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Camelid single domain antibodies (VHHs) as neuronal cell intrabody binding agents and inhibitors of *Clostridium botulinum* neurotoxin (BoNT) proteases

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ABSTRACT

Botulinum neurotoxins (BoNTs) function by delivering a protease to neuronal cells that cleave SNARE proteins and inactivate neurotransmitter exocytosis. Small (14 kDa) binding domains specific for the protease of BoNT serotypes A or B were selected from libraries of heavy chain only antibody domains (VHHs or nanobodies) cloned from immunized alpacas. Several VHHs bind the BoNT proteases with high affinity (K_D near 1 nM) and include potent inhibitors of BoNT/A protease activity (K_i near 1 nM). The VHHs retain their binding specificity and inhibitory functions when expressed within mammalian neuronal cells as intrabodies. A VHH inhibitor of BoNT/A protease was able to protect neuronal cell SNAP25 protein from cleavage following intoxication with BoNT/A holotoxin. These results demonstrate that VHH domains have potential as components of therapeutic agents for reversal of botulism intoxication.

1. Introduction

Botulinum neurotoxins (BoNT) act on the peripheral nervous system to inhibit the release of acetylcholine from pre-synaptic nerve terminals at the neuromuscular junction, causing flaccid paralysis. The holotoxins are about 150 kDa consisting of three domains that are separately responsible for neuron receptor binding, translocation and catalysis. Once internalized into motor neurons, the mode of action of these toxins involves proteolytic cleavage of SNARE proteins that play key roles in neurotransmitter release. SNARE proteolysis is mediated by the 50 kDa metalloproteinase domain of the toxin, also called the light chain (Lc). The proteases for several of the seven known botulinum serotypes, notably BoNT/A and BoNT/B, are remarkably stable once in nerve cell cytosol and intoxication can last for several months before normal function returns. Because of its extreme potency, persistence and relative ease of production, BoNTs are considered among the most serious (CDC Category A) of the current bioterrorism threats.

Antitoxin agents are available that can prevent BoNT intoxication if administered prior to the development of major symptoms. Currently there is no antidote that can reverse the symptoms of intoxication once they have occurred. As a result, botulism patients must be maintained on respirators, often for many months, before motor

Abbreviations: HcAbs, heavy chain only antibodies; VHH, heavy chain only V_H domain; BoNT, botulinum neurotoxin; Lc, light chain; SNARE, soluble NSF attachment protein; GST, glutathione-S-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; SBP, streptavidin binding protein; CMV, cytomegalovirus.

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function eventually returns. Reversal of nerve intoxication must involve inhibition and/or elimination of the protease from intoxicated neurons. Several research teams are working to develop small molecule drugs that inhibit BoNT Lc proteases and reverse intoxication. Biomolecules that bind BoNT proteases with high affinity, particularly those that inhibit its enzyme activity, could also have value in the development of botulism therapeutic agents.

Camelids such as camels, llamas and alpacas produce a class of heavy chain only antibodies (HcAbs) that lack a light chain and thus bind antigens entirely through their V_H domain. The V_H domain from HcAbs is called the VHH domain. Recombinant VHHs (also called nanobodies) generally express to high levels in a soluble and functional form within microbial host systems (Arbabi Ghahroudi et al., 1997), probably because the domain naturally folds and functions independent of V_L interactions. VHHs also generally have improved hydrodynamic properties and stability as compared to conventional recombinant antibodies (Dumoulin et al., 2002; van der Linden et al., 1999). Furthermore, VHHs appear to have an improved ability to bind enzyme active site pockets leading to biomolecular inhibitors of catalytic function (Lauwereys et al., 1998). Evidence is growing that VHHs are often functional as intracellular antibodies termed "intrabodies" when expressed in the reducing environment of eukaryotic cytosol (Jobling et al., 2003; Verheesen et al., 2006). The unique features of VHHs have begun to be exploited for therapeutic and other possible commercial applications (Gibbs, 2005). Here we report the identification and characterization of recombinant VHHs, prepared from alpacas immunized with BoNT/A and BoNT/B proteases, which bind the BoNT proteases with high affinity. Some of the BoNT VHHs potently inhibit BoNT protease function and remain functional when expressed within neurons.

2. Materials and methods

2.1. Preparation of recombinant BoNT/A and BoNT/B proteases

The coding sequences of BoNT/A protease (A-Lc), encoding amino acids 1–448 of BoNT/A holotoxin, and BoNT/B protease (B-Lc), amino acids 1–442 of BoNT/B holotoxin, were synthesized employing codons optimized for expression within *Escherichia coli* (Midland Certified Reagent Company, Inc.). These DNAs were cloned into pET14b (Novagen) in frame with the hexahistidine coding sequence at the amino terminus. The plasmids were transformed into Rosetta-gami *E. coli* cells (Novagen) and induced for expression as recommended by the manufacturer. The yield of soluble protein was significantly improved by culturing the cells overnight at 15 °C following induction with IPTG. Recombinant proteases were purified from the soluble cell extract by immobilized metal affinity chromatography using standard procedures. Fusions of A-Lc and B-Lc were also produced to glutathione-*S*-transferase (GST). For this, the Lc coding regions were introduced into a derivative of the pGEX vector (GE Healthcare) in frame with GST coding DNA. The GST fusion proteins were purified by standard glutathione affinity methods. The recombinant BoNT Lc proteins were >95% pure as estimated by SDS-PAGE and remained soluble and enzymatically active for several months at 4 °C or for at least several years when stored at >1 mg/ml in 50% glycerol at −20 °C.

2.2. Immunization of alpacas with BoNT/A and BoNT/B proteases

Purified recombinant A-Lc was used to immunize two alpacas essentially as previously described (Maass et al., 2007). Two alpacas were purchased locally and maintained in pasture. Alpacas were given four immunizations of recombinant A-Lc at two-week intervals. Following the final boost, B cells were harvested (see below). Twelve months later, the same alpacas were similarly immunized with recombinant B-Lc. The final immunization prior to B cell harvest contained both B-Lc and A-Lc in an effort to boost cross-reactive epitopes.

2.3. Identification of VHHs that bind to BoNT proteases

Two VHH-display libraries were produced for this work. The first library was prepared from B cells obtained from the alpacas following immunization with A-Lc. Procedures for alpaca VHH identification from this library were virtually identical to those we previously reported for another antigen using the HQ2-2 vector (Maass et al., 2007).

The second VHH-display library was prepared from B cells obtained from the same alpacas after immunization with B-Lc and boosting for A-Lc. This library was prepared in the JSC vector generally as described by Sepulveda and Shoemaker (2008). PCR amplification employed the improved primer design as we reported in Maass et al. (2007). The single forward primer used was AlpVh-F1 (GATCGCCGGCCAGKTGCAGCTCGTGGAGTCNGGNGG) and the two reverse primers were AlpVHH-R1 (GATCACTAGTGGGGTCTTCGCTGTGGTGCG) and AlpVHH-R2 (GATCACTAGTTTGTGGTTTTGGTGTCTTGGG). The reverse primers prime mRNA for the short and long hinge VHH coding regions, respectively. Amplification was typically performed with 35 cycles of 95 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min. Amplified VHH cDNA (0.4 kb) was purified, digested with NgoMIV and SpeI and ligated into the similarly digested JSC vector. Using high efficiency *E. coli* transformation methods, more than 10^6 independent clones were obtained and pooled to make both VHH-display libraries. At least 18 random clones were picked and characterized by DNA fingerprinting and >90% had inserts of the proper size.

Panning for VHH-displayed phage that binds to A-Lc or B-Lc was done mostly as described previously (Maass et al., 2007) using target protein coated onto single wells of a 12-well plate. Diminishing concentrations of target protein (from 20 to 0.01 μg/ml), reduced incubation times and longer washing times were employed in subsequent panning cycles in an effort to select for phage with higher affinity to the target protein. Bound phage was recovered from wells in two steps. First, 500 μl of a fresh overnight culture of ER2738 *E. coli* cells were added to the well for 15 min at 37 °C and removed. In the second step, phage remaining on the plastic following the *E. coli* infection was subjected to an additional elution in 0.2 M glycine pH 2.2