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Engineering Clostridia Neurotoxins with elevated catalytic activity

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ABSTRACT

BoNT/B and TeNT cleave substrate VAMP2 at the same scissile bond, yet these two toxins showed different efficiency on substrate hydrolysis and had different requirements for the recognition of P2′ site of VAMP2, E^{78}. These differences may be due to their different composition of their substrate recognition pockets in the active site. Swapping of LC/T S1′ pocket residue, L^{230}, with the corresponding isoleucine in LC/B increased LC/T activity by ~25 fold, while swapping of LC/B S1′ pocket residue, S^{201}, with the corresponding proline in LC/T increased LC/B activity by ~10 fold. Optimization of both S1 and S1′ pocket residues of LC/T, LC/T ($K^{168}E$, $L^{230}I$) elevated LC/T activity by more than 100-fold. The highly active LC/T derivative engineered in this study has the potential to be used as a more effective tool to study mechanisms of exocytosis in central neuron. The LC/B derivative with elevated activity has the potential to be developed into novel therapy to minimize the impact of immunoresistance during BoNT/B therapy.

1. Introduction

The Clostridia Neurotoxins (CNTs) are among the most potent protein toxins for humans. These include the Botulinum Neurotoxins (BoNT) that are responsible for botulism, and the Tetanus Neurotoxin (TeNT) that causes spastic paralysis. CNTs are 150 kDa dichain proteins with typical **A–B** structure–function properties, where the **B** (binding) domain binds to the surface components on mammalian cell and translocates the **A** (active) domain to an intracellular location (Montecucco and Schiavo, 1994). CNTs are organized into three functional domains: an N-terminal catalytic domain (light chain, LC), an internal translocation domain (heavy chain, HCT), and a C-terminal receptor binding domain (heavy chain, HCR) (Davletov et al., 2005). The CNTs are zinc metalloproteases that cleave SNARE (Soluble NSF Attachment REceptor) proteins, which are known to have the ability to interfere with synaptic vesicle fusion to the plasma membrane and ultimately block neurotransmitter release in nerve cells (Montecucco and Schiavo, 1994, 1993). Mammalian neuronal exocytosis is driven by the formation of protein complexes between the vesicle SNARE protein, VAMP2, and the plasma membrane SNAREs, SNAP25 and syntaxin 1a (Brunger, 2005). There are seven BoNT serotypes (termed A–G) that cleave specific residues on one of the three SNARE proteins: BoNT serotypes B, D, F, G, and TeNT cleave VAMP2, BoNT serotypes A and E cleave SNAP25, and BoNT serotype C cleaves SNAP25 and syntaxin 1a (Montecucco and Schiavo, 1993; Schiavo et al., 1992, 1994a, 1994b).

BoNTs are the most widely used protein therapeutic agents. BoNT/A was approved by the US FDA to treat strabismus, blepharospasm, and hemificial spasm and then for cervical dystonia, cosmetic use, glabellar facial lines and axillary hyperhidrosis as early as 1989. The efficacy of

Abbreviations: CNTs, Clostridia Neurotoxins; BoNT/B, Botulinum Neurotoxin; TeNT, Tetanus Neurotoxin; LC, light chain; VAMP2, vesicle associated membrane protein-2; SNARE, soluble NSF attachment receptor.

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BoNT/A in treating dystonia and other disorders related to involuntary skeletal muscle activity, coupled with the satisfactory safety profile, has prompted empirical/off-label use in a variety of ophthalmological, gastrointestinal, urological, orthopedic, dermatological, secretory, and painful disorders (Glogau, 2002; Benedetto, 2004; Ascher and Rossi, 2004; Cheng et al., 2006; Abrams and Hallett, 2013; Naumann et al., 2013; Hallett et al., 2013; Esquenazi et al., 2013; Chancellor et al., 2013). On December 11, 2000, a Botulinum Neurotoxin serotype B product (MYOBLOC™) was approved by the FDA in the United States as a treatment for patients with cervical dystonia to reduce the severity of abnormal head position and neck pain associated with cervical dystonia (Figgitt and Noble, 2002; Brashear, 2001).

The therapeutic benefits of BoNT for treatment of conditions associated with involuntary muscle spasm and contractions, cosmetic use, and other applications are transient and repeated injections are necessary. In some patients, BoNT could elicits neutralizing antibodies against the corresponding toxin, thus reducing the beneficial effects or rendering the patient completely unresponsive to further treatment (Goschel et al., 1997). The exact percentage of patients who may develop immunoresistance to BoNT treatment is unknown, but it is commonly believed that there are fewer patients who develop blocking antibodies when treated with BoNT/A than with BoNT/B (Atassi, 2004; Jankovic, 2006). This is probably due to the use of lower dosages of BoNT/A complex than BoNT/B complex (Brashear et al., 1999). The development of blocking antibodies is also more common in patients who receive treatment of cervical dystonia or spasticity, which requires larger dosages and periodic administration of toxin; on the other hand it is less common in patients who are treated for laryngeal dystonia, blepharospasm or cosmetic use, all of which require smaller dosages for treatments (Atassi, 2004; Swope and Barbano, 2008; Comella, 2008). Thus reducing the treatment dosage may help to reduce the development of immunoresistance.

In current study, comparative characterization of the substrate recognition pockets in the active sites of LC/B and LC/T was performed, producing results that lead to optimization of the substrate recognition of LC/B and LC/T and formation of LC derivatives with elevated catalytic activity. The highly active LC/T derivative engineered in this study has the potential to be used as a more effective tool to study mechanisms of exocytosis in central neuron. The LC/B derivative with elevated activity has the potential to be developed into novel therapy to minimize the immunoresistance issue of BoNT/B therapy.

2. Experimental

2.1. Plasmid construction for protein expression

Plasmids for the expression of LC/T (1–436) and VAMP2 (1–97) and subsequent protein expression and purification were performed as previously described (Chen and Barbieri, 2007; Chen et al., 2007; Chen, Wan). Site directed mutagenesis of pLC/T and pVAMP2 were performed using QuickChange (Stratagene) protocols as previously described (Chen and Barbieri, 2007; Chen et al., 2007). Plasmids were sequenced to confirm the mutation and that additional mutations were not present within the ORFs. Mutated proteins were produced and purified as described above (Chen and Barbieri, 2007; Chen et al., 2007, 2008; Chen and Wan).

2.2. Linear velocity and kinetic constant determinations for VAMP2 cleavage by LC/B and LC/T

Linear velocity reactions (10 μl) were performed as previously described (Chen and Barbieri, 2007; Chen et al., 2007, 2008). VAMP2 proteins (5 μM) were incubated with varying concentrations of LC/T, LC/B or derivatives in 10 mM Tris–HCl (pH 7.6) with 20 mM NaCl at 37 °C for 10 min. Reactions were stopped by adding SDS-PAGE buffer, and VAMP2 and cleavage product were resolved by SDS-PAGE. The amount of VAMP2 cleaved was determined by densitometry. K_m and k_{cat} determinations were performed with the same assay where VAMP2 concentrations were adjusted to between 1 and 300 μM to achieve ~10% cleavage by LC/T and derivatives. Reaction velocity versus substrate concentration was fit to the Michaelis–Menten equation and kinetic constants were derived using the GraphPad Program (San Diego, CA). At least five independent assays were performed to determine the kinetic constants for each protein.

2.3. Compensatory assay

Effect of compensatory mutations within LC/T on the cleavage of VAMP2 and mutated forms of VAMP2 was performed as previously described with modification (Chen et al., 2007). Briefly, 5 μM VAMP2 or VAMP2 derivatives were incubated with LC/T or derivatives and uncleaved and cleaved VAMP2 were resolved by SDS-PAGE. The amount of wild type LC/T or derivatives in the reaction were plotted verses % cleavage and the amount of LC required to cleave 50% of VAMP2 or VAMP2 derivative were calculated.

2.4. LC crystallization and structure determination

LC/T derivative was crystallized by the hanging drop vapor diffusion method. The concentrated protein solution was in a buffer of 10 mM Tris, 20 mM NaCl PH 7.9 and at a concentration of 7.5 mg/ml. Each drop contained 1 μl protein solution and 1ul well buffer, which was composed of 250 mM $Mg(NO_3)_2$ and 15% PEG 3350. Crystals were grown at 16 °C for 4–5 days until maturation. For data collection, crystals were harvested and cryoprotected in well buffer plus 20% glycerol. Data was collected at 100K on Rigaku MicroMaxTM-007HF x-ray machine and processed using iMOSFLM (Powell, 1999). The crystals belonged to the monoclinic group C222 with a cell parameter of $a = 105.38$ Å, $b = 176.83$ Å, $c = 57.36$ Å and diffracted to 2.6 Å. There was one molecule per asymmetric unit (Table 1). The structure of LC/T ($E^{168}E$, $L^{230}I$) was solved by molecular replacement using the PHASER module in the CCP4i suite of programs with LC/T (PDB ID: 1Z7H) as search model (Molecular replacement. Pr, 2008). The subsequent