Multiple Pocket Recognition of SNAP25 by Botulinum Neurotoxin Serotype E*

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Botulinum neurotoxins (BoNTs) are zinc proteases that cleave SNARE proteins to elicit flaccid paralysis by inhibiting the fusion of neurotransmitter-carrying vesicles to the plasma membrane of peripheral neurons. There are seven serotypes of BoNT, termed A–G. The molecular basis for SNAP25 recognition and cleavage by BoNT serotype E is currently unclear. Here we define the multiple pocket recognition of SNAP25 by LC/E. The initial recognition of SNAP25 is mediated by the binding of the B region of SNAP25 to the substrate-binding (B) region of LC/E comprising Leu166, Arg167, Asp127, Ala128, Ser129, and Ala130. The mutations at these residues affected substrate binding and catalysis. Three additional residues participate in scissile bond cleavage of SNAP25 by LC/E. The P3 site residues, Ile178, of SNAP25 interacted with the S3 pocket in LC/E through hydrophobic interactions. The S3 pocket included Ile147, Ile164, and Ile182 and appeared to align the P1’ and P2 residues of SNAP25 with the S1’ and S2 pockets of LC/E. The S1’ pocket of LC/E included three residues, Phe191, Thr159, and Thr208, which contribute hydrophobic and steric interactions with the SNAP25 P1’ residue Ile181. The S2 pocket residue of LC/E, Lys224, binds the P2 residue of SNAP25, Asp179, through ionic interactions. Deletion mapping indicates that main chain interaction(s) of residues 182–186 of SNAP25 contribute to substrate recognition by LC/E. Understanding the mechanism for substrate specificity provides insight for the development of inhibitors against the botulinum neurotoxins.

The Clostridium botulinum neurotoxins (BoNTs) are the most potent protein toxins for humans (1). BoNTs are zinc proteases that cleave SNARE proteins that inhibit fusion of neurotransmitter-carrying vesicles to the plasma membrane of peripheral neurons. BoNTs are 150-kDa single chain proteins that are activated by proteolysis to generate disulfide-linked di-chain proteins. BoNTs are organized into three functional domains as follows: an N-terminal catalytic light chain (LC) and a C-terminal heavy chain (HC) that is further divided into a translocation domain and a C-terminal receptor binding domain (2, 3). Intoxication of neurotransmitter-carrying junction by BoNTs occurs by multiple step mechanisms involving the following: (a) binding to presynaptic neuronal cell membrane; (b) internalization to endosomes by receptor-mediated endocytosis and/or synaptic vesicle uptake; (c) membrane translocation of the light chain from mature endosomes into neuronal cytoplasm, and (d) target recognition and cleavage of neuronal substrate. The tropism for neurons is because of the affinity of BoNT for receptors on peripheral neurons (2). BoNT-receptor interactions are sometimes enhanced by gangliosides, which act as co-receptors (4).

There are seven BoNT serotypes, A–G, with serotypes A, B, and E responsible for most natural human intoxications (5). Human intoxication by BoNT/E is most commonly related to eating contaminated fish (6). BoNT serotypes are defined by the specificity of antibody neutralization where antibodies that neutralize one serotype fail to neutralize other serotypes. The crystal structures of the LC of BoNTs are very similar, whereas the amino acid sequences of these LCs display low overall homology, the HEXXH motif, characteristic of the catalytic pocket of Zn2+-endopeptidase, is conserved. BoNT cleaves one of the three SNARE proteins, SNAP25, VAMP, and Syntaxin. VAMP is cleaved by BoNT/B, -D, -F, and -G at distinct sites. SNAP25 is cleaved by BoNT/A, -C, and -E at distinct sites. Syntaxin is also cleaved by BoNT/C (7). Unlike other metalloproteinase, BoNTs recognize extended regions of the substrate for cleavage. The substrate specificity of BoNTs has become a subject of investigation because the utilization of BoNT for clinical therapies has intensified.

There are two licensed vaccines against botulism, a pentavalent vaccine against serotypes A–E (8) and a heptavalent vaccine against serotypes A–G (9). These vaccines are chemically inactivated BoNT produced in C. botulinum and are in limited supply. Upon intoxication, BoNT has significant morbidity and mortality (10, 11). Thus, there is a need to develop more efficient vaccines and therapies against botulism. In addition, because of their specific recognition and cleavage of SNAP2 proteins, BoNTs are widely utilized as therapeutic agents to treat various neurological disorders of dystonia and spinal spasticity, including blepharospasm, torticollis, and spasmodicus (12) and as well as being utilized as tools for investigating intracellular vesicular trafficking and the mechanism of membrane fusion (13–15). Understanding the substrate specificity of BoNT will provide insight into the development of therapies against BoNT intoxication and the modification of BoNT to optimize therapeutic potential and extend our tools to study.

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2The abbreviations used are: BoNT, botulinum neurotoxins; LC, light chain; HC, heavy chain; GST, glutathione S-transferase; SNAP, soluble NSF attachment receptor; SNARE, soluble NSF attachment protein; ELISA, enzyme-linked immunosorbent assay; SSR, SNARE secondary recognition; AS, active site; B, binding.
intracellular vesicular trafficking and the mechanisms of membrane fusion.

The mechanism of substrate recognition of BoNT/A has recently been addressed. LC/A recognizes SNAP25 through noncovalent interactions between residues within the belt region of LC/A and a region of SNAP25 that is distanced from the active site (16). Active site interactions includes four pockets within LC/A which aligned the P1 residue for cleavage (17). The recent crystal structure of LC/E (18) provided a platform to define LC/E recognition of SNAP25. Saturation mutagenesis of SNAP25 identified two sub-sites within SNAP25, which included an active site (AS) region that contributed to substrate recognition and cleavage and a binding (B) region, which contributed to substrate affinity. This prompted the characterization of SNAP25 cleavage by LC/E.

MATERIALS AND METHODS

Molecular Modeling

Molecular modeling was performed using SWISS-MODEL (20), available on line. The structure of SNAP25-(146–202) was obtained from the LC/A-SNAP25 complex structure (Protein Data Bank code 1GTX) and used as a template to model SNAP25. Briefly, the primary amino acid sequence of SNAP25-(129–185) was saved as text file and input together with the structure of SNAP25-(146–202) into Swiss PDB-viewer, where SNAP25-(129–185) was modeled in project mode. The initial model of SNAP25-(129–185) was then refined through adjusting the alignment based on the known biochemical and structural properties of SNAP25 and LC/E. The project SNAP25-(129–185) structure was sent to the SWISS-MODEL data base where the SNAP25-(129–185) structure was modeled. After the structure of SNAP25-(129–185) was completed, the LC/A-SNAP25, LC/E, and SNAP25-(129–185) structures were input into the Swiss PDB-viewer. The LC/E structure and SNAP25-(129–185) were fitted into LC/A-SNAP25-(146–206) complex structure to generate a LC/E-SNAP25-(129–185) complex. Two hydrophobic and one basic pocket in the active site of LC/E were defined as candidate sites for LC/E-SNAP25 interactions (termed the S1, S2, and S3 pockets). Based upon the position of these pockets, SNAP25 P sites residues were adjusted to optimally dock into S1, S2, and S3 using the PyMol program. LC/E substrate-SNAP25 binding region was also identified and optimized as described above to align the 5 pockets of LC/E and P site residues of SNAP25.

Plasmid Construction and Protein Expression

BoNT LC/E was constructed by amplifying DNA encoding LC/E-(1–408) of C. botulinum serotype E Beluga (21) and subcloning into pET-15b. Site-directed mutagenesis on LC/E or SNAP25 was performed using QuickChange (Stratagene), and protein expression and purification were performed as described in a previous study (22).

Cleavage of SNAP25 by BoNT-LCs

Standard Linear Velocity Reaction—Reactions contained (10 μl) the following: 5 μM SNAP25 was incubated with indicated concentrations of LC/E and LC/E derivatives in 10 mM Tris-HCl (pH 7.6) with 20 mM NaCl. Reactions were incubated for 10 min at 37 °C, stopped by adding SDS-polyacrylamide buffer, and heated for 5 min. Samples were subjected to SDS-PAGE, and gels were stained. The amount of SNAP25 cleavage was determined by densitometry.

Standard Time Course Assay—5 μM SNAP25 was incubated with 0.5 μM LC/E or the indicated LC/E derivative in 100-μl reactions. Ten-μl aliquots were withdrawn at specified times and processed as described above.

Kinetic Parameters—Km and kcat determinations were performed for WT-LC/E and LC/E derivatives using SNAP25-(141–206) as substrate. LC concentrations were adjusted to cleave <20% substrate at several concentrations of substrate ranging from 1.5 to 18 μM. The reaction was carried out in 10 μl of toxin reaction buffer, incubated at 37 °C for 10 min, and stopped by adding 10 μl of 2× SDS-polyacrylamide buffer. The reaction mixture was subjected to SDS-PAGE, and the amount of cleaved product was calculated by densitometry. Reaction velocity versus substrate concentration was fit to the Michaelis-Menten equation, and kinetic constants were derived from Lineweaver-Burk Plot using SigmaPlot (Chicago, IL).

Mutations Complementation Assay—5 μM of WT-SNAP25, SNAP(I181F), or SNAP(D179A) were incubated with LC/E (0.025–10 nM) or LC/E derivatives (0.01–1 μM) in 10-μl reactions at 37 °C for 10 min. Reactions were stopped by adding SDS-polyacrylamide buffer, heated for 5 min, and subjected to SDS-PAGE. Gels were stained, and the amount of SNAP25 cleaved was determined by densitometry. The concentration of WT-LC/E or LC/E derivatives was plotted versus % cleavage, and the concentrations of WT-LC/E or LC/E derivatives required for 50% cleavage of SNAP25 were calculated.

Trypsin Digestion Assay of LC/A

To address the possibility that the individual mutations had global effects on the structural elements of LC/E, a partial trypsin digestion assay was performed as described previously (17). Mutations to LC/E that resulted in decreased hydrolysis activity, such as F191A, T159A, K224A, and L167R, had identical trypsin digestion profile as wild type (WT)-LC/E (Fig. 1). Other LC/E single and double mutations also had similar trypsin digestion profiles as WT-LC/E (data not shown).

GST Pulldown Assay

The GST pulldown assay (300 μl) was performed by preincubating 6 μM of GST-SNAP25 (I181E) with 2 μM of WT-LC/E or derivatives for 30 min and then adding 30 μl of glutathione-Sepharose bead for 30 min, pre-blocked in 10% bovine serum albumin. The beads were pelleted and washed, and proteins in the pellet were detected by Western blotting using anti-LC/E and GST antibody (Sigma). GST-SNAP25, WT-LC/E, or derivatives alone were used as positive and negative control.

ELISA Binding Assay

The binding of SNAP25 to LC/E or derivatives was further characterized using ELISA binding assay using Alexa Fluor 488-labeled fluorescent SNAP25. Briefly, 1.5 μg of LC/E or derivatives were coated into wells of a 96-well nitrocellulose acid-ELISA plate (Qiagen) in 100 μl of phosphate-buffered saline at
Recognition of SNAP25 by BoNT/E

FIGURE 1. Trypsin digestion LC/E and LC/E derivatives. Five μM WT-LC/E and indicated LC/E derivative were incubated with 0.1 μM trypsin at 37 °C for 30 min. Reactions were stopped by adding SDS-polyacrylamide buffer, heating for 5 min, and subjecting to 13.5% SDS-PAGE, and gels were stained with Coomassie Blue.

4 °C overnight. Wells were washed three times in phosphate-buffered saline and incubated with different concentrations (1–50 μM) of Alexa Fluor 488 (Invitrogen)-labeled GST-SNAP25. After 1 h of incubation and shaking at room temperature, the plates were washed three times using a plate washer (Thermo). The fluorescence signals were recorded using a Victor III fluorescence detector (PerkinElmer Life Sciences). The LC/B-coated and blank wells were used as negative control to subtract background signal.

RESULTS

Molecular Modeling of LC/E-SNAP25 Complex Structure—Saturation mutagenesis identified two regions, B and AS, within SNAP25 that contributed to the substrate binding and catalysis, respectively (19). This provided a starting point for the characterization of SNAP25 recognition by LC/E. Structural modeling predicted that the active site region of LC/E had three interaction sites, termed S3, S2, and S1’, with the AS of SNAP25 and that LC/E made several contacts with the substrate binding region of SNAP25 that were distanced from the active site of LC/E (Fig. 2A).

Enzymatic Activity of Mutated LC/E Derivatives—Molecular modeling identified LC/E residues that composed the S3, S2, and S1’ pockets. Mutation to residues residing in each substrate recognition pocket and the residues that interacted with the B region of LC/E had specific effects on the velocity of substrate hydrolysis (Fig. 3). Mutated LC/E that had changes in the efficiency of SNAP25 cleavage were subjected to more detailed analysis.

Binding of the B Region of SNAP25 to the Substrate B Region of LC/E—Molecular modeling predicted that residues Asp127, Ile164, Asn169, and Gly168 of LC/E interacted with residues within the B region of SNAP25 (Fig. 2B). In the model of LC/E-SNAP25, Ile164 of SNAP25 was predicted to have hydrophobic interactions with Leu166 of LC/E, whereas Asp172 of SNAP25 was predicted to have an ionic interaction with Arg167 of LC/E (Fig. 2B). Replacement of Leu166 with Ala increased the $K_m$ of LC/E by ~7-fold and reduced the $k_{cat}$ by ~20-fold, whereas replacement of Arg167 of LC/E with Ala increased the $K_m$ by ~5-fold and reduced the $k_{cat}$ by 30-fold (Table 1). The LC/E-SNAP25 model also predicted that residues Asp127, Ala128, Ser129, and Ala130 of LC/E formed a pocket, where the side chain of Asn169 of SNAP25 docked into the pocket with Gly168 of SNAP25 located at the edge of the pocket (Fig. 2B). Two point mutations in LC/E, D127A and S129A resulted in an increased $K_m$ by ~6-fold and a reduced $k_{cat}$ of ~2- and ~5-fold for SNAP25 cleavage, respectively, relative to wild type LC/E (Table 1). The unexpected reduction of $k_{cat}$ for the L166A and R167A mutation within LC/E implicates a role in substrate catalysis, probably indirect and involving the proper alignment of residues involved in the alignment of the AS residues of LC/E with SNAP25.

S3 Pockets of LC/E Play Dual Roles on P3 Residue Binding and Alignment—Molecular modeling identified a hydrophobic pocket within LC/E, termed S3, which was predicted to interact with Ile178, the SNAP25 P3 residue (Fig. 2C). The S3 pocket of LC/E was composed of three Ile, Ile47, Ile164, and Ile182. Substitution of each Ile with Ala resulted in LC/E derivatives that possessed an ~2-fold higher $K_m$ and ~3–15-fold lower $k_{cat}$ for the cleavage of SNAP25 relative to WT-LC/E (Table 1). The magnitude of these effects was consistent with mutations at the P3 residue Ile178 of SNAP25 that reduced primarily the $k_{cat}$ for LC/E cleavage of SNAP25 (19), suggesting that S3 pocket-P3 site interactions are involved in the alignment of SNAP25 for optimal LC/E cleavage. Multiple Ile mutations within the P3 pocket were anticipated to have an additive effect on catalysis, but this was not assessable because of the insolubility of the double or triple Ile-mutated LC/E proteins.

LC/E S1’ Pocket Residues Recognize Ile181, the P1’ Residue in SNAP25—Four residues compose the S1’ pocket of LC/A (17). An overlay of the LC/A and LC/E structures predicted the composition of the LC/E S1’ pocket to contain five residues (Thr159, Phe191, Thr208, Tyr354 and Tyr356) (Fig. 2D). The role of these LC/E residues as components of the S1’ pocket was next determined. LC/E-Phe191A showed ~80-fold lower $k_{cat}$ values for SNAP25 cleavage than wild type LC/E (Table 1). Replacement of LC/E-Phe191 with hydrophobic residues, such as Leu and Tyr, had less of an effect on substrate hydrolysis, with ~20- and ~4-fold lower $k_{cat}$ values, respectively, than wild type LC/E. Replacement of residue Phe191 to Asn, which introduced a polar environment, lowered the $k_{cat}$ ~100-fold. Replacement of LC/E-Thr159 and LC/E-Thr208 with Ala lowered the $k_{cat}$ ~30- and ~20-fold relative to WT-LC/E, respectively (Table 1). Replacement of LC/E-Phe191, Thr159, or Thr208 with Arg lowered the $k_{cat}$ by ~2000-, ~1500-, and ~8000-fold, respectively, showing that in addition to hydrophobicity, the size and/or charge within the pocket influenced interactions between Ile181, the P1’ residue, and the S1’ pocket residues (Table 1). Mutations at LC/E-Phe191, Thr159, or Thr208 did not affect the $K_m$ value for SNAP25 cleavage. In contrast, replacement of Tyr354 and Tyr356, which are located near Thr159, Thr208, and Phe191 in the S1’ pocket of LC/E, did not affect the catalytic activity of LC/E, indicating that Tyr354 and Tyr356 do not contribute to S1’ pocket formation and P1’ site recognition.
FIGURE 2. Recognition of SNAP25 by specific pockets in LC/E.

A, co-structure of LC/E-SNAP25 was modeled using SWISS-MODEL and PyMol programs, where the substrate recognition and binding pockets S1', S2, S3, and the B region of LC/E were identified. The P1', P2, P3, and B sites, which are important in substrate recognition and binding, were labeled. The structural model was made using complex structure of LC/A-SNAP25 (Protein Data Bank code 1XTG) and LC/E structure (Protein Data Bank code 1T3A) as templates. B, binding and recognition of B region of SNAP25 by B pocket of LC/E. The B pocket was composed of Arg167, Leu166, Asp127, Ala128, Ser129, and Ala130, where Arg167 were predicted to form a salt bridge with Asp172 of SNAP25, and Leu166 was predicted to have hydrophobic interactions with Ile171. Residue Asp127, Ala128, Ser129, and Ala130 form a pocket where the side chains of Asn169 dock into this pocket, and the Gly168 sits on the edge of the pocket.

C, recognition of the SNAP25 P2 and P3 residues by the S2 and S3 pockets of LC/E, respectively. The basic S2 pocket contained a positively charged residue Lys224 that interacts with P2 Asp179, forming a salt bridge. The S3 pocket plays a dual role in both substrate recognition and catalysis. The hydrophobic S3 pocket was composed of Ile164, Ile164, and Ile164. Hydrophobic interactions between Phe191 and Ile181 as well as the steric interaction of the pocket were predicted to contribute to P1' site recognition.

D, recognition of the SNAP25 P1' residue by the S1' pocket of LC/E. The S1' pocket was composed of residues Phe191, Thr199, and Thr208. Hydrophobic interactions between Phe191 and Ile181 as well as the steric interaction of the pocket were predicted to contribute to P1' site recognition.
The loss of activity by mutation at Phe191 could be complemented more efficiently than WT-LC/A (Table 2). This indicated that respectively, whereas LC/E(F191A) cleaved SNAP25(I198F) WT-SNAP25 at was tested. LC/E(K224A) and LC/E(K224R) cleaved LC/E S1 respectively, whereas LC/E(F191A) cleaved SNAP25(I198F) more efficiently than WT-LC/A (Table 2). This indicated that the loss of activity by mutation at Phe191 could be complemented by converting P1’ Ile181 to Phe and suggested that Phe191 played a direct role in P1’ recognition. In addition, LC/E(T159A) and LC/E(T208A) cleaved SNAP25(I181F) at rates similar to WT-LC/E (Table 2). Together, these data implicate Phe191, Thr159, and Thr208 as components of the S1’ pocket that contribute to recognition of the P1’ residue of SNAP25.

S2 Pockets Specifically Recognize the P2 Residue of SNAP25, Asp179—In addition to the P1’ residue, the P2 residue of SNAP25, Asp179, contributed to SNAP25 cleavage by LC/E (19). Previous studies showed that mutation of Asp179 to Ala or Val lowered the $k_{cat}$ of LC/E cleavage of SNAP25 by ~70- and ~300-fold, respectively (19). Molecular modeling of SNAP25 within the LC/E structure (using LC/A-SNAP25 co-crystal structure as a template) identified an S2 pocket opposite the S1’ pocket in the AS cavity of LC/E; where Lys224 was predicted to form a salt bridge with Asp179, the P2 residue of SNAP25 (Fig. 2C). Mutation of Lys224 to Ala or Arg reduced the $k_{cat}$ of SNAP25 cleavage by ~100- and ~300-fold, respectively, whereas the $K_m$ value remained unchanged (Table 1). These results support a model where Lys224, the S2 pocket residue, contributes to the proper alignment of the scissile bond for cleavage rather than contributing to substrate affinity.

Association of the S2 pocket with the P2 residue, Asp179, was further assessed by examining the complementation properties of LC/E mutations on the cleavage of SNAP25(D179A). LC/E(K224A) and LC/E(K224R) cleaved SNAP25(D179A) more efficiently or at the same rate as WT-LC/E (Table 2) suggesting that the P2 residue, Asp179, is recognized by the S2 pocket residue Lys224.

Effect of Double Pocket Mutations on SNAP25 Cleavage—The relationship of residues within the S1’ and S2 pockets was assessed by double mutation analysis. A double mutated LC/E derivative at the S1’ and S2 pockets, LC/E(F191A,K224A), was >20,000-fold less active than WT-LC/E. In contrast, a double mutation within the same pocket, (LC/E F191A,T159A) had similar $K_m$ and $k_{cat}$ values as individual mutations within the S1’ pocket (Table 1). Thus, mutations at two individual pockets caused additive effects on the efficiency of SNAP25 cleavage, whereas two mutations within the same pocket had properties that were similar to single mutation within that pocket. These data also indicate that the S2 and S1’ pockets of LC/E play unique roles in SNAP25 recognition and catalysis.

Specificity of SNAP25 Cleavage by LC/E—To address the specificity of single site mutations on LC/E cleavage of SNAP25, two mutations were engineered at residues that were predicted not to interact with SNAP25, LC/E(N247A) and LC/E(E52A). The catalytic properties of these mutated proteins were similar to WT-LC/E (Table 1).

The Affinity of LC/E and Derivatives to SNAP25—A GST pulldown was used to determine the affinity of SNAP25 to LC/E and derivatives. The GST-SNAP25(I181E), an inactive substrate of LC/E, showed the same or slightly higher ability to pull down S1’ mutants, F191A, T159A, T208A, and S2 pocket mutant, K224A, as the WT-LC/E suggesting that the mutation at S1’ and S2 pocket primarily contribute to the substrate catalysis rather than substrate affinity, which was consistent with their unchanged $K_m$ of these mutated proteins (Fig. 4). The double mutations of S1’ and S2 pocket, LC/E(F191A,K224A)
and LC/E(Phe\textsuperscript{191}, Thr\textsuperscript{159}), also showed the same affinity to bind SNAP25 as WT-LC/E suggesting that double mutation in same or different S pockets affected primarily on substrate catalysis instead of substrate affinity (Fig. 4). The ability of GST-SNAP25 to pull down S3 pocket mutations, I\textsubscript{164}A, I\textsubscript{184}A, and the binding pocket mutations, L\textsubscript{166}A, R\textsubscript{167}A, and S\textsubscript{129}A, was about 2–6-fold less efficient than WT-LC/A, which was consistent with their increased $K_m$ values when mutated to these residues (Fig. 4). To further quantify the binding efficiency of LC/E and derivatives to GST-SNAP25, an ELISA was developed to determine the apparent $K_v$ value for WT-LC/E and LC/E(F\textsubscript{191}A), LC/E(I\textsubscript{182}A), and LC/E(L\textsubscript{166}A). The apparent $K_v$ value of WT-LC/E was similar to that of LC/E(F\textsubscript{191}A) but about 3–6-fold smaller than those of LC/E(I\textsubscript{164}A) and LC/E(L\textsubscript{166}A), respectively (data not shown). These results were consistent with the pulldown assays. These data also confirmed that the residues in the substrate recognition pockets had limited effect on substrate affinity, whereas the residues at the B region of LC/E contributed both to the substrate binding and alignment.

### DISCUSSION

The specificity of BoNTs for their SNARE protein substrate was proposed to be due to the ability of each BoNT serotype to recognize and interact with nine-residue SNARE secondary recognition (SSR) motif (23). The SSR motif was conserved across species, which implicated an essential role in SNARE function (24). Peptides corresponding to the nine-residue SSR motifs in VAMP inhibit BoNT serotype B and G function (25). The role of SSR in BoNT/A and /E recognition was less apparent because deletion of the SSR motif within SNAP25 remained a substrate for LC/A and /E cleavage (26). Recent studies have made progress toward understanding how LC/A recognizes and cleaves SNAP25. The structure of a noncatalytic LC/A complexed with SNAP25-(146–204) provided a model for BoNT/A-SNAP25 interactions and defined an H\textsubscript{9251} exosite and a H\textsubscript{9252} exosite as accessory sites for catalysis, although limited information was obtained regarding the exact binding mode of SNAP25 to the LC (16, 27). The co-crystal of the inhibitor L-ArgHX to LC/A showed that the carbonyl- and N-hydroxyl groups of L-ArgHX formed a bi-dentate ligand with the zinc ion, and the guanidinium group of the Arg side chain formed a salt bridge with the carbonyl R group of Asp\textsuperscript{370}, suggesting that the inhibitor-bound structure mimicked a catalytic intermediate for the P1 residue, Arg\textsuperscript{198}, at the active site. This provided a starting point to identify and characterize the role of S1\textsuperscript{1} pocket in substrate recognition (27). Recent studies revealed the molecular mechanism of LC/A recognition and cleavage of SNAP25 that involve several steps in SNAP25 recognition and active site organization (17).

In BoNT/A, initial interactions with SNAP25 involve a discontiguous surface between residues within the belt region of LC/A and the B region residues of SNAP25. The “Velcro-like”

### TABLE 2

| LC/E derivatives/SNAP25 derivatives | 50% cleavage of SNAP25 | WT/LC/E derivatives | 50% cleavage of SNAP25(I181F) | WT/LC/A derivative |
|------------------------------------|------------------------|----------------------|---------------------------|-------------------|
| WT                                | 0.5                    | 1                    | 150                      | 1                 |
| F191A                             | 40                     | 80                   | 2000                     | 2                 |
| F191R                             | 1000                   | 2000                 | 1000                     | 6.7               |
| T159A                             | 15                     | 30                   | 150                      | 1                 |
| T208A                             | 10                     | 20                   | 300                      | 2                 |

| LC/E derivatives/SNAP25 derivatives | 50% cleavage of SNAP25 | WT/LC/E derivatives | 50% cleavage of SNAP25(D179A) | WT/LC/A derivative |
|------------------------------------|------------------------|----------------------|-------------------------------|-------------------|
| WT                                | 0.5                    | 1                    | 35                           | 1                 |
| K224A                             | 50                     | 100                  | 200                          | 0.6               |
| K224R                             | 100                    | 200                  | 40                           | 1.1               |

### FIGURE 4

Binding of WT-LC/E and LC/E derivatives to SNAP25 (I181E).

The affinities of LC/E and LC/E derivatives were determined by a GST pull-down assay. Six μM of GST-SNAP25(I181E) (a noncleavable substrate for LC/E) was incubated with 2 μM WT-LC/E or LC/E derivatives for 30 min and then incubated with glutathione-Sepharose bead for an additional 30 min. Proteins in the pulldown were detected by Western blotting (WB) using LC/E or GST antibody. The GST-SNAP25, WT-LC/E, or LC/E derivatives alone were used as positive and negative control. A is a representative experiment showing the binding of WT-LC/E, LC/E(F191A), and LC/E(I166A) to SNAP25(I181E) and controls. B is the quantification of A results for all mutants. Data represent the mean (± S.D.) of five independent experiments.
The binding of SNAP25 to LC/A aligns the P5 residue, Asp193, with Arg177, a S5 pocket residue, to form a salt bridge at the periphery of one side of the active site. In contrast to LC/A, substrate recognition of LC/E involved a relatively short B region of SNAP25 and the B pocket of LC/E. The interactions involved substrate recognition and included a hydrophobic interaction between Leu166 of LC/E and Ile178 of SNAP25, a salt bridge between Arg167 of LC/E and Asp172 of SNAP25, and interactions between the pocket of LC/E, composed of Asp127, Ala128, Ser129, and Ala130 with Asn169 and Gly168 of SNAP25, where the side chain of Asn169 docked into the pocket and Gly168 was located at the edge of the pocket. Mutations to LC/E at Leu166 and Arg167 affected both the $K_m$ and $k_{cat}$ values, suggesting that the binding of SNAP25 to the B region of LC/E contributes not only the substrate affinity but also aligns LC/E to facilitate recognition of SNAP25 at the active site of LC/E.

Outwardly, the extended recognition of SNAP25 for efficient cleavage by LC/A and LC/E is analogous to the association of SNAP25/syntaxin with VAMP during the fusion of neurotransmitter vesicles with the plasma membrane (reviewed in Ref. 28). In the case of vesicle SNARE and target SNARE recognition, the N termini of the proteins appear to initiate recognition and continue to associate by a “Zipper”-like mechanism to contribute to the movement of the neurotransmitter vesicle toward the plasma membrane for fusion (29, 30).

In LC/A, recognition of SNAP25 is facilitated by recognition of the P4 site by the S4 pocket of LC/A, which packs the loop250 to loop370 of LC/A, broadens the active site cavity of LC/A, and subsequently enables the recognition of P1 site by the S1’ pocket. In contrast to LC/A, there is no single residue that plays the same role as Lys201 of SNAP25 in LC/E substrate recognition, because mutations at residues 182–186 of SNAP25 did not show significant effect on substrate hydrolysis. However, deletion of residues 182–186 of SNAP25 completely inhibited the ability of LC/E to cleave truncated SNAP25 substrates (26), suggesting that a main chain interaction of a P4 residue (residues 182–186) in SNAP25 may contribute to substrate binding and/or alignment by LC/E. Similar to the S4’-P4’ interaction in LC/A, the interactions between LC/E and residues 182–186 of SNAP25 may change the configuration of the active site cavity to allow the recognition of P1’ and P2 site by S1’ and S2 pockets of LC/E and to facilitate cleavage of SNAP25.

The S1’ pocket of LC/E is formed by Phe191, Thr159, and Thr208. The hydrophobic interaction between LC/E (Phe191) and Ile181 of SNAP25 plays a role in P1’ residue recognition, because mutation of Phe191 to Ala reduced the substrate hydrolysis efficiency. In addition, the physical size of the S1’ pocket may play an important role in the docking of the P1’ residue, because replacement of residues that formed the S1’ pocket, such as Phe191, Thr159, and Thr208 with Arg, had a dramatic effect on substrate hydrolysis. The docking of P1’ residue, Ile181, facilitated proper alignment into the Zn$^{2+}$-binding domain for scissile bond cleavage. Overall, the S1’ pocket residues of BoNT/A and /E correlate in size and hydrophobicity with the cognate P1’ residue of SNAP25 implying the role for S1’ pocket in SNAP25 recognition.

FIGURE 5. Multiple pocket recognition and cleavage of SNAP25 by LC/E. A, cytosolic LC/E initially interacts with the B site of SNAP25 at the plasma membrane of neurons through the B region of LC/E (A and B). This interaction ends at the hydrophobic S3 pocket, which specifically recognizes P3, Ile178, through hydrophobic interactions (C). The binding of SNAP25 to LC/E is facilitated through P1’ and P2 site interactions with the S1’ and S2 pockets of LC/E (D). Proper docking of P1’ and P2 sites into S1’ and S2 pockets initiates a final alignment that precisely aligns the substrate scissile bond for cleavage (E). After cleavage, the products of cleavage leave the LC/E targets to another SNAP25 molecule starting another cycle of substrate recognition and cleavage (F). B, schematic representation of the residues of SNAP25 that interact with the B region and S pockets of LC/E and to facilitate cleavage of SNAP25.
Recognition of SNAP25 by BoNT/E

Although sharing a common theme, substrate recognition by LC/A and LC/E follows unique steps to provide for cleavage site specificity. In LC/E (Fig. 5) initial substrate binding is distanced from the active site, where SNAP25 recognition by LC/E is accomplished by binding to the B site of SNAP25. The initial binding of SNAP25 to LC/E ends at the recognition of the P3 residue of SNAP25 to the S3 pocket of LC/E, which facilitates the alignment of the P1’ and P2 sites of SNAP25 for S1’ and S2 pocket binding. The S1’ pocket is formed by Phe\textsuperscript{191}, Thr\textsuperscript{159}, and Thr\textsuperscript{208}, where the hydrophobic interaction between Phe\textsuperscript{191} and Ile\textsuperscript{181} as well as the physical size of the S1’ pocket play an important role on P1’ site recognition and docking. The basic S2 pocket contains a positively charged residue, Lys\textsuperscript{224}, which specifically recognizes P2 Asp\textsuperscript{179} through a salt bridge. The proper docking of these two sites into the active site pocket of LC/E optimally aligns the scissile bond into the cleavage site of LC/E. Cleavage of the scissile peptide bond appears to follow a general base-catalyzed mechanism. Arg\textsuperscript{362} and Tyr\textsuperscript{365} interact with the carbonyl oxygens of the P1 and P1’ residues of SNAP25, respectively, and stabilize the oxyanion in the transition state (31, 32).

Effective therapies against botulism are limited, due in part to a lack of understanding of BoNT substrate specificity. Understanding the steps involved in substrate recognition and hydrolysis will provide insight into strategies to design inhibitors that block catalysis.

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