A Single Mutation in the Recombinant Light Chain of Tetanus Toxin Abolishes Its Proteolytic Activity and Removes the Toxicity Seen after Reconstitution with Native Heavy Chain†

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ABSTRACT: Specific proteolysis by the tetanus toxin light chain of a vesicle-associated membrane protein (VAMP) involved in exocytosis is thought to underlie its intracellular blockade of neurotransmitter release. To substantiate this mechanism, recombinant light chain was expressed as a maltose binding protein–light chain fusion product in *Escherichia coli*. After purification by affinity chromatography and cleavage with factor Xa, the resultant light chain was isolated and its identity confirmed by Western blotting and N-terminal sequencing. It exhibited activity similar to that of the native light chain in proteolyzing its target in isolated bovine small synaptic vesicles and in hydrolyzing a 62-residue synthetic polypeptide spanning the cleavage site of the substrate. The importance of Glu^{234} in the catalytic activity of the light chain, possibly analogous to Glu^{143} of thermolysin, was examined using site-directed mutagenesis. Changing Glu^{234} to Ala abolished the protease activity of the light chain, but its ability to bind the polypeptide substrate was retained. Each recombinant light chain could be reconstituted with the heavy chain of tetanus toxin, yielding the same level of disulfide-linked species as the two native chains. Whereas the toxin formed with wild-type light chain exhibited appreciable neuromuscular paralysis activity and mouse lethality, the equivalent dichain material containing the Ala^{234} mutant lacked neurotoxicity in both the *in vitro* and *in vivo* assays. Thus, these results demonstrate directly, for the first time, that the lethality of tetanus toxin and its inhibition of exocytosis in intact neurons are attributable largely, if not exclusively, to endoprotease activity.

Tetanus toxin (TeTx[1]) is responsible for the clinical symptoms of tetanus, a potentially fatal spastic paralysis due to a specific and irreversible blockade of transmitter release at inhibitory synapses (Simpson, 1981). TeTx is produced by *Clostridium tetani* as a single-chain protein with an M_r of 150 000; proteolytic cleavage of the latter generates the active dichain form that consists of a light chain (LC) and a heavy chain (HC) linked by one interchain disulfide bond (Weller et al., 1989). The HC mediates the binding of toxin to the appropriate acceptor(s) and the translocation of LC into cells, where the latter is responsible for the intracellular blockade of exocytosis [reviewed by Simpson (1989); Dolly, 1990, 1992].

TeTx, together with botulinum neurotoxin B, recently has been found to be a potent zinc-dependent endoprotease that cleaves rat vesicle-associated membrane protein 2 (VAMP-2), a key component in the exocytosis of synaptic vesicles (Schiavo et al., 1992a; Link et al., 1992; de Paiva et al., 1993). In addition, its LC has been shown to cleave cellubrevin, a ubiquitous nonneuronal protein with homology to VAMP (McMahon et al., 1993). A number of researchers (e.g., Devault et al., 1987; Stocker et al., 1988) have noted that certain zinc-dependent endoproteases contain the consensus sequence HExxH. In thermolysin, zinc binding is achieved via His^{142} and His^{146} within this motif, together with Glu^{166}; the fourth ligand is water [reviewed by Valee and Auld (1990)]. Comparison of the sequence of LC with those of thermolysin and other zinc endoproteases has revealed the presence of the same consensus motif (Jongeneel et al., 1989), suggesting that Glu^{234} in LC may correspond to Glu^{143} in thermolysin, an essential amino acid in this enzyme's catalysis (Hangauer et al., 1984; Möual et al., 1991).

The role of Glu^{234} within this motif in the LC of TeTx has been studied using site-directed mutagenesis and an *in vitro* assay for the proteolysis of cellubrevin. It was demonstrated that cellubrevin was not cleaved when COS cells were cotransfected with mutant LC (Glu^{234} substituted by Gln) and cellubrevin DNA constructs (McMahon et al., 1993). However, it is essential to reaffirm the functional relevance of such findings by assaying the biological activities of the purified recombinant LC and its mutant in intact neurons and the whole animal, after their reconstitution with native HC. Herein, using the cloned TeTx-LC gene (Fairweather & Lyness, 1986), reliable and efficient methodologies were adapted for the expression of recombinant LC in *Escherichia coli* as a maltose binding protein–LC fusion product (MBP–LC) and for the subsequent purification of LC by affinity chromatography after cleavage with factor Xa. Furthermore,

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[1] Abbreviations: TeTx, tetanus toxin; LC and HC, light and heavy chains of TeTx, respectively; LD_{50}, amount of toxin that kills 50% of injected mice within 4 days; factor Xa, activated factor X protease; SSVs, small synaptic vesicles; VAMP, vesicle-associated membrane protein (isoforms 1 and 2); HV62, 62 amino acid peptide corresponding to residues 33–94 of human VAMP-2; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MBP, maltose binding protein; MCS, multiple cloning site; PCR, polymerase chain reaction; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; IPTG, isopropyl β-D-thiogalactoside; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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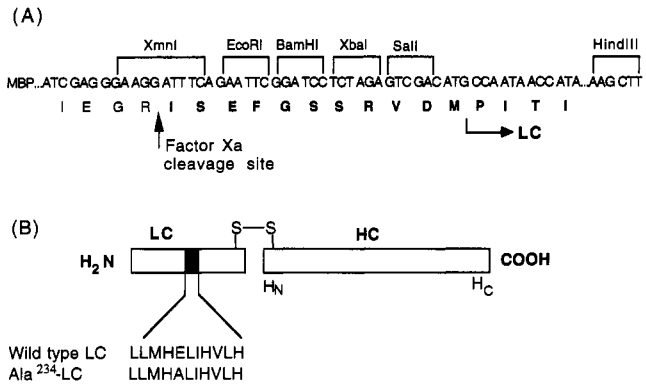

FIGURE 1: Schematic representations of TeTx and the DNA constructs used for expression of MBP–LC. (A) MCS of pMAL-c2 and the sequence between MBP and LC, including the factor Xa cleavage site (R–I), are shown. DNA encoding LC was subcloned using *Sal*I and *Hind*III sites. The single-letter codes represent the amino acid sequence of the first 15 residues of the purified recombinant LC and Ala^{234}-LC, determined by N-terminal microsequencing; the sequence of authentic LC starts from P. (B) Diagram of the structure of TeTx showing the HC disulfide-linked to LC and the zinc binding domain; E changed to A in the mutant is also indicated.

the purified LC was associated with HC, isolated from *C. tetani*-derived TeTx, to generate a dichain form displaying activity resembling that of the native toxin. In this study, it is also proven that mutation of Glu^{234} to Ala in LC abolishes its ability to cleave VAMP or a synthetic substrate and, accordingly, its neurotoxicity upon reconstitution with HC. Collectively, these results demonstrate the feasibility of expressing LC in heterologous cells and the importance of Glu^{234} in protease activity, essential for its action *in vivo*, and they create opportunities for the construction of various nontoxic mutants of TeTx and, probably, botulinum neurotoxins.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases and DNA-modifying enzymes were from Promega or Stratagene and used as recommended by the manufacturer. AccuGel 40 for DNA sequencing was purchased from National Diagnostics. The DNA sequence kit was from Cambridge Bioscience; pMAL vectors, maltose affinity resin, anti-MBP antibodies, and factor Xa were obtained from New England Bio-Labs. Native LC and HC were prepared as detailed in Weller et al. (1989).

Methods

Recombinant DNA Techniques for Preparation of the MBP–LC Construct. E. coli K-12 strain TG1 was used in this work. Plasmid pMAL-LC (wild-type LC gene) was constructed by polymerase chain reaction (PCR) amplification of a 1371-bp fragment encoding LC from pTet87 (Fairweather et al., 1993), using primers a (5′-GAGATGGTCGACATGCCAATAACCATAAATAAT-3′) and d (5′-ACGCGAAGCTTTTATCATGCAGTTCTATTATA-3′), followed by ligation of this product to pMAL-c2 (Figure 1A) that had been cleaved by *Sal*I and *Hind*III (their respective sites are underlined in the primers). For site-directed mutagenesis, two additional primers, b (5′-TAGTACATGTATAAGTGCGTGCATTAATAG-3′) and c (5′-TTATACATGTACTACATGGT-3′), each possessing the *Afl*III cleavage site underscored above, were used to mutate a Glu to an Ala at position 234 of LC; PCR amplification of pTet87 was accomplished with primer pairs a/b and c/d, used separately. The fragment from pair a/b was digested with *Sal*I and *Afl*III, and that from pair c/d was digested with *Afl*III and *Hind*III. After purification with the Magic DNA clean-up system (Promega), the samples were ligated to pMAL-c2 cut with *Sal*I and *Hind*III, creating pMAL-LC-Ala^{234} (the mutated LC gene).

Expression of the Fusion Proteins and Purification of LC and Ala^{234}-LC. After transformation, *E. coli*-containing plasmids pMAL-LC or pMAL-LC-Ala^{234} were grown at 37 °C in L-broth (100 μg/mL ampicillin and 2 mg/mL glucose) to a cell density of about 2×10^8 cells/mL ($A_{600nm} \sim 0.5$); induction was initiated by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.3 mM. Cells were harvested 2 h later and centrifuged at 6000*g* for 30 min, and the resultant pellets were resuspended in column buffer [10 mM Tris-HCl, 200 mM NaCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, and 1 mM dithiothreitol (DTT) (pH 7.4)] containing 1 mM phenylmethanesulfonyl fluoride and lysed by sonication. After centrifugation, crude extracts were applied to an amylose affinity column (2.5 × 10 cm, 40 mL of resin). Following the removal of nonbound proteins by washing with buffer, the bound MBP–LC was eluted with column buffer containing 10 mM maltose (Maina et al., 1988). The fusion proteins were concentrated to 0.5–1 mg/mL using an Amicon Centricon [protein was measured with the Bradford assay (Bradford, 1976)] and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting, using anti-MBP polyclonal and anti-LC monoclonal antibodies. Both MBP–LC preparations were cleaved at 23 °C for 24 h with factor Xa at an enzyme:protein ratio of 0.5–1:100 (w/w), yielding free MBP and either LC or its mutant. After extensive dialysis against the column buffer to remove maltose, LC or Ala^{234}-LC was then purified by readsorption onto a new affinity column. Fractions were monitored for A_{280nm} and checked again by SDS–PAGE and Western blotting. For amino acid sequencing, recombinant LC or its mutant was run on SDS–PAGE and transferred onto a poly(vinylidene difluoride) membrane (Tous et al., 1989), with automated Edman degradation performed on a Model 4000 protein sequencer (Chelsea Instruments, London).

Measurement of LC-Induced Proteolysis of VAMP in Bovine Small Synaptic Vesicles (SSVs) in Vitro. Native, recombinant (wild-type or Ala^{234}) LCs were incubated with SSVs (0.5 mg/mL) for 90 min at 37 °C in 50 mM Hepes, 400 mM NaCl, 5 mM DTT, 2 μM $ZnSO_4$ (pH 7.4). The reactions were terminated by the addition of SDS–PAGE sample buffer followed by boiling for 3–5 min. Samples were subjected to SDS–PAGE and detected by Western blotting using affinity-purified anti-HV62 antibody raised [as outlined for a similar antigen in de Paiva et al. (1993)] against a 62 amino acid synthetic polypeptide corresponding to residues 33–94 of human VAMP-2 (Shone et al., 1993).

Determination of the Cleavage of HV62 Polypeptide by Recombinant LC and Its Mutant. A stock solution of peptide (40 μM final concentration, 60 μL final volume) in 20 mM Hepes and 200 mM NaCl (pH 7.4) containing 5 mM DTT was incubated at 37 °C with LC preparations. At time intervals, the reactions were terminated by the addition of 60 μL of 5 mM ethylenediaminetetraacetic acid and 1% (v/v) trifluoroacetic acid (TFA) (pH 2) followed by centrifugation. Samples were stored at −20 °C until analysis. The amount of hydrolysis of HV62 was measured by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a Micropax

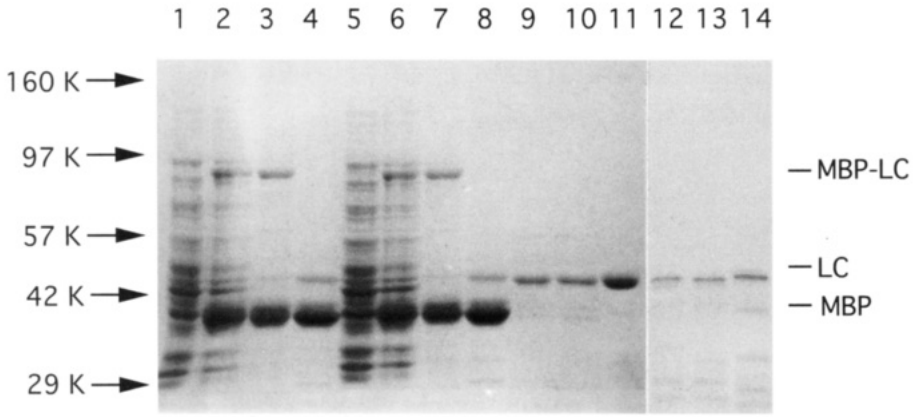

FIGURE 2: SDS–PAGE analysis of the purification of MBP–LC and the cleaved LC: Western blotting of LC preparations. *E. coli* containing recombinant vector for wild-type or mutant LCs were induced with 0.3 mM IPTG. Cells were lysed by sonication, and crude extracts were loaded onto amylose affinity resin. Following the removal of nonbound proteins by washing, bound fusion proteins were eluted by 10 mM maltose. The isolated products were digested with factor Xa at 23 °C for 24 h, and the resultant LC preparations were purified by passing the digested samples through new aliquots of the affinity resin. Samples at each stage of the purification procedures were subjected to SDS–PAGE, followed by Coomassie staining (lanes 1–11) and Western blotting (lanes 12–14) with an anti-LC monoclonal antibody. Wild-type samples: lanes 1 and 2, crude extracts of noninduced and induced cells; lane 3, eluate of the affinity column; lane 4, the latter after cleavage with factor Xa; lanes 9 and 12, purified LC. Equivalent mutant samples: lanes 5–8, 10, and 13, respectively; lanes 11 and 14, native LC.

C_{18} column equilibrated in 0.05% TFA using a 0–60% acetonitrile gradient, while monitoring A_{220nm}. N-Terminal sequencing of the cleavage product revealed a single proteolytic site between Gln^{76} and Phe^{77}, in accordance with previously published observations (Schiavo et al., 1992b). The percentage of total HV62 hydrolysis was calculated from the peak height of one breakdown product (residue 77–94); a linear standard curve that related peak height to known quantities of product was used for quantitation. For the estimation of the ability of Ala^{234}-LC to attenuate HV62 cleavage by native LC, 9 μM HV62 was preincubated with 4.5 μM Ala^{234}-LC in the reaction buffer at 37 °C for 1 h before the addition of 150 nM native LC. The sample was analyzed as above.

Association of Recombinant LC and Its Mutant LC with Native HC. To recreate the dichain species, equimolar amounts of native, recombinant LC or Ala^{234}-LC were mixed separately with the same amount of HC purified from TeTx, as detailed previously (Weller et al., 1989). The mixtures were dialyzed against 2 M urea, 20 mM DTT, 1 M NaCl, and 50 mM Tris-HCl (pH 8.4) with stirring for 18 h and then further dialyzed without agitation against 50 mM Tris-HCl and 600 mM glycine (pH 8.4) for 72 h. The extent of covalent reconstitution was checked by nonreducing SDS–PAGE (Weller et al., 1989).

Bioassay of Reassociated Dichain Toxins. Mice (20 g) were injected (200 μL/mouse) subcutaneously into the dorsal neck region with dichain toxin or other samples (Fairweather et al., 1990) and the LD_{50} values were determined, as described before (Maisey et al., 1988).

Measurements of Neuromuscular Transmission. The inhibition of acetylcholine release by the reconstituted dichain from mouse left phrenic nerve–hemidiaphragm preparations (de Paiva & Dolly, 1990) was measured as a reduction of the nerve-evoked muscle tension. The time to paralysis was recorded as the period from the addition of toxin to when the muscle tension decreased to 10% of the original amplitude (de Paiva et al., 1993).

RESULTS

Subcloning the Structural Gene for Wild-Type and Mutated LCs into E. coli Expression Vectors. The DNA encoding the LC was generated by PCR from a vector (pTet87) containing this sequence and cloned into the *Sal*I–*Hind*III sites of pMAL-c2, conserving the factor Xa cleavage site between MBP and LC (Figure 1A). After subcloning, colonies resistant to ampicillin were grown, the plasmid DNA was purified, and the construct (pMAL-LC) was confirmed, using restriction enzymes and DNA sequencing of the whole insert. Thus, agarose electrophoresis of the *Sal*I and *Hind*III digests gave a fragment of 1387 bp, the expected size for this insert. DNA sequencing revealed the nucleotide sequence at the junction where the 5′-end of the LC gene was fused to the multiple cloning site (MCS) and the factor Xa cleavage site (Figure 1A) and also showed the LC sequence to be in the correct reading frame with the MBP gene. For structure–function studies, site-directed mutagenesis of the LC DNA was employed to change Glu^{234} to Ala (Figure 1B). The mutant construct (pMAL-LC-Ala^{234}) was processed and analyzed as above, with confirmation of the mutation being achieved by obtaining the expected nucleotide sequence for Ala^{234}-LC.

Expression of the MBP–LC Fusion Protein: Purification of Recombinant LC and Its Ala^{234} Mutant. Cultures of *E. coli* containing pMAL-LC or pMAL-LC-Ala^{234} were induced with IPTG for different times and at 25, 30, 37, and 42 °C. SDS–PAGE of both cell extracts showed that a new protein band (M_r ~90 000), which was absent from the Coomassie staining pattern of the noninduced cultures, appeared following induction (Figure 2). Its size is in accordance with that expected from a fusion of MBP and LC [M_r ~40 000 (Duplay et al., 1984) and 50 000, respectively]. Moreover, the identities of the adducts and free MBP were established by Western blotting with antibodies to LC and MBP. Notably, pMAL-c2 constructs produced more free MBP than either fusion protein (Figure 2). In contrast to pMAL-c2, which expresses protein into the cytoplasmic space of *E. coli*, pMAL-p2 yielded less fusion protein, although this is transported into the periplasmic space (data not shown). Therefore, pMAL-c2 constructs were used for all subsequent work because they gave high yields (14 mg of fusion protein from 500 mL of culture), constituting about 18.6% of the total *E. coli* protein. The optimal conditions established for expressing recombinant LC and Ala^{234} mutant using pMAL-c2 were 2 h of induction with IPTG at 37 °C; neither a longer induction time nor inclusion of protease inhibitors increased the yield of product. Both fusion proteins were soluble in aqueous buffer (up to 0.5

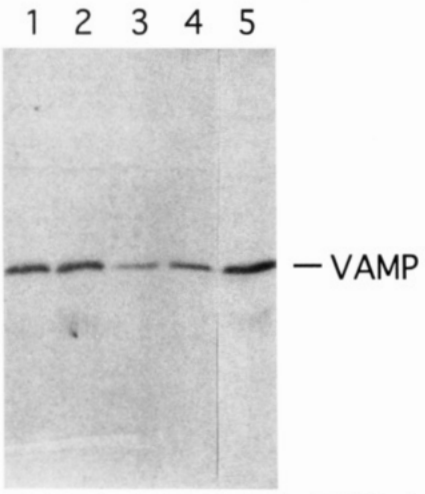

FIGURE 3: Proteolysis of VAMP in bovine SSVs *in vitro* by native and recombinant LCs, but not the Ala^{234} mutant. The various LC preparations were incubated with bovine SSVs (0.5 mg/mL) for 90 min at 37 °C, and the reaction mixtures were subjected to SDS–PAGE and Western blotting with anti-VAMP antibody. Lanes 1 and 2, samples incubated without toxin at 37 and 4 °C, respectively. Incubations at 37 °C: lane 3, with 50 nM native LC; lane 4, with 100 nM recombinant LC; lane 5, with 2.5 μM expressed Ala^{234}-LC.

mg/mL) and stable when stored in the latter at –20 °C for up to 8 months.

Large-scale purification of the two fusion proteins was accomplished by affinity chromatography of *E. coli* cytoplasmic extracts on amylose resin. There was near-complete adsorption of the MBP–LC samples to the column, and after the removal of unwanted proteins by washing, each fusion protein was eluted efficiently using maltose. The resultant samples showed substantial enrichment of the fusion proteins and free MBP, as revealed on SDS–PAGE followed by Coomassie staining and Western blotting as above (Figure 2). Cleavage of the two concentrated fusion proteins with factor Xa gave complete conversion to the respective wild-type and Ala^{234}-LC with the liberation of MBP, as seen on SDS–PAGE (Figure 2). Further purification of both samples of recombinant LC was achieved by readsorption of MBP onto the amylose resin, leaving the LC in the column wash; electrophoretic purity was demonstrated by protein staining, and their identities were established by Western blotting, using an anti-LC monoclonal antibody (Figure 2). Furthermore, this was confirmed by protein microsequencing of the two products, which revealed four residues identical to those of the N-terminus of native LC preceded by the eleven expected amino acids encoded by the MCS nucleotide sequence present (Figure 1A). Recoveries of purified LC samples from the isolated fusion protein ranged from 20 to 40% (n = 5).

Functional Properties of Wild-Type and Mutated LCs

Recombinant LC, Unlike Its Ala^{234} Mutant, Cleaves Vesicular VAMP and a Polypeptide Therefrom. Measurement of the documented zinc-dependent protease action of native LC (see introduction) was employed as a convenient assay for the biological activity of recombinant LC. Incubation of the latter (100 nM) or authentic LC (50 nM) with bovine SSVs resulted in proteolytic cleavage of VAMP, as semi-quantitatively assessed by antibody reactivity (Figure 3) or protein staining (de Paiva et al., 1993) of the digests following SDS–PAGE. However, Ala^{234}-LC proved inactive even at a concentration of 2.5 μM (Figure 3), highlighting the fact that Glu^{234} is essential for enzymatic activity (see below). For a more accurate quantitation of their relative activities, RP-HPLC was used to measure the cleavage of a synthetic 62-residue polypeptide, HV62, corresponding to residues 33–94 of human VAMP-2. By quantitation of the separated cleavage product (residues 77–94), time- and concentration-dependent hydrolysis of the polypeptide by native LC was recorded (Figure 4). Recombinant LC (250 nM) also cleaved this substrate (40 μM), confirming its biological activity. However, a 2.5-fold higher concentration than that of the authentic LC was needed to exhibit the same level of hydrolysis (n = 4) (Figure 4). For the specified conditions used, the initial rates (n = 4) of substrate cleavage observed at 37 °C with 100 nM native and recombinant LC were 45.6 ± 3.6 and 21.6 ± 2.4 pmol/min, respectively (see Discussion). More importantly, proteolysis of the polypeptide (40 μM) could not be detected with Ala^{234}-LC (Figure 4) when incubated for 3 h at 2.5 μM, emphasizing the paramount importance of Glu^{234} to the catalytic activity of LC. The lack of proteolytic activity in Ala^{234}-LC could be due to an inability either to cleave the peptide bond (Gln–Phe) or to bind the substrate. To distinguish between these possibilities, HV62 was preincubated with Ala^{234}-LC before the addition of native LC; this reduced the activity of the native LC by more than 50%, indicating that the mutant LC retained the ability to bind peptide, although experimental difficulties precluded measurement of the affinity (Figure 4, inset).

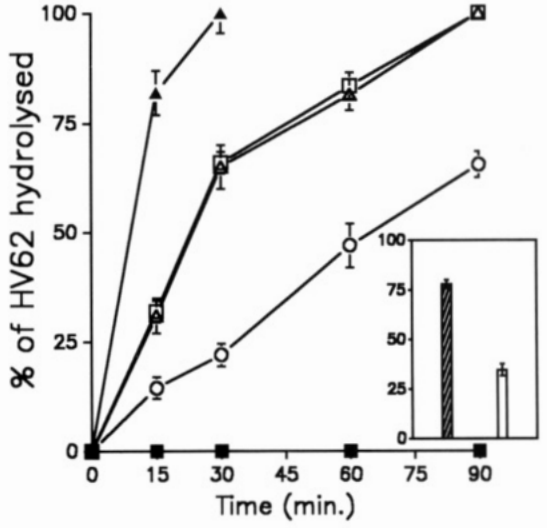

FIGURE 4: Cleavage of HV62 peptide by native, recombinant, or mutant LCs. A solution of 40 μM HV62 was incubated with LC samples in 20 mM Hepes, 200 mM NaCl, and 5 mM DTT (pH 7.4) at 37 °C for different time intervals. Extents of peptide hydrolysis were monitored by quantitation of the smaller of the two specific cleavage products (see Experimental Procedures): (○), 33, (□), 100, and (▲) 250 nM native LC; (△) 250 nM recombinant LC; (■) 2.5 μM Ala^{234}-LC. The inset shows the ability of Ala^{234}-LC to reduce the apparent hydrolysis of HV62 substrate by native LC. After 9 μM HV62 was preincubated in the same buffer with 4.5 μM Ala^{234}-LC at 37 °C for 1 h, native LC was added to a final concentration of 150 nM and the reaction was allowed to proceed at 37 °C for 0.5 h (open bar). A control incubation was carried out using the same conditions, except for the omission of Ala^{234}-LC (hatched bar). HV62 hydrolysis was determined (±SD) in quadruple experiments, as detailed in the Experimental Procedures.

Dichain Toxin Reconstituted Using HC and Expressed, Wild-Type LC but Not Its Mutant Causes Neuroparalysis: Further Evidence for a Proteolytic Mechanism. When an equimolar mixture of purified HC and recombinant LC or the Ala^{234} mutant in 2 M urea and 20 mM DTT was dialyzed over 3 days into nondenaturing buffer, SDS–PAGE of the resultant samples showed that reassociation occurred (Figure 5). With recombinant wild-type and mutated LCs, the relative amounts of the dichain species [comigrated with native TeTx

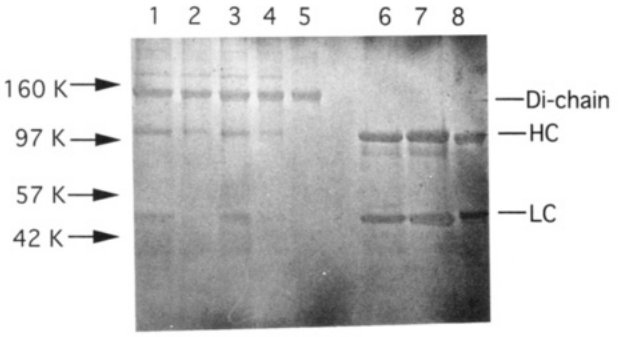

FIGURE 5: Electrophoretic demonstration of the formation of disulfide-linked dichain protein from recombinant LC preparations and the native HC of TeTx. Reconstitution was accomplished by dialyzing, for 72 h at 4 °C, an equimolar mixture of recombinant LC or its Ala^{234}-LC and HC (isolated from TeTx) in buffer containing 2 M urea and 20 mM DTT into a renaturing medium (see Experimental Procedures). An aliquot (300 μg) was loaded onto an HPLC DEAE column in 25 mM Tris-HCl buffer (pH 8.4) and eluted with a NaCl gradient (0–1 M) in the same buffer. The samples were analyzed by SDS–PAGE under nonreducing (lanes 1–5) and reducing (lanes 6–8) conditions, with detection of the proteins by silver staining. Lanes 1 and 2, reconstituted recombinant LC and native HC, before and after HPLC, respectively; lanes 3 and 4, samples reconstituted using Ala^{234}-LC and HC, before and after HPLC, respectively; lane 5, native TeTx; lanes 6–8, materials as in lanes 2, 4, and 5 but reduced before electrophoresis.

(M_r ~150 000)] formed were 55.1 and 56.8%, respectively, as determined by densitometric scanning of the silver-stained gel; native HC and LC gave a similar level of reconstitution (Table 1). The latter involved interchain disulfide formation as, upon reduction by DTT, the toxin was converted back to free HC and LC.

The documented local action of TeTx in blocking neuromuscular transmission (Habermann et al., 1980) was exploited to assess the biological activity of the reconstituted samples relative to that of the intact toxin. At 10 nM, TeTx abolished nerve-evoked muscle tension within 150 min, whereas toxin generated from native HC and LC required 240 min to achieve paralysis (Figure 6); this is in accord with the reported lower neuromuscular blocking activities of reconstituted chains from TeTx and botulinum neurotoxin A, relative to those of their native toxins (Weller et al., 1989; Maisey et al.,1988). When recombinant LC was reassembled with HC, the resultant dichain exhibited about one-half the expected potency; 40 nM recombinant dichain required the same paralysis time as 20 nM reconstituted native dichains (Figure 6, inset), consistent with the reduced enzymatic activity of expressed LC noted above. With the mouse bioassay, TeTx also proved more toxic (15-fold) than the refolded native chains (Table 1), and again, when recombinant LC was employed in the reconstitution, there was a further drop in lethality (Table 1), approximating that observed previously (Fairweather et al., 1993).

Convincingly, the dichain toxin reconstituted using Ala^{234}-LC and native HC proved inactive on neuromuscular transmission over 5 h at 100 nM (Figure 6), reaffirming the essential need for the enzymatic activity in the toxin's action. Likewise, it showed no detectable toxicity in mice, as also recorded for HC alone (Table 1).

DISCUSSION

It has been demonstrated that the LC of TeTx, like those of botulinum neurotoxin types B and F, proteolyzes rat VAMP-2 contained within synaptic vesicles (Schiavo et al., 1992a, 1993; Link et al., 1992). More recently, botulinum

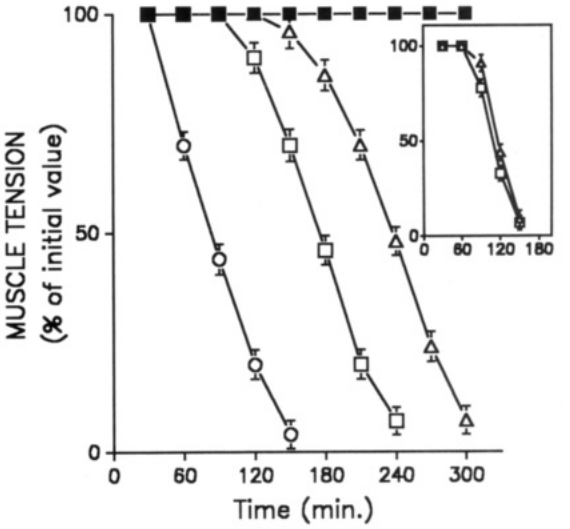

FIGURE 6: Effects of reconstituted HC and recombinant LC or the Ala^{234} mutant on neuromuscular transmission: comparison with TeTx and reconstituted native chains. Mouse phrenic nerve hemidiaphragms were exposed to the various HPLC-purified samples at 37 °C, and the resultant changes in nerve-evoked muscle tension were measured over time as detailed in de Paiva et al. (1993): (○) 10 nM TeTx; (□) reconstituted native HC and LC (10 nM); (△) recombinant LC assembled with native HC (10 nM); (■) Ala^{234}-LC refolded with HC (100 nM). Values are the means (±SD) obtained from three experiments. The inset shows the results obtained with reconstituted native HC and LC (□, 20 nM) and native HC assembled with recombinant LC (△, 40 nM). Note that the stated concentrations of reconstituted samples have not taken into account the minor content of noncovalently linked chains.

Table 1: Mouse Lethalities of TeTx and Reconstituted Samples Formed from Native HC and Recombinant LC or the Ala^{234} Mutant

sample	lethality in mice (LD_{50}/mg)[a]	covalent dimer[b] (%)
TeTx	0.5×10^8	100
reconstituted using		
native HC and LC	3.3×10^6	55.4
native HC and recombinant LC	3.3×10^5	55.1
native HC and Ala^{234}-LC	<50	56.8
native HC alone used for reconstitution	<50	

[a] Measured over 4 days, as outlined in the Experimental Procedures; mean values are shown for triplicate experiments. [b] HC purified from TeTx was reconstituted with equimolar amounts of native LC, recombinant LC, or Ala^{234}-LC to form dichains. The proportion of total protein present as a covalent dimer was determined by SDS–PAGE and densitometric scanning of silver-staining gels.

neurotoxin A has been reported to cleave SNAP-25 (Blasi et al, 1993), another neuronal protein intimately concerned with exocytosis (Söllner et al., 1993). Despite the convincing evidence for such proteolytic activity, its involvement in the *in vivo* actions of these toxins has yet to be demonstrated directly. In the case of TeTx, confirmatory evidence for this was obtained herein by construction of its active dichain form from purified native HC and LC synthesized in *E. coli* and by showing the loss of toxicity in mice and neuroparalysis when the material was prepared similarly, except for the use of a LC mutant lacking protease activity.

To express the LC in heterologous cells for this purpose and to examine the role of residues within its putative zinc binding motif (HExxH) by site-directed mutagenesis, the LC gene was incorporated into the pMAL-c2 vector. Recombinant LC was synthesized and purified from *E. coli* while fused to MBP, the latter being used as an affinity tag to facilitate the purification and subsequent isolation of free LC. The availability of this preparation allowed a quantitative com-

parison to be made, for the first time, between its activities and those of native LC using several different model systems. Cleavage of the HV62 substrate and VAMP within bovine SSVs by recombinant LC was ~40% of the activity of native LC. This is in accord with a previous report that recombinant LC has a lower potency than native LC as an inhibitor of exocytosis in permeabilized chromaffin cells (Fairweather et al., 1993). In that study, free LC was synthesized, as opposed to a fusion protein, and was purified using ion-exchange rather than affinity chromatography. Thus, the lowered activities of the two LC preparations may be attributable to the different methods of expression or purification. Such an acceptable degree of inactivation is not surprising in view of the multiple and lengthy protocols involved in LC isolation.

Another contributory factor could be the presence of MBP and the absence of HC during the initial folding of expressed LC, in contrast to the situation in *C. tetani* where LC and HC are synthesized as a single-chain protein; note that the conditions used in the subsequent reconstitution of both chains in 2 M urea should have allowed much of their original tertiary structures to be retained (Eisenstein & Schachman, 1990). Finally, the observed difference in enzymatic activity may arise from C-terminal proteolytic degradation, which probably occurs in *E. coli*, as documented for the free LC (Fairweather et al., 1993). Although native and expressed LC samples exhibited indistinguishable mobilities on SDS–PAGE, a decrease in size due to the truncation of recombinant LC could be masked by the 11 additional residues at the N-terminus (see Figure 1A). Hence, mass spectrometric analysis will be employed in the future to ascertain whether such proteolysis does occur. Should this be the case, it ought to be possible to eliminate the problem by use of protein engineering.

The integrity of recombinant LC generated here was analyzed further by its association with native HC, followed by monitoring its ability to block neuromuscular transmission and to be lethal in mice. The purified, covalently linked species displayed significant activity in both assays. However, the activities of dichain toxins formed using recombinant LC were reduced (2- and 10-fold in the two assays, respectively) compared to the activity of that formed from native LC. Of course, the 50% reduction noted in its neuromuscular blocking potency seems likely to have resulted from the equivalent deficit observed for the enzymatic activity of expressed LC. This proposal is substantiated by the demonstrated ability of the reconstituted recombinant LC–HC complex to bind the toxin's ecto-acceptors and undergo subsequent internalization at motor nerve endings and, consequently, cause neuroparalysis (Figure 6). On the other hand, it is suggested that the much lower *in vivo* toxicity (and associated spastic paralysis) may also reflect less efficient retrograde transport of this dichain species to the central nervous system, the major site of action for TeTx (Dolly, 1992). Additionally, imperfect folding of expressed LC (see above) could increase the turnover of the reconstituted protein in the whole animal and, thereby, result in diminished toxicity. Such a speculative abnormality was not, however, reflected in any increased susceptibility to reduction by DTT of the interchain disulfide of toxin reconstituted with recombinant LC or its Ala^{234} mutant compared with intact TeTx or its reassociated chains (data not shown).

The ability to create active toxin from *E. coli* derived LC has allowed the exploration of the importance of Glu^{234} within the zinc binding region. This residue occurs within a region containing the homologous sequence HExxH known to contain two of the amino acids involved in the zinc coordination and the catalytic activity of thermolysin and other zinc metalloendoproteases (Jongeneel et al., 1989). The loss of proteolytic and neuroparalytic activities of the Ala^{234}-LC mutant probably is not due to a gross alteration in the structure, because it gave the same level of refolding plus covalent assembly with HC and the resultant dichain had stability upon storage at 4 °C similar to that of reconstituted native chains. Notably, the lack of neurotoxicity in the dichain protein reconstituted using Ala^{234}-LC is not due to an inability to bind ecto-acceptors and, presumbaly, undergo internalization at the motor nerve ending, because it was found to be capable of antagonizing the neuromuscular paralysis activity of TeTx (data not shown). Moreover, it is unlikely that the lack of activity of this mutant LC is due to an inability to bind the substrate, because Ala^{234}-LC attenuated native LC-induced cleavage of the HV62 peptide. This demonstrates that Glu^{234} serves a functional rather than a structural role in LC; thus, Glu^{234} of the LC of TeTx is probably equivalent to Glu^{143} in thermolysin, which plays a catalytic role in the proteolysis of its substrate.

On the basis of these findings derived from three different assays, one can conclude that Glu^{234} is indispensable for the toxin's intracellular action, although the molecular basis of a reputed (Facchiano & Luini, 1992) secondary activity in TeTx (i.e., the activation of transglutaminase) remains to be deciphered. Clearly, the construction of nontoxic mutants of TeTx using a combination of native and recombinant fragments will allow further studies of the structure and function of TeTx. This should be of great value in the design of new drugs and new vaccines for clinical applications.

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