Expression and Purification of the Light Chain of Botulinum Neurotoxin A: A Single Mutation Abolishes Its Cleavage of SNAP-25 and Neurotoxicity after Reconstitution with the Heavy Chain†

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ABSTRACT: Botulinum neurotoxin type A (BoNT/A) selectively and irreversibly inhibits acetylcholine release from peripheral nerve endings. While the toxin's heavy (H) chain contributes to neuronal binding and internalization, its light (L) chain is a Zn^{2+}-dependent endoprotease that intracellularly cleaves synaptosomal-associated protein of M_r = 25 kDa (SNAP-25). For research and clinical exploitation of this uniquely-acting neurotoxin, recombinant wild-type L chain was produced together with a mutant in which His^{227} in the Zn^{2+}-binding motif was substituted by Tyr. The PCR-amplified wild-type and mutant L chain genes were cloned, fused to the gene for maltose-binding protein, and expressed at high levels in *Escherichia coli.* The soluble fusion proteins were purified using amylose affinity chromatography, and, after factor X_a cleavage, the free L chains were isolated. The wild-type was shown to proteolyze SNAP-25 at a rate approaching that of the native chain while the mutant was inactive. Reconstitution of the pure wild-type L chain with native homogeneous H chain yielded a disulfide-linked dichain form that inhibited neuromuscular transmission *in vitro* and produced the symptoms of botulism *in vivo.* After reconstitution with the H chain, the Tyr^{227} mutant L chain failed to show any neuroparalytic activity in either of these assays. This methodology allows, for the first time, routine preparation of recombinant forms of the L chain that are needed to decipher the molecular details of its interaction with substrate and, thereby, assist the design of effective inhibitors. Moreover, the generation herein of a nontoxic dichain that retains ability to bind, internalize, and translocate to the cytosol of motor nerve terminals, by reconstituting inactive L chain with its native partner, could provide a targeted vehicle for the transport of drugs into peripheral cholinergic neurons.

Seven antigenically distinct forms (types A-G) of botulinum neurotoxin (BoNT)[1] are produced by the gram-positive, spore-forming bacterium *Clostridium botulinum.* These preferentially inhibit the release of acetylcholine from peripheral nerve endings and result in neuromuscular paralysis in humans and animals (Tacket & Rogawski, 1989). BoNT is synthesized as a single polypeptide chain and subsequently cleaved by endogenous proteases to yield the fully active dichain form in which a 50 kDa light (L) chain and a 100 kDa heavy (H) chain are linked by a disulfide bond [reviewed in Dolly (1992)]. The mechanism of action of BoNT has been proposed to be triphasic (Simpson, 1980) involving binding to ectoacceptors located exclusively on peripheral cholinergic nerve endings (Black & Dolly, 1986a), followed by acceptor-mediated internalization and intracellular inhibition of the exocytotic release of transmitter (Black & Dolly, 1986b; de Paiva & Dolly, 1990). The C-terminal half of the H chain is important for toxin binding to the cell surface acceptors because its removal from BoNT/A abolishes the ability to bind at the neuromuscular junction (Poulain et al., 1989). However, the H chain, once isolated from the toxin, is unable to bind avidly to these acceptors because it fails to antagonize the neuromuscular paralyzing action of intact BoNT/A at the mouse nerve−muscle junction (Maisey et al., 1988); the active conformation of the H chain for binding is apparently maintained by its association with L chain (de Paiva et al., 1993b). The subsequent membrane translocation of L chain appears to be mediated by a domain within the N-terminal half of the H chain (Niemann, 1991), via a process involving the interchain disulfide bond (de Paiva et al., 1993b). Only the L chain of BoNT/A is required for the ultimate intracellular inhibition of exocytosis in vertebrates, if membrane binding and internalization are bypassed through permeabilization or liposomal delivery (Dayanithi et al., 1990; de Paiva & Dolly, 1990; Stecher et al., 1989).

More recently, the L chains of all these clostridial neurotoxins have been shown to be Zn^{2+}-dependent proteases, an advance afforded by the cloning of their genes (Binz et al., 1990; Thompson et al., 1990; Whelan et al., 1992a,b). Notably, each L chain contains the Zn^{2+}-binding

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[1] Abbreviations: BoNT/A, B, botulinum neurotoxin type A, B; L and H chain, light and heavy chain of BoNT/A; wt-L chain, recombinant wild-type L chain of BoNT/A; DTT, DL-dithiothreitol; MBP, maltose-binding protein; SNAP-25, synaptosomal-associated protein of M_r = 25 kDa; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; IPTG, isopropyl-β-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LB, Luria broth; MCS, multiple cloning site; GST, glutathione *S*-transferase; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′N′*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TeTx, tetanus toxin.

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domain, HExxH, characteristic of Zn^{2+}-dependent endoproteases, and, importantly, their substrates have been identified as proteins involved in the fundamental process of exocytosis. BoNT/B, D, F, and G, together with tetanus toxin (TeTx), cleave synaptobrevin (Schiavo et al., 1992a), whereas BoNT/A and E act on SNAP-25 (Blasi et al., 1993a; Schiavo et al., 1993) and type C_1 proteolyzes syntaxin 1A/B (Blasi et al., 1993b). Evidence for the neuroparalytic action of BoNT/A being reliant on its endoprotease activity was obtained from the antagonism caused by chelating the Zn^{2+} bound to the L chain (Schiavo et al., 1992c; de Paiva et al., 1993a); also, mutation of the catalytic Glu residue in the latter abolished its cleavage of SNAP-25 (Blasi et al., 1993a). In elegant recent studies of the interactions of recombinant SNAP-25, syntaxin, and synaptobrevin, truncation of SNAP-25 by BoNT/A was shown to reduce the formation of the SDS stable ternary complex (Chapman et al., 1994; Hayashi et al., 1994).

To facilitate further structure/activity studies and clinical applications of BoNT/A, the genes encoding its L chain and a single amino acid mutant thereof were fused with that of maltose-binding protein (MBP) and expressed in a heterologous host. The adequate level of expression achieved herein followed by the purification of proteolytically-active wild-type (wt) L chain provide a basis for deciphering the details of its enzymic action, as well as allowing assessment of the functional roles of the substrate, SNAP-25. Furthermore, production of enzymically-inactive recombinant L chain that can be reconstituted with H chain should allow this preparation to be applied as a cholinergic-specific transporter for selective delivery of drugs into the peripheral nervous system.

EXPERIMENTAL PROCEDURES

Materials. The cloning vector, plasmid pBluescript II SK^+, was purchased from Stratagene (Cambridge, U.K.); the expression vectors, pMal-C2 and pMal-P2, as well as the amylose resin were from New England BioLabs (Hitchin, U.K.). Restriction endonucleases, other DNA-modifying enzymes, and factor X_a (molecular biology grade) were obtained from Promega (Southampton, U.K.). The Sequenase DNA-sequencing kit and ECL system were from Amersham (Amersham, U.K.). BoNT/A was supplied by List Biologicals Inc. (Campbell, CA).

General Recombinant DNA Procedures. DNA isolation, agarose gel electrophoresis, and the transformation of *E. coli* [strains XLI-blue and TG1 grown in Luria broth (LB)] were carried out as described by Sambrook et al., (1989). Restriction endonucleases and other DNA-modifying enzymes were used in accordance with the manufacturer's instructions.

Cloning and Sequencing of BoNT/A L Chain. The DNA sequence encoding L chain of BoNT/A was PCR amplified using, as primers, the synthetic oligonucleotides 5′-AAAGGCCTTTTGTTAATAAACAA-3′ and 5′-GGAATTCTTACTTATTGTATCCTTTA-3′. The latter were designed with specific restriction site sequences to allow unidirectional cloning. The restriction sites *Stu*I and *Eco*RI were added to the 5′ and 3′ ends of the L chain gene fragment, respectively, while a stop code was also introduced at the C-terminus. Chromomosal DNA from *C. botulinum* (strain 63A) served as a template in the following protocol; PCR reactions were performed in a total volume of 100 μL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 50 pmol of each primer, 200 ng of genomic DNA, and 2.5 units of *Taq* polymerase. The reaction mixture was subjected to 35 consecutive cycles consisting of denaturation (1 min at 94 °C), annealing (2 min at 37 °C), and polymerization (2 min at 72 °C). Finally, the reaction was extended for another 5 min at 72 °C. The resultant products were purified by agarose gel electrophoresis and then treated with *Stu*I and *Eco*RI, and cloned into *Sma*I- and *Eco*RI-digested pBluescript II SK^+ to yield pSAL. The cloned L chain gene was verified by sequencing using the dideoxynucleotide termination method of Sanger et al. (1977), according to a protocol recommended for the Sequenase DNA kit. Double-stranded plasmid DNA was first denatured by alkali and then neutralized by spin dialysis (Murphy & Kavanagh, 1988). To generate overlapping sequences, oligonucleotides, where required, were synthesized, and the complete L chain gene was thus analyzed.

Construction of the Expression Recombinant Vector. The L chain gene was cloned into pBluescript II SK^+, cleaved with *Bam*HI and *Sal*I and inserted between the *Bam*HI and *Sal*I sites of the expression vectors pMal-C2 and pMal-P2. These plasmids harbor the *malE* gene encoding MBP that is controlled by a strongly inducible promoter, P_{tac}. pMal-P2 contains a *malE* signal sequence, which directs the fusion protein through the membrane into the periplasmic space. A multiple-cloning site (MCS) within these plasmids permits subcloning of the L chain gene at the 3′ end of *malE*. Importantly, a cleavage sequence specific for factor X_a is present between *malE* and the fused L chain gene.

Generation of BoNT/A L Chain Mutant. A single amino acid (His^{227} to Tyr) mutant of the L chain was generated by "recombinant PCR" (Higuchi, 1990), using the cloned L chain as a template. In "primary" amplification, the synthesized sense and antisense oligonucleotide primers 5′-AACTTATATATGCTGGAC-3′ and 5′GTCCAGCATATATAAGTT-3′ were used together with the two oligonucleotides described earlier. "Secondary" PCR, also utilizing the oligonucleotides used above as for the amplification of the L chain gene, was undertaken to amplify the complete mutant gene. The expression vector for the Tyr^{227} mutant was constructed as for the wt-L chain.

Expression and Purification of the Fusion Proteins. Routinely, each single fresh bacterial colony containing one of the expression constructs was inoculated into LB medium with 100 μg of ampicillin/mL plus 2% (w/v) glucose and grown overnight at 30 °C. The culture was then diluted 1:10 in fresh LB medium with ampicillin at 100 μg/mL; after a 2 h incubation, expression of the fusion protein was induced by addition of 0.1 mM isopropyl β-thiogalactopyranoside (IPTG). Following a further 4 h incubation at 30 °C, cells were collected by centrifugation at 6000*g* for 10 min. The pellets of cells expressing wt or the mutated L chain were then resuspended in column buffer [10 mM Tris, pH 8.0, 200 mM NaCl, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), and 1 mM DL-dithiothreitol (DTT)] containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM benzamidine and lysed by sonication. These lysates were centrifuged at 15 000*g* for 15 min at 4 °C, and the supernatants were applied to an amylose affinity column [2 × 10 cm, 30 mL of resin]; unbound proteins were washed from the resin with column buffer until no proteins were detected in the eluate, by measuring the absorption at 280 nm. The bound MBP−L chain fusion protein was subsequently eluted with the column

buffer supplemented with 10 mM maltose. Those fractions containing fusion protein were pooled and dialyzed against 20 mM Tris-HCl, pH 8.0, supplemented with 150 mM NaCl, 2 mM $CaCl_2$, and 1 mM DTT for 24 h at 4 °C. Each fusion protein was cleaved by addition of factor X_a (enzyme: substrate weight ratio of 1:100) and incubation while dialyzing into the latter Tris-HCl medium over an additional 24 h at 4 °C, yielding MBP and either wt or mutant L chain. Following the inhibition of factor X_a by adding soybean trypsin inhibitor (50 μg/mL), the resultant mixture was then loaded onto a 10 mL amylose column equilibrated with the above noted column buffer. The flow through fractions containing pure L chain were collected and stored at −20 °C, after aliquots were prepared for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Total *E. coli* extract or the purified proteins were solubilized in SDS sample buffer and subjected to PAGE according to Laemmli (1970). When required, proteins were transferred from slab gels to PVDF membrane and Western blotting performed using a rabbit polyclonal anti-L chain antibody (1:500 dilution) characterized elsewhere (Cenci di Bello et al., 1994); reactive bands were visualized using the ECL detection system.

Evaluation of the Proteolytic Activities of Recombinant L Chains toward SNAP-25. Abilities of the wt and mutant L chains to cleave SNAP-25 were compared with that of native BoNT/A, using a quantitative immunological assay with polyclonal antibodies directed toward the C-terminal region (residues 195−206) of SNAP-25 (to be detailed elsewhere). The substrate utilized for this assay was obtained by preparing a glutathione *S*-transferase (GST)−SNAP-25 fusion protein containing a cleavage site for thrombin, expressed using the pGEX-2T vector and purified by affinity chromatography on glutathione agarose (to be detailed elsewhere). The SNAP-25 was then cleaved from the fusion protein using thrombin in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 2.5 mM $CaCl_2$ (Smith & Johnson, 1988) (enzyme:substrate weight ratio 1:100) while the latter was still bound to the gel. The SNAP-25 subsequently eluted with the latter buffer was dialyzed into 100 mM *N*-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 7.5, for 24 h at 4 °C, and the total protein concentration was determined using a modified Bradford's assay (Maisey et al., 1988).

Before assessing their enzymic activities, all recombinant preparations containing L chain or its mutant were dialyzed overnight at 4 °C into 100 mM HEPES, pH 7.5, containing 0.02% Lubrol and 10 μM zinc acetate. BoNT/A, previously reduced with 20 mM DTT for 30 min at 37 °C as well as these dialyzed samples was then diluted to different concentrations in the latter HEPES buffer supplemented with 1 mM DTT. Reaction mixtures comprising 5 μL substrate (giving a final concentration of 8.5 μM) and 20 μL reduced BoNT/A, wt-L chain, or the Tyr^{227} mutant were incubated at 37 °C for 1 h before 25 μL aqueous 2% TFA containing 5 mM ethylenediaminetetraacetic acid (EDTA) was added, which causes immediate inactivation of the proteolytic activity (Foran et al., 1994). Aliquots of each were prepared for SDS−PAGE and Western blotting with the polyclonal SNAP-25 antibody (1:1000 dilution) by adding SDS−PAGE sample buffer and boiling. Antibody reactivity was monitored with biotinylated anti-rabbit immunoglobulin (1:500) followed by streptavidin-biotinylated horseradish peroxidase complex (1:500), using an ECL detection system and quantified by densitometric scanning.

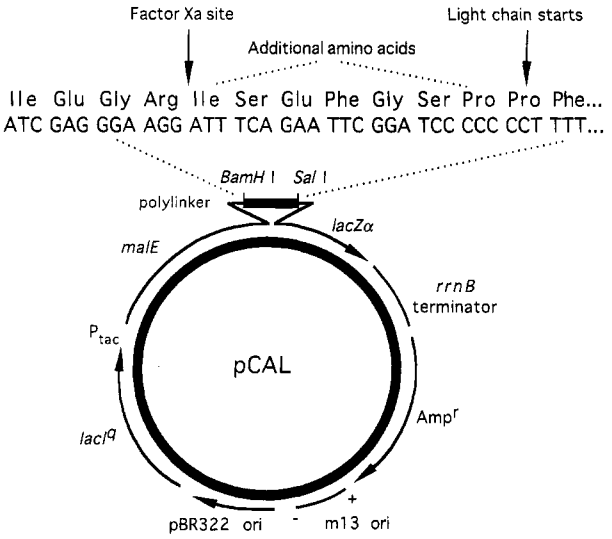

FIGURE 1: Schematic representation of the L chain expression construct, pCAL. This was produced by insertion of the L chain gene between *Bam*HI and *Sal*I restriction sites at the polylinker of the vector pMal-C2. The vector contains the inducible P_{tac} promoter positioned to transcribe *malE*−*lacZα* gene fusion. The *lacI*^{q} gene encodes the lac repressor which turns off transcription from P_{tac} until induction by IPTG. The *rrnB* terminator prevents transcription from interfering with plasmid replication. Amp^r encodes β-lactamase for ampicillin resistance. M13-ori and pBR322ori indicate the origins of DNA replication. The factor X_a cleavage site and L chain start are denoted by arrows.

Reconstitution of Native, Recombinant wt or Tyr^{227} Mutant L Chain with Purified H Chain. The native chains of BoNT/A were isolated and purified using an established chromatographic procedure (Maisey et al., 1988). The resultant purified H chain was added to equimolar amounts of native L chain, recombinant wt, or the Tyr^{227} mutant L chain. Reconstitution was carried out by dialysis of the samples at 4 °C over 4 days against 25 mM Tris, pH 8.0, and 150 mM NaCl supplemented with 50 μM zinc acetate. Formation of the 150 kDa disulfide-linked dichain toxin was assessed by SDS−PAGE under nonreducing conditions.

Assessment of the Mouse Lethality of Reconstituted Toxins and Their Effect on Neuromuscular Transmission. The ability of the reconstituted samples to induce botulism in mice was evaluated following their intraperitoneal injection and expressed as the number of doses, lethal within 4 days, present per milligram of protein (LD_{50}/mg) (Maisey et al., 1988). Excised mouse phrenic nerve-hemidiaphragms (Balb/C; 20−25 g) were bathed in aerated Krebs−Ringer medium, and muscle twitch was evoked by supramaximal stimulation of the phrenic nerve and measured using a force-displacement transducer (de Paiva et al., 1993b).

RESULTS

Cloning of BoNT/A L Chain and Construction of Expression Vectors for the MBP−wt and Mutant L Chain Fusion Proteins. A DNA fragment encoding the L chain of BoNT/A was amplified by PCR with sense and antisense primers annealed to its beginning and end; specific restriction sites, *Stu*I and *Eco*RI, for unidirectional cloning and a stop codon were introduced into the amplified gene. This was then cloned into *Sma*I- and *Eco*RI-digested plasmid pBluescript II SK$^+$ to form pSAL. The identity of the entire nucleotide sequence of the cloned gene was verified by double-stranded plasmid DNA sequencing and found to be identical to that

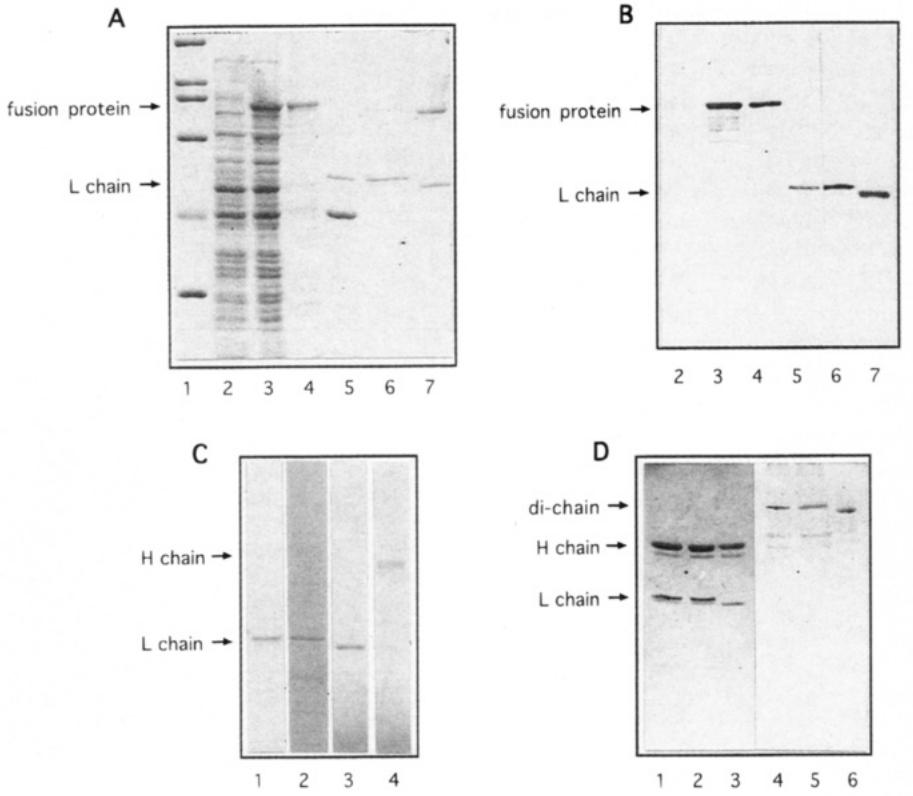

FIGURE 2: Electrophoretic analysis of the expression and purification of the recombinant L chains, isolated native L chain, and their reconstitution with H chain. SDS−PAGE was performed on 9% acrylamide gels under reducing conditions except where specified. In panels A, C, and D protein was visualized by staining with Coomassie Blue R-220. (A) Lane 1, molecular weight standards (205, 116, 97.4, 66, 45, and 29 kDa); lane 2, total protein from *E. coli* cells bearing the expression construct pCAL grown in the absence of IPTG; lane 3, same as lane 2 but the sample was taken 4 h after IPTG induction; lane 4, affinity-purified fusion protein; lane 5, as lane 4 but digested with factor X_a; lane 6, isolated wt-L chain; lane 7, reduced native BoNT/A. (B) Western blotting of panel A, lanes 2 −7, with a polyclonal antibody raised against the L chain of BoNT/A, following SDS−PAGE and protein transfer to PVDF membrane. Antibody binding was detected using an anti-rabbit alkaline phosphatase-conjugated immunoglobulin and developed as described before (Cenci di Bello et al., 1994). Note that a somewhat larger M_r was obtained for the recombinant L chains relative to that for native L chain, due to the extra residues present at the N- and C-termini. (C) Lane 1, wt-L chain; lane 2, Tyr^{227} mutant L chain; lane 3, Isolated native L chain; lane 4, native H chain of BoNT/A. (D) Electrophoresis under reducing (lanes 1−3) and nonreducing (4−6) conditions. Lanes 1 and 4, expressed Tyr^{227} mutant L chain and native H chain following their reconstitution by removal of DTT and urea to reform the dichain protein; lanes 2 and 5, reconstitution of wt-L chain with native H chain; lanes 3 and 6, reconstitution of native L and H chain. Note that a minor faster migrating component can be seen in the H chain preparation and, also, the electrophoretic mobilities of both bands are different under reducing and nonreducing conditions, as reported previously (de Paiva et al., 1993b).

of the authentic L chain [residues 1−448 as published by Binz et al. (1990)].

To achieve an adequate level of expression, the cloned gene was restricted with *Bam*HI and *Sal*I from the plasmid pSAL and inserted into the expression vector pMal-C2 between the same restriction sites in the MCS to form pCAL (Figure 1). This clone was screened by restriction digestion, and subsequent agarose gel electrophoresis revealed the presence of a fragment 1.3 kb in size. DNA sequence analysis confirmed its correct orientation when compared to the sequence of L chain. Sequencing also revealed that the 5′ end of the L chain gene was fused to the MCS and factor X_a cleavage site via a short sequence encoding seven additional amino acids (Figure 1). Moreover, the position of this site showed the L chain gene to be in a proper reading frame with the *malE* gene; the whole construct was controlled by the strongly inducible promoter P_{tac} which can be turned on by IPTG. As for the wt-L chain, the sequence of the mutant L chain made by recombinant PCR (see later) was verified. The Tyr^{227} mutant expression vector was constructed in the same way as for the wt adduct.

Expression and Purification of Fusion Proteins and Recombinant L Chains. Initially, small (10 mL) cultures of the recombinant clone-containing plasmid pCAL were induced in *E. coli* with IPTG and aliquots of the solubilized cells analysed by SDS−PAGE, thereby revealing the presence of a major band with M_r = 90 kDa (Figure 2A). Its size is consistent with that expected of a fusion protein composed of MBP (40 kDa) and BoNT/A L chain (50 kDa). When compared with noninduced cultures, those cells exposed to IPTG contained very much larger amounts of this fusion protein (Figure 2A). Such expression in induced-cell extracts was also confirmed by Western blotting with a polyclonal anti-L chain antibody (Figure 2B). In addition to the predominant fusion protein, this antibody, shown before to be specific for the L chain of BoNT/A (Cenci di Bello et al., 1994), recognized a number of very faint bands of lower M_r, suggesting the occurrence of limited enzymatic degradation in these cells. Despite the inability of protease inhibitors (1 mM PMSF and 10 mM benzamidine) to eliminate this breakdown (data not shown), the final yield of intact fusion protein remained sufficient (see below).

To obtain the expressed L chain protein and its mutant, two different constructs were initially evaluated. The first (pMal-P2), which was abandoned and, thus, has not been detailed in this paper, encoded an MBP signal peptide responsible for the transport of the fusion protein into the

periplasmic space. While free MBP was present in large quantities in the periplasm of *E. coli* containing this plasmid, the MBP−L chain fusion product was hardly detectable in either this compartment or whole cell extracts (data not shown). The second construct (pMal-C2), which was utilized for the complete study, encoded a fusion protein lacking such a signal peptide. When using this protocol, the yields of cytoplasmic MBP-L chain were far higher. Indeed, on a preparative scale (1 L), cells induced with IPTG yielded 5−10 mg of total MBP−wt-L chain (estimated from the stained SDS−PAGE gels; see Figure 2A); a similar recovery was observed for the mutant. These L chain-containing fusion proteins were subsequently purified from the cells after subjecting cytoplasmic extracts from a 1 L culture to amylose affinity chromatography (Figure 2A,B).

In order to isolate the wt-L chain or mutant L chains from the sugar-binding moiety, the fusion proteins were firstly incubated with factor X_a, yielding free MBP, the L chains, and a trace of uncleaved fusion protein (Figure 2A). Each mixture was then loaded onto a second amylose column which bound both the MBP and any remaining fusion protein while the liberated L chain was eluted (~50 μg/mL) in the wash through. After pooling the L chain-containing fractions (total yield from 1 L was ~0.5 mg), analysis by SDS−PAGE revealed high levels of purity with the full-length wt and mutant L chains accounting for >90% of the total protein (Figure 2A−C). It is noteworthy that the recombinant L chains show a slightly slower electrophoretic mobility than the native L chain, and this seems to equate to the extra size contributed by both the additional amino acids incorporated at its N-terminus (see Figure 1) and residues Thr^{438}−Lys^{447} at its C-terminus (see Discussion).

Isolated wt-L Chain Enzymically Cleaves SNAP-25 While the Mutant Protein is Inactive. In order to allow a meaningful comparison of the enzymic activities of the recombinant L chain products with that of reduced BoNT/A, an assay was developed based on the use of an antibody reactive toward the intact C-terminal region of SNAP-25 (containing the site cleaved by BoNT/A). As revealed by Western blot, prepared after SDS−PAGE of the BoNT/A plus SNAP-25 incubation mixtures, neither of the two products was recognized by this antibody (not shown). The absence of immunoreactivity toward the larger N-terminal SNAP-25 fragment can be reconciled with the very small fraction of the antigenic region remaining (Figure 3A). On the other hand, the lack of antibody recognition of the C-terminal fragment may arise from a conformational change. This sensitive immunoassay demonstrated that, over a period of 60 min at 37 °C, increasing concentrations (0−200 nM) of reduced BoNT/A cleaved SNAP-25 yielding a near linear plot ($EC_{50} \approx 75$ nM; Figure 3B,E). Importantly, the purified wt-L chain also proteolyzed the recombinant SNAP-25 albeit slightly less effectively ($EC_{50} \approx 150$ nM; Figure 3C,E) than the reduced native toxin. In contrast, the Tyr^{227}-substituted form of recombinant L chain did not cause any diminution of SNAP-25 antibody binding even at concentrations as high as 400 nM (Figure 3D,E). These results demonstrate, for the first time, that an enzymically-active form of the L chain of BoNT/A can be produced in adequate amounts by recombinant means that allows its purification and, thereby, afford other applications. Moreover, substitution of a single amino acid in the Zn^{2+} motif abolishes ability to degrade its neuronal target protein.

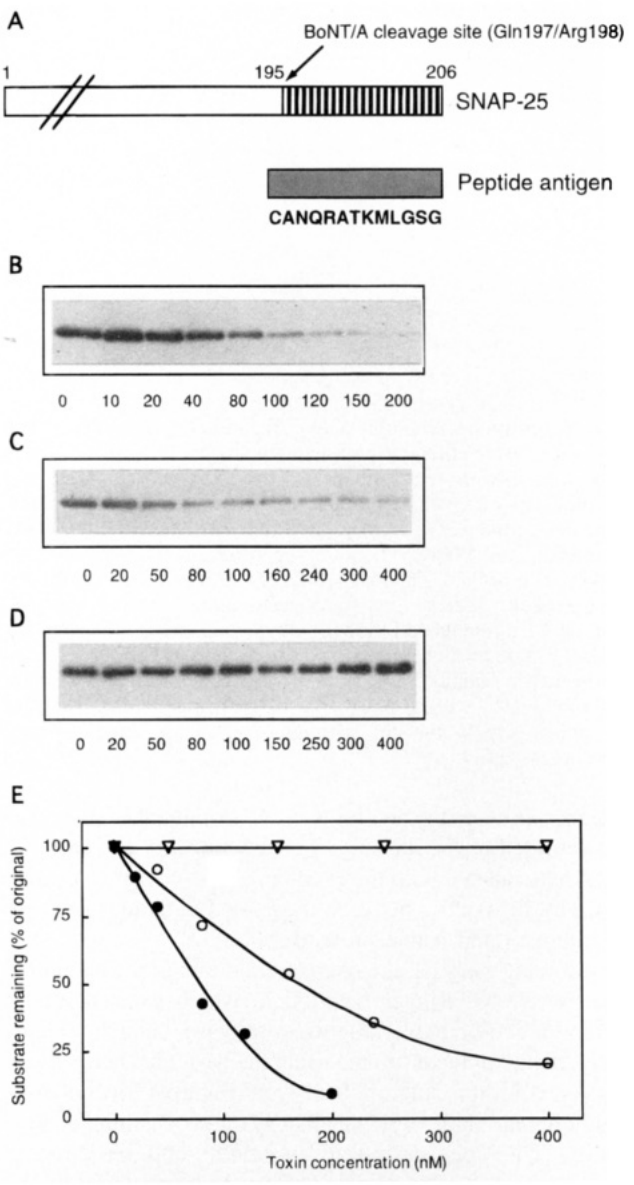

FIGURE 3: wt but not mutant L chain cleaves SNAP-25. (A) Schematic representation of the C-terminal fragment of SNAP-25 encompassing the BoNT/A cleavage site, against which the polyclonal antibody utilized was raised. (B) Western blot of expressed SNAP-25 (8.5 μM) following incubation for 1 h at 37 °C with various concentrations of reduced native BoNT/A. Antibody binding was detected using an anti-rabbit biotinylated second antibody and the ECL system. (C) As for B but SNAP-25 was incubated with wt-L chain at the concentrations indicated. (D) Also as in panel B except SNAP-25 was exposed to the Tyr^{227} mutant L chain. (E) Values obtained from densitometric scanning of the Western blots represented in panels B−D. Reduced native BoNT/A (●) and purified wt-L chain (○) effectively cleaved SNAP-25, while the Tyr^{227} mutant was devoid of such proteolytic activity (▽).

Recombinant L Chains Can Be Reconstituted with BoNT/A H Chain Yielding a Dichain Form: Only the Resultant wt Blocks Neuromuscular Transmission and Is Lethal in Mice. To assess the activity of the purified wt and Tyr^{227} mutant L chain at mammalian motor nerve endings and *in vivo*, they were first reconstituted with H chain isolated (Figure 2C) from native BoNT/A. Following the removal of DTT and urea by dialysis, the association of the recombinant L chain and native H chain to form a disulfide-linked 150 kDa protein was monitored by SDS−PAGE (Figure 2D) and quantified by densitometric scanning. As is evident in this figure, the proportion of dichain formed from the recombinant L chains was marginally lower than that with the native L chain.

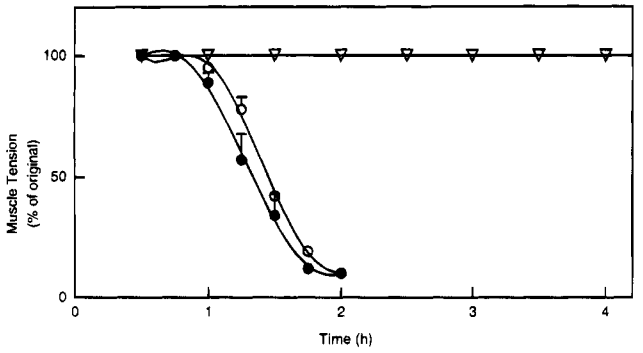

FIGURE 4: Effects of native and recombinant wt and mutant L chain on nerve-evoked neuromuscular transmission at motor end-plates once reconstituted with the native H chain of BoNT/A. When applied to mouse phrenic nerve-hemidiaphragms, BoNT/A H chain reconstituted with recombinant L chain (1.8 nM; ○) blocked neuromuscular transmission with only a fractionally lower potency compared to the native reconstituted L and H chains (2.0 nM; ●). In contrast, even a larger amount of the dichain containing the Tyr^{227} mutant form of the L chain (10 nM; ▽) was incapable of affecting nerve-evoked muscle twitch. The concentrations of the reconstituted material were calculated following the quantification of the amount of the 150 kDa dichain material present by SDS–PAGE and densitometric scanning. The tissues were bathed in Krebs–Ringer medium aerated with 95% O_2 and 5% CO_2 maintained at 24 °C. All points shown are the average of at least three separate experiments ±SD.

Indeed, ~70% of the recombinant wt or mutant L chain was reconstituted to the dichain whereas >90% of the native L chain reassociated with the H chain. This may be indicative of slight differences between the folded structures of these recombinant and native proteins.

Following dialysis, the reconstituted material was diluted into the Krebs–Ringer medium in which excised mouse phrenic nerve-hemidiaphragms were being bathed. Importantly, the toxin reconstituted using the wt-L chain effectively blocked neuromuscular transmission (Figure 4); its potentcy was only marginally less than that of the reconstituted native chains. The blockade of transmission by both these reconstituted proteins was reversed upon the application of 0.3 mM 4-aminopyridine, a blocker of voltage-gated K^+ channels which is known to temporarily restore nerve-evoked muscle tension at BoNT/A-poisoned synapses (Simpson, 1988). This confirms that the inhibition by the recombinant L chain-containing sample results from a presynaptic blockade of transmitter release and, thus, mimics that caused by BoNT/A. In contrast, the dichain material containing the Tyr^{227} mutant of the L chain had no effect on nerve-evoked muscle twitch even when tested at high concentrations (Figure 4), thereby reaffirming the lack of proteolytic activity observed with the mutant in the SNAP-25 cleavage assay (see above) at this clinically-relevant model of the toxin's site of action.

Notably, similar results to those detected with the *in vitro* test were obtained when the reconstituted samples were injected into mice; the toxicity of the dichain material containing wt-L chain (6×10^7 LD_{50}/mg, two separate preparations) was comparable to that of the reconstituted native chains ($7 \times 10^7 LD_{50}$/mg, two separate preparations), while those mice injected with the Tyr^{227} mutant reconstituted protein (up to 5 μg) showed no signs of botulism within 4 days. Therefore, by all *in vitro* and *in vivo* assays used in this study, the wt-L chain expressed in *E. coli* approaches the potency exhibited by its native counterpart while the mutated Tyr^{227} L chain is devoid of activity. Importantly, it can be concluded from these novel findings that the dichain toxin formed using recombinant L chain binds to ectoacceptors on motor nerve endings, becomes internalized, translocates to the cytosol and blocks acetylcholine release. Clearly, its efficiency in accomplishing all steps in this multiphasic intoxication approximate to those of native BoNT/A.

DISCUSSION

In recent years, all of the genes encoding the various serotypes of BoNT and TeTx have been cloned and sequenced [e.g., Binz et al. (1990)]. This led to the successful expression and purification of a recombinant form of the L chain of TeTx (Li et al., 1994) which was found to be proteolytically active toward synaptobrevin as well as being capable of inhibiting neurotransmitter release once reconstituted with native H chain, both *in vitro* at the neuromuscular junction and *in vivo*, where it causes spastic paralysis. Importantly, point mutation of Glu^{234} within that recombinant L chain resulted in loss of protease and biological activity, thereby confirming initial biochemical evidence for the importance of these residues in the action of TeTx (Schiavo et al., 1992b).

Technical difficulties hitherto in expressing the L chain of BoNT/A in a heterologous host and subsequent isolation of the viable protein have limited molecular studies to those in the sea hare, *Aplysia californica*, where microinjection of L chain mRNA into the buccal ganglionic neurons caused a blockade of acetylcholine release (Kurazono et al., 1992). This technique allowed the importance of certain N- and C-terminal residues to be established from the observed loss of the neuroparalytic activity resulting from injection of mRNA encoding L chain deletion mutants. Two later studies demonstrated the cleavage of SNAP-25, translated *in vitro*, by recombinant L chain of BoNT/A expressed in the same reaction mixture (Binz et al., 1994; Blasi et al., 1993a). Utilizing this system, two Glu^{224} point mutants of BoNT/A L chain were shown to be enzymically inactive, thereby illustrating the importance of this residue for protease activity. While these models have proved valuable in uncovering the intracellular actions of the L chain of BoNT/A, more widespread (e.g., in the mammalian nervous system) biochemical studies with purified preparations of the wt or mutant proteins have not been feasible to date. This limitation has now been overcome by the successful generation of functional recombinant L chain of BoNT/A by PCR amplification, fusion with the *malE* gene, and expression as a maltose-binding protein in *E. coli*. Importantly, isolation of the expressed protein as a maltose-binding protein was found to not only increase the solubility and, therefore, yield of the recombinant L chain, but also to facilitate its subsequent isolation from the cells. The L chains of BoNT/A, once enzymically-cleaved from the fusion protein, included seven additional N-terminal amino acids derived from the vector plus the 10 C-terminal residues (Thr^{438}–Lys^{447}) that are usually removed from the native L chain upon its enzymic processing by proteases endogenous to *C. botulinum* (Krieglstein et al., 1994). It was not deemed necessary to investigate the effects of these residues on the properties of the recombinant L chain as its behavior, both before and after reconstitution with H chain, was qualitatively identical to that of the native counterparts. However, the proteolytic activity of wt-L chain and ability to form a dichain toxin with the H chain were somewhat lower than that of

native L chain. These differences may arise from the extended length of recombinant L chain and/or result from a slightly altered folded conformation; in relation to the latter suggestion, it ought to be noted that the toxin is produced initially in *C. botulinum* as a single protein and later processed to yield the L and H chains.

As already discussed, the L chains of all the clostridial neurotoxins are metalloendoproteases that share the motif, HExxH, containing those residues involved in Zn^{2+} binding and catalytic activity of many other such proteases. The importance of His^{227} in this motif of BoNT/A was explored further by its substitution with Tyr, through site-directed mutagenesis. The absence of proteolytic activity from the mutant L chain confirms that this residue is essential in the cleavage of SNAP-25; furthermore, its importance in the toxin's toxicity was established from the disappearance of neuroparalytic activity *in vivo* and *in vitro* at a clinically-relevant site, e.g., phrenic nerve-endings, when BoNT reconstituted using mutant L chain was tested. Therefore, the production of biologically active plus an inert form of the L chain of BoNT/A shall provide means of studying the proteolysis of SNAP-25 in mammalian tissues and will also aid in gaining a greater understanding of the role of SNAP-25 as a component of the exocytotic pathway both in neurons and other cells.

An additional advance afforded by the expression of the L chain of BoNT/A is the provision, by generating nontoxin derivatives of the toxin, of a potential vehicle for the selective transportation of drugs into the cholinergic peripheral nervous system. Such a molecule includes all those features known to be essential for the productive binding and internalization of the toxin, i.e., the entire H chain, maintained in a conformation capable of productive interaction with the acceptors by its association with the L chain [see de Paiva et al. (1993b)], and the presence of an interchain disulfide bond known to be essential for translocation (de Paiva et al., 1993b; Dolly et al., 1992) but containing a mutated, proteolytically-inactive L chain. By attaching suitable therapeutic agents to the latter, it may be possible to provide targeted and novel treatments for disorders affecting peripheral motor neurons.

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