnte die Verdopplung der Spaltrate durch die Deletionsmutante SNAP-25 Δ187 darauf zurückzuführen sein, dass es zu einer Interaktion zwischen SNAP-25 Glu183 und dem His25 in BoNT/C kommt.

3.3. Mechanismus der Substraterkennung clostridieller Neurotoxine

Anhand der gewonnen Ergebnisse dieser Arbeit kann ein generelles Modell für die Substraterkennung clostridieller Neurotoxine erstellt werden. Alle CNT benötigen neben der eigentlichen Spaltstelle einen weiteren Bereich starker Wechselwirkung zur optimalen Substraterkennung, die α-exosite. Dabei unterscheiden sich die Größe der Interaktionsfläche und die Abstände zwischen α-exosite und Spaltstelle bei den einzelnen Neurotoxinen deutlich voneinander (Abb.8).

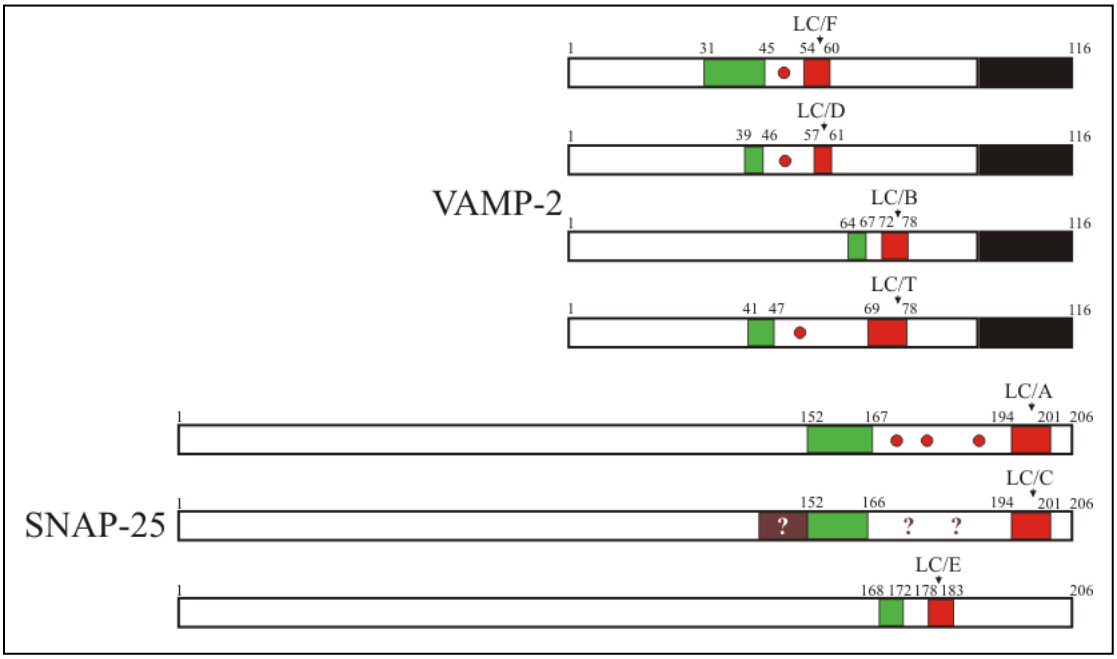

Abb.8 Abstände zwischen α-exosite und Spaltstelle bei VAMP-2 und SNAP-25. Die roten Kästen stellen Bereiche mit starker Wechselwirkung in der Spaltstelle dar. Die grünen Kästen symbolisieren die α-exosite. Die roten Punkte symbolisieren einzelne Aminosäurereste, die an der Interaktion beteiligt sind. Bei SNAP-25 und BoNT/C sind vermutlich noch weitere nicht untersuchte N-terminal von der α-exosite liegende Aminosäuren sowie Bereiche zwischen α-exosite und Spaltstelle für die Substraterkennung wichtig (brauner Kasten und Fragezeichen).

3.3.1. Spaltstellen

Bei allen Serotypen hat ein Austausch an der P_1' Position einen drastischen negativen Effekt auf die Spaltbarkeit. Der Grund dafür ist die stringente Anforderung der entsprechenden S_1' Taschen was Größe und Ladung angeht. Die Interaktion zwischen den Aminosäuren der P_1' Position und der S_1' Tasche bildet den entscheidenden Schritt der Substratpositionierung. Dies erklärt, warum die P_1' Aminosäure bei allen Neurotoxinen wichtig ist.

Die restlichen Aminosäuren der Spaltstelle spielen vermutlich eine wichtige Rolle bei vorangehenden Wechselwirkungen zur korrekten Ausrichtung der P_1' Position in die S_1' Tasche (Chen *et al.*, 2007a; Chen et al., 2007b). Die Interaktionspunkte, die bei dieser Ausrichtung eine Rolle spielen, variieren zwischen den Neurotoxinen (Tab. 3).

Tab. 3 Effekte von Aminosäureaustauschen in der Spaltsstelle

	P_5	P_4	P_3	P_2	P_1	**P_1'**	P_2'	P_3'	P_4'	P_5'
BoNT/A[1]	D	E	A	N	Q	**R**	A	T	K	M
BoNT/B	A	G	A	S	Q	**F**[3]	E	T[3]	S	A
BoNT/C	*E*	*A*	*N*	*Q*	R[3]	**A**	*T*	*K*	*M*	*L*
BoNT/D	E	R	D	Q	K	**L**	S	E	*L*	D
BoNT/E[1]	R	Q	I	D	R	**I**	M	E	K	A
BoNT/F	L	E	R	D	Q	**K**	L	S	E	*L*
BoNT/G	*F*	*E*	*T*	*S*	*A*	**A**[4]	*K*	*L*	*K*	*R*
TeNT	A	G	A	S	Q	*F*	E	*T*	S	A

A: ≥ 75% Reduktion A: 50%-75% Reduktion A: 25%-50% Reduktion *A*: nicht analysiert

[1]Chen und Barbieri, 2006 [3]Shone und Roberts, 1994 [3]Vaidyanathan *et al.*, 1999
[4]Yamasaki *et al.*, 1994

3.3.2. α-exosites

Eine Erklärung warum sich die α-exosites so stark voneinander Unterscheiden findet sich in den gewonnen Strukturdaten der leichten Ketten. Diese besitzen zwar eine relativ einheitliche Gesamtstruktur, allerdings findet man gerade im Interaktionsbereich mit dem Substrat starke Unterschiede in der Oberflächenstruktur (Abb. 9). Bei allen Serotypen erkennt man deutlich den Spalt, in dem das Substrat bindet und der in der S_1' Tasche endet.

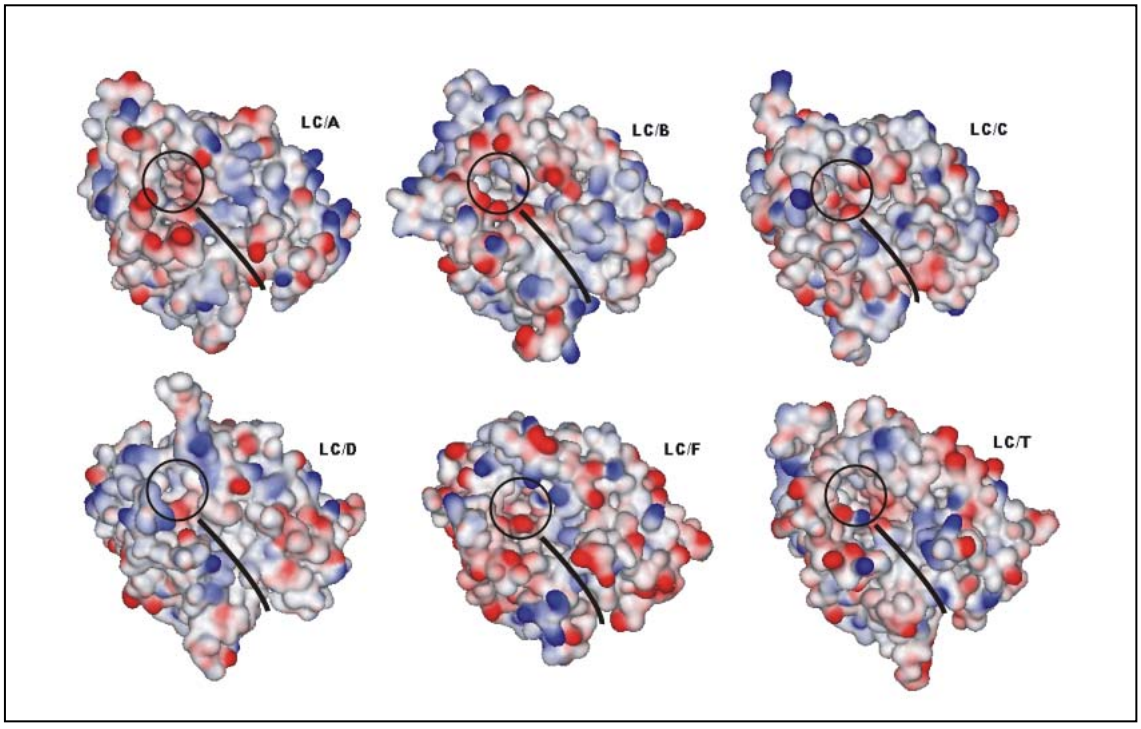

Abb.9 Kristallstrukturen von ausgewählten leichten Ketten. Die berechnete Oberfläche ist nach ihrem elektrostatischen Potenzial gefärbt. Rot: negativ geladene Reste; Blau: positiv geladene Reste. Der Kreis markiert die S_1' Tasche. Der schwarze Balken markiert die Position des Substrates.

3.3.3. Allgemeines Modell

Zusammenfassend lässt sich ein allgemeines Modell der Substraterkennung clostridieller Neurotoxine erstellen (Abb. 10). Dabei kommt es zu einer ersten Interaktion im Bereich der α-exosite. Diese erste Bindung ist entscheidend für eine effektive Spaltung des Substrats. Das Substrat wird anschließend in Richtung Spaltstelle dirigiert. Dies geschieht durch weitere einzelne Interaktionspunkte (Abb. 8). Die korrekte Einfädelung der P_1' Position in die S_1' Tasche wird durch angrenzende Aminosäureinteraktionen bewirkt und führt schließlich zur Spaltung des Substrates.

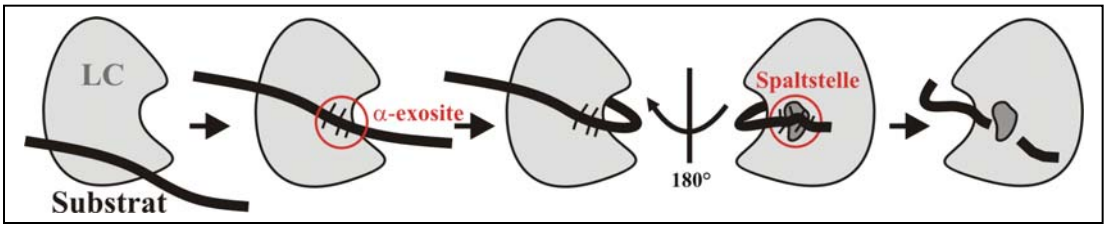

Abb.10 Modell der Substraterkennung clostridieller Neurotoxine. Nach der initialen Bindung an der α-exosite wird das Substrat durch weitere Interaktionspunkte zur Spaltstelle geleitet. Hier vermitteln definierte Wechselwirkungen die korrekte Einfädelung des Substrates in das aktive Zentrum.

3.4. Ausblick

Da im Zuge dieser Arbeit nur die Seite des Substrates untersucht wurde, wäre es jetzt sinnvoll auch die Bindungsstellen im Enzym näher zu charakterisieren. So können anhand der vorliegenden Strukturdaten die möglichen Wechselwirkungspartner in den leichten Ketten identifiziert werden. Dort könnten Mutationen durchgeführt und deren Auswirkung auf die Spaltung untersucht werden. Möglicherweise gelingt es so leichte Ketten zu generieren, die in der Lage sind, zuvor nichtspaltbare SNARE Proteine zu hydrolysieren. Diese Mutanten könnten dann eingesetzt werden, um vesikuläre Transportwege in der Zelle näher zu untersuchen.

Die in dieser Arbeit erstellten BoNT/B- und BoNT/D-sensitiven TI-VAMP Mutanten könnten als Basis für ein konditionelles „loss-of-function"-Phänotyp Model dienen um weitere funktionelle Studien, z.B. zum Neuriten-Wachstum, durchzuführen.

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Publikationen

Originalpublikationen in „peer reviewed“ Zeitschriften:

“Identification of the amino acid residues rendering TI-VAMP insensitive toward botulinum neurotoxin B.”
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Poster

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