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Production of biologically active light chain of tetanus toxin in *Escherichia coli*

Evidence for the importance of the C-terminal 16 amino acids for full biological activity

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The activity of the light (L) chain of tetanus toxin, and of mutants constructed by site-directed mutagenesis, was studied by expression and purification of the proteins from *E. coli*. Wild-type recombinant L chain (pTet87) was active in the inhibition of exocytosis from cultured bovine adrenal chromaffin cells, although at a level 5–15% of that of L chain purified from tetanus toxin. L chain mutants which terminated at Leu-438 (pTet89), or which contained a Cys-to-Ser mutation at residue 439 (pTet88) were equally as active as the full-length recombinant protein. The reduced activity of pTet87 L chain correlated with C-terminal proteolysis of the protein upon purification. A tryptic fragment derived from native light chain and which terminated at Leu-434 also showed reduced activity in the exocytosis assay, consistent with a requirement of the C-terminal region of the L chain for maximal activity. pTet87 L chain, but neither of the mutants, could be associated with purified H (heavy) chain to form a covalent dimer which induced the symptoms of tetanus in mice. The ability to form biologically active toxin using recombinant L chain will be of great value in structure–function studies of tetanus toxin.

Tetanus toxin; Exocytosis; *E. coli*, Chromaffin cell; Site directed mutagenesis; Recombinant protein

1. INTRODUCTION

Tetanus toxin, produced by *Clostridium tetani*, causes spastic paralysis by blocking transmitter release at inhibitory synapses. The toxin is synthesized as a single 150 kDa polypeptide chain which is cleaved by a host protease to produce a di-chain molecule, with a disulphide bond between the N-terminal 50 kDa light (L) chain and the 100 kDa C-terminal heavy (H) chain [1]. Neurotoxicity in animals requires the entire toxin, as none of the purified chains exhibits toxicity. The toxin is considered to act in three distinct steps; binding to a cell-surface receptor, internalization, and finally inhibition of neurotransmitter release (for a review see [2]). The H chain is involved in the binding to sensitive cells through ganglioside [3] and perhaps proteinaecous receptors [4]. The L chain alone is sufficient to block neurotransmitter release, if injected into neurones or brought into the cytosol through a permeabilized membrane [5–7]. Recent data indicate that the L chain has a zinc-dependent protease activity [8,9]. It cleaves synaptobrevin [10], one component of the pre-synaptic vesicles. Possibly this enzymatic activity is responsible for the pharmacological action of the toxin.

The intracellular activity of purified tetanus toxin L chain was first demonstrated using permeabilized chromaffin cells [5] and injected *Aplysia* neurones [7]. The activities of the L chains of both tetanus and botulinum toxins have also been studied in *Aplysia* neurones using micro-injection of mRNA encoding the relevant proteins [11]. These experiments showed a reduction in the post-synaptic response after injection of mRNA encoding tetanus L chain, demonstrating that the translated products were responsible for the inhibition of transmitter release [11].

We have previously described the use of bacterial expression systems for the production of immunogenic fragments of tetanus toxin which have application in vaccine development [12]. In this paper, we describe the construction, expression and characterization of recombinant forms of derivatives of tetanus toxin L chain from *E. coli*. We demonstrate that purified recombinant L chain inhibits exocytosis in bovine adrenal cells and can form biologically active toxin when associated with purified H chain.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer-Mannheim and were used as recommended by the manufacturer. [α-^{35}S]dATP and the in vitro site-directed mutagenesis kit were supplied by Amersham, UK. Chromatography columns were from Pharmacia, Freiburg, Germany.

2.2. *Recombinant DNA techniques*

Escherichia coli K-12 strain TG1 was used throughout this work. DNA encoding tetanus toxin was derived from pTet20 [13], which contains a 1.6 kb *Hin*dIII–*Eco*RI fragment encoding the L chain and part of the H chain of tetanus toxin. A stop codon was inserted at the C-terminus of the L chain in pTet20 after Ala-457 by site-directed mutagenesis using the oligonucleotide 5′-TCCTCCTAAATCTGTTTATCATGCAGTTCTATTAT-3′. The resulting 1.6 kb *Eco*RI–*Hin*dIII fragment was cloned into pUC19 so that the expression of the L chain was under the control of the *lac* promoter. Oligonucleotides, 5′-CGATGGATCCATTTTAATTAGGAGATGATACGTATGCTGCAGAATTCCG-3′ and 5′-GATCCGGAATTCTGCAGCATCATACGTATCATCTCCTAATTAAAATGGATCCAT-3′, were cloned into Bluescript SKII$^+$ to facilitate the cloning of a 1.6 kb *Sna*BI–*Eco*RI L chain fragment to form pTet86. The resulting 1.6 kb *Bam*HI fragment was then cloned into pTETtac115 cut with *Bam*HI and *Bgl*II to form pTet87. Similarly, oligonucleotides were used to change Cys-439 to either Ser-439 (pTet89) or to a stop codon (pTet88). Recombinant DNA techniques were performed as described by Maniatis et al. [14]. The DNA sequence of all mutants was confirmed by direct DNA sequencing of plasmids.

2.3. *Expression and purification of recombinant proteins*

E. coli cells containing plasmids were grown overnight in L-broth [14] with ampicillin (100 μg/ml). Cells were diluted 1 in 5 into fresh medium containing 60 μg/ml isopropyl-thio-galactopyranoside (IPTG) and grown for 4 h to an A_{650} of approximately 2.0. Cells from 2 l of culture were harvested by centrifugation, washed in buffer 1 (50 mM Tris-HCl, pH 7.5, 50 mM NaCl) and resuspended in 20 ml buffer 1 containing 10 mM benzamidine and 1.0 μg/ml DNase. The cells were then lysed by double passage through a pre-cooled French pressure cell (15,000 lb · in.$^{-2}$). DTT, EDTA and PMSF were each added to final concentrations of 1 mM, and intact cells were removed by centrifugation (10,000 × *g*, 10 min). The lysate (20 ml) was then stored at −70°C before purification.

Lysate (5–6 ml) was mixed with an equal volume of buffer (40 mM piperazine-HCl, 2 M ammonium sulphate, 1 mM DTT, pH 5.8), stirred at 4°C for 30 min and centrifuged (50,000 × *g*, 30 min, 4°C). After filtration through a 0.2 μm filter, the supernatant was loaded on to a phenyl-Sepharose HP 16/10 column. Fractions were eluted with a gradient of 40 mM piperazine-HCl, pH 5.8, 2 M urea, 1 mM DTT, and tested for L chain immune reactivity by SDS-PAGE and Western blotting. Positive fractions were pooled, dialysed against 30 mM bis-Tris-HCl, 1 mM DTT, pH 5.8, and loaded on to a MonoQ 10/10 column equilibrated with the same buffer. The column was eluted with a linear gradient of 300 mM NaCl in 30 mM bis-Tris-HCl, pH 5.8, 1 mM DTT. Fractions containing L chain were pooled and mixed with an equal volume of buffer (100 mM sodium phosphate, 2.5 M ammonium sulphate, 1 mM DTT, pH 5.8) and applied to an alkyl-Superose 5/5 column equilibrated with 50 mM sodium phosphate, 1.25 M ammonium sulphate, 1 mM DTT, pH 5.8. The column was eluted with a linear gradient of 50 mM sodium phosphate, pH 5.8. The yield of purified L chain was 1–2 mg/l culture, L chain purified from toxin and subjected to these purification steps retained full activity.

2.4. *[^{3}H]Noradrenaline release from digitonin-permeabilized adreno medullary cells*

Chromaffin cells from bovine adrenal medulla were cultured, preloaded with [^{3}H]noradrenaline, permeabilized with digitonin and incubated with L chain preparations or with potassium glutamate buffer alone [5,9]. The Ca^{2+}-stimulated release of [^{3}H]noradrenaline was measured in the presence of ATP [5,9].

2.5. *Association of H and L chains and determination of toxicity*

Native chains of tetanus toxin were isolated and purified from tetanus toxin [15]. Purified H chain was mixed with equimolar amounts of native or recombinant L chain, and reconstitution of toxin was attempted by overnight incubation at 4°C against 50 mM Tris, 600 mM glycine, pH 8.4 [15]. The LD_{50} of the mixtures was determined [16] and formation of covalently linked heterodimers was assessed by SDS-PAGE under non-reducing conditions [15].

2.6. *Trypsin digestion of L chain of tetanus toxin*

Purified L chain (6 mg/ml) was digested with trypsin (E.C. 3.4.21.4) at an enzyme:substrate ratio of 1:200 at 37°C for 1 h. Protease activity was then blocked by diisopropylfluorophosphate (1 mM). Cleavage products were purified by HIC on alkyl-Superose 5/5 (see section 2.3.) and treated with BrCN followed by C_{18} reverse phase HPLC. Peaks appearing de novo in the peptide pattern were collected [19] and subjected to Edman degradation using a Pulsed Liquid Phase ABI477A sequencer.

3. RESULTS

Synthesis of the L chain of tetanus toxin in *E. coli* was achieved by using a clone (pTet20) which encodes the entire L chain and part of the H chain [13]. A stop codon was introduced by site-directed mutagenesis in order to make Ala-457 the C-terminal amino acid of the recombinant L chain (see Fig. 1). To obtain expression of the L chain, the gene was cloned into pTETtac115, a construct we have used previously for the high-level expression of tetanus toxin fragment C [12]. The resulting plasmid (pTet87) expresses the L chain using the ribosome binding site from the *E. coli trpE* gene, rather than those of tetanus toxin. Two derivatives of pTet87 were constructed by site-directed mutagenesis: pTet88 which has a stop codon introduced in place of Cys-439, and pTet89 in which Cys-439 was changed to Ser (see Fig. 1).

E. coli containing plasmids pTet87, 88 and 89 were grown and induced for the synthesis of L chain. Fig. 2 shows that all strains produce a protein which reacts with monoclonal antibody specific for L chain. pTet87 and pTet89 both give bands of molecular weight approximately equal to native L chain, whereas pTet88, which is 19 amino acids shorter (Fig. 1) produces a band

	2	434	439	450	457
pTet87	P.....K L I G L C K K I I P P T N I R E N L Y N R T A				
pTet88	P.....K L I G L S K K I I P P T N I R E N L Y N R T A				
pTet89	P .....K L I G L				
Tryptic peptide	P .....K				

Fig. 1. Predicted C-terminal amino acid sequence of the recombinant L chain proteins, and the determined C-terminal sequence of trypsinized L chain. The absence of the Met residue before Pro-2 was confirmed in pTet87 (see text). The sequence between Pro-2 and Lys-434 is represented by dots (...). The C-terminal amino acid of toxin-derived L chain is Glu-450 [17]. The Cys-439 residue which was changed to Ser is underlined.

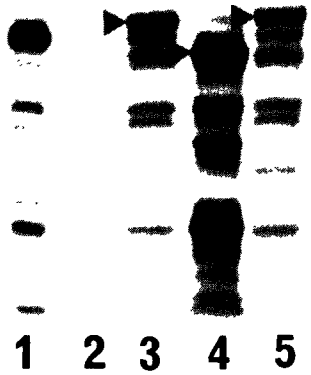

Fig. 2. Western blot of native and recombinant L chain preparations with rabbit anti-L chain sera. Lanes 1, native L chain; 2, *E. coli* pTet83; 3, pTet87; 4, pTet88; 5, pTet89. *E. coli* SDS lysates were prepared from freshly grown cells and are shown before purification. The arrows point to the mature L chain translation products from *E. coli*.

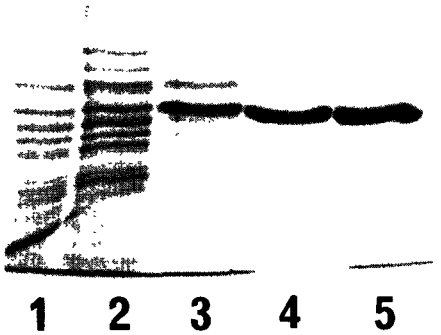

Fig. 3. 8% SDS-PAGE analysis of the purification of L chain from *E coli*. Lanes 1: *E. coli* pTet87 lysate; 2 ammonium sulphate precipitation; 3, phenyl-Sepharose HP; 4, Mono-Q; 5, alkyl-Superose. Gels were run under reducing conditions and were silver stained. Amounts of protein loaded are: lanes 1 and 2, 1 μg; lane 3, 0.5 μg; lanes 4 and 5, 0.3 μg.

of lower molecular weight. In all strains, proteolytic breakdown of the light chain was evident from the appearance of several bands of lower molecular weight than the mature protein.

The three recombinant proteins were purified from *E. coli* lysates as described in section 2 (see Fig. 3). SDS-PAGE analysis of the different recombinant L chains revealed that purified pTet87 protein (Fig. 4, lane 4) had increased mobility compared to L chain derived from toxin (lane 3); this preparation has previously been shown to have Glu-450 as the C-terminal amino acid [17]. Native L chain treated with a control *E. coli* lysate also exhibited increased mobility (lane 6), suggesting the presence of protease activity inherent in the *E. coli* lysate. This proteolysis could be only partially prevented by the addition of high concentrations of the protease inhibitors pepstatin and leupeptin (data not shown). Purified pTet87, 88 and 89 all exhibited similar mobilities (lanes 4–6), in contrast to that observed in the *E. coli* lysates (see Fig. 2). This suggests that the originally larger proteins (pTet87 and pTet89) have been shortened during cell lysis or purification to derivatives having more similar molecular weights. N-Terminal amino acid sequencing of the first 20 amino acids of purified pTet87 protein revealed the predicted sequence, starting with Pro-2, the first amino acid of native L chain (data not shown). Thus it is probable that a proteolytic activity in *E. coli* lysates is acting at the C-terminus of the L chain, whether derived from tetanus toxin or from *E. coli*. Interestingly, L chain purified from L-H_N chain ran at an intermediate position between native L chain and L chain which had been exposed to *E. coli* lysates (lane 7). This preparation had the expected N-terminus, whereas its C-terminus was shortened by three or four amino acids, ending at Thr-446 or Asn-447 [17].

To ascertain whether the recombinant L chain retained biological activity, H chain purified from tetanus toxin was mixed with pTet87, pTet88 and pTet89 proteins purified from *E. coli*. After dialysis to allow association of the chains, the mixtures were injected into mice. Symptoms of tetanus were seen only in mice injected with the pTet87–H chain mixture, demonstrating

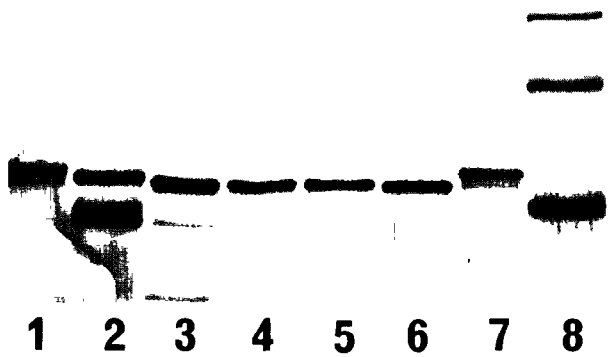

Fig. 4. Comparison of the molecular weights of L chains from different origins. Lanes: 1 and 7, native L chain, 2, fragment L-H_N composed of L chain (upper band) and H_N (lower band); 3, native L chain treated with pTet83 lysate; 4, pTet88; 5, pTet89; 6, pTet87; 8, molecular weight markers (top to bottom; 94 kDa, 67 kDa, 43 kDa)

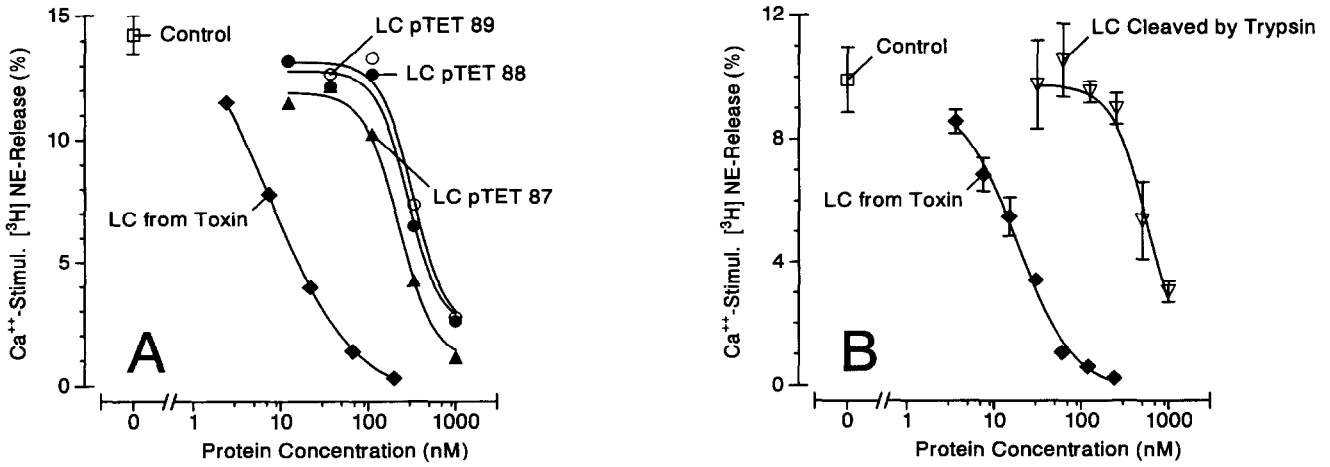

Fig. 5. Inhibition of Ca^{2+}-stimulated catecholamine release from digitonin-permeabilized chromaffin cells by recombinant L chain. Permeabilized adrenal medullary cells were treated with increasing concentrations of purified preparations of L chain and derivatives in Ca^{2+}-free buffer for 25 min at 37°C. (A) L chain from tetanus toxin (♦); pTet87 (▲); pTet88 (●); pTet89 (○). (B) L chain from tetanus toxin (♦); trypsin-treated L chain (▽). The control value represents the % release observed in the absence of added protein. Control values (□) are evoked release due to 20 μM free Ca^{2+} without addition of L chain and were the mean of triplicate determinations. The data points of L chain and derivatives represent the mean of duplicate determinations. Basal (spontaneous release) of 0.9% (A) and 1.5% (B) was subtracted.

that the recombinant L chain was active as a component of biologically active neurotoxin (Table I). No symptoms of tetanus were seen using the pTet88 and pTet89 mixtures. pTet87 was the only recombinant protein which could be shown by SDS-PAGE to form a covalent heterodimer with the H chain via a disulphide bond. These results indicate that the Cys-439 of the L chain is essential for reassociation of the L and H chains, and that disulphide linkage of separated L and H chains is essential for toxicity in vivo.

The bovine adrenal chromaffin cell system was used to study the ability of the L chain preparations to inhibit the release of catecholamines. The purified recombinant preparations were equally as active as inhibitors of transmitter release (Fig. 5A), however, *ten* times less than the native L chain. A similar reduction in potency was observed using native L chain, purified from tetanus toxin, which had been treated with a control *E. coli* lysate prior to purification (data not shown). This modified L chain, and all the purified recombinant L chains, showed increased mobility on SDS-PAGE (Fig. 4).

As the N-terminal amino acid sequence is conserved, increased mobility suggested a loss of C-terminal amino acid residues. A comparable degree of shortening, as evidenced by increased electrophoretic mobility, was achieved by treatment of L chain with high concentrations of trypsin (Sanders, D., Ph.D. Thesis, Gießen, 1993). The resulting purified tryptic core protein had an unmodified N-terminus. To identify its C-terminus, it was cleaved with cyanogen bromide. When compared to that of native L chain, the resulting peptide pattern displayed a loss of one peptide (#11) which starts with (417) RVNT and is known to terminate at Glu-450, the C-terminus of native L chain [17,19]. A new peptide appeared which shared the same N-terminus as peptide #11, however, Edman degradation stopped at Lys-434, indicating that this was the C-terminal amino acid. As shown in Fig. 5B, this fragment had 10–30 times less activity in the adrenal cell system than native L chain, consistent with a requirement for amino acid residues after Lys-434 for full activity of the L chain in this assay.

4. DISCUSSION

Our results demonstrate that it is possible to produce active L chain of tetanus toxin in *E. coli*. Biological activity of this protein was demonstrated in two ways: (i) association with H chain to produce neurotoxic activity in mice, and (ii) inhibition of catecholamine release in permeabilized bovine adrenal cells. In the chromaffin cells, the activity of recombinant pTet87 protein

Table I

Association of recombinant L chains with the H chain of tetanus toxin

Source of L chain	Toxicity of reassociated chains (LD_{50})[a]	Covalent dimer[c] (%)
Native[b]	0.1	100
Native[b], trypsinized	not tested	0
pTet87	6	25
pTet88	> 1,000	0
pTet89	> 1,000	0

[a]H chain from toxin was mixed with equimolar amounts of L chain from either toxin or *E. coli* to associate to holotoxin. Toxicity was determined as described [16] and is given as μg/kg (mouse). H and L chains alone purified from toxin had LD_{50}s of > 10 μg/kg.
[b]Prepared from tetanus toxin.
[c]Protein present as a covalent dimer (%) was determined by SDS-PAGE.

was 5–15% that of native L chain. This reduction is consistent with C-terminal proteolytic degradation of recombinant L chain which was observed upon purification of the protein, and which appeared to involve an *E. coli* protease. The requirement for this C-terminal region of L chain for maximal activity in the exocytosis assay was reinforced by our data showing the reduced activity of a trypsin-derived fragment of native toxin L chain which was found to terminate at Lys-434. Analysis of the C-terminal sequence of purified pTet87 L chain would reveal the exact extent of degradation at this site and could help further define the sequence of L chain which is essential for maximum activity of the L chain. Production of L chain in protease-deficient strains of *E. coli*, or under conditions where these enzymes are inactive, will allow the purification of the mature form of this protein and increase its activity.

The lower activity (60-fold) of holotoxin, formed by association of purified native H chain and recombinant L chain pTet87, in contrast to holotoxin formed by mixing purified native chains, is striking. This can be partially explained by the lower association efficiency of L chain pTet87 (Table I). Thus the amino acids C-terminal to Cys-439 may be important for the association of the chains. In addition, loss of toxicity may also be due to the lower potency of the shortened L-chains, which is reflected by the lower inhibitory potency of exocytosis in vitro. Our results contrast to those obtained using mRNA encoding various L chain sequences expressed in *Aplysia* [11]. In this system, it was found that mRNA encoding L chain terminating at Thr-392 was still active in depressing the post-synaptic response to presynaptic stimuli, whereas we find that larger proteins terminating at Leu-438 (pTet88) or at Lys-434 (trypsin derived core fragment) have reduced activity. Differences between the *Aplysia* and the bovine adrenal cell systems, or the use of mRNA compared to purified protein, may account for some or all of these differences. However, neither system represents the in vivo activity of tetanus toxin, and we think it attractive to use mutant toxin derivatives in an animal model. The use of recombinant L chain together with toxin-purified H chain provides a powerful method for the analysis of the effect of site-directed mutations within the L chain in vivo. The reassociation of *E. coli*-derived L chain with toxin-derived H chain has demonstrated that disulphide linkage of these two chains is essential for in vivo toxicity. This supports the conclusions of Schiavo et al. [18], who used reduction of the inter-chain disulphide bond to demonstrate a requirement for mouse toxicity.

Our observation that L chain synthesized in *E. coli* can be purified and retains biological activity makes it attractive to study further derivatives of L chain-containing mutations of the histidine residues contained within the Zn^{2+}-binding domain, to test the hypothesis in animals that Zn^{2+}-binding mediated by these amino acids is essential for toxin activity. L chain and derivatives constructed by site-directed mutagenesis purified from *E. coli* can be used to further characterize the protease activity now found to be associated with this protein [8–10].

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