Recombinant Forms of Tetanus Toxin Engineered for Examining and Exploiting Neuronal Trafficking Pathways*

Received for publication, April 20, 2001, and in revised form, June 5, 2001
Published, JBC Papers in Press, June 11, 2001, DOI 10.1074/jbc.M103517200

Yan Li‡, Patrick Foran‡, Gary Lawrence§, Nadiem Mohammed‡, Chan-Ka-Ning Chan-Kwo-Chion‡, Godfrey Lisk‡, Roger Aoki§, and Oliver Dolly‡

From the ‡Department of Biochemistry, Imperial College, London SW7 2AZ, United Kingdom and §Allergan Inc., Irvine, California 92623-9534

Tetanus toxin is a fascinating, multifunctional protein that binds to peripheral neurons, undergoes retrograde transport and trans-synaptic transfer to central inhibitory neurons where it blocks transmitter release, thereby, causing spastic paralysis. As a pre-requisite for exploiting its unique trafficking properties, a novel recombinant single chain was expressed at a high level in Escherichia coli as a soluble, easily purifiable protein. It could be activated with enterokinase to produce a dichain that matched native toxin in terms of proteolytic and neuroinhibitory activities, as well as induction of spastic paralysis in mice. Importantly, nicking was not essential for protease activity. Substitution of Glu234 by Ala created a protease-deficient atoxic form, which blocked the neuroparalytic action of tetanus toxin in vitro, with equal potency to its heavy chain; but, the mutant proved >50-fold more potent in preventing tetanus in mice. This observation unveils differences between the intoxication processes resulting from retrograde transport of toxin in vivo and its local uptake into peripheral or central nerves in vitro, dispelling a popularly held belief that the heavy chain is the sole determinant for efficient trafficking. Thus, this innocuous mutant may be a useful vehicle, superior to the heavy chain, for drug delivery to central neurons.

Many potential treatments for disorders of the central nervous system are hindered by the impermeability of the blood-brain barrier to large therapeutic molecules. An alternative entry route entails uptake into peripheral nerve endings followed by retrograde axonal transport to their cell body and trans-synaptic transfer to central neurons. Such fascinating trafficking routes are exploited efficiently by growth factors (1), neurotrophic viruses (2), and tetanus toxin (TeTx1 (1, 3, 4)).

Thus, insights could be gained into the fundamental processes of protein and membrane trafficking by investigating the cellular and molecular mechanisms responsible for such movements, for example, of TeTx; also, novel means of preventing tetanus should emerge.

TeTx, a protein from Clostridium tetani composed of a heavy chain (HC) and light chain (LC) linked by a disulfide and non-covalent bonds, causes spastic paralysis by targeted delivery to the spinal cord and lower brain where it blocks transmitter release from inhibitory nerve endings (5). The HC is required for binding to ecto-acceptors on peripheral and central neurons (6, 7, and reviewed in Ref. 8), but functional antagonism of whole toxin by recombinant HC (9) has not been demonstrated in vivo. The LC functions independently as a Zn2+-dependent protease and cleaves specifically synaptobrevin, a synaptic vesicle protein essential for Ca2+-elicited neurotransmitter release (10, 11). Clearly, this toxin and its chains or derivatives can provide invaluable research tools for elucidating poorly understood intra- and inter-neuronal trafficking pathways/mechanisms. Moreover, TeTx has unique potential as a central nervous system delivery vehicle because of its proven ability to co-transport attached “cargo” such as complexed anti-toxin (12) or conjugated enzymes (13). Because TeTx is one of the most poisonous substances known, ablation of this toxicity is an essential pre-requisite for such exploitation; this can be achieved by mutagenesis of the protease active site in LC but requires subsequent reconstitution with HC, which gives a low yield of dichain (9, 14). Due to its ease of preparation (15, 16), the C-terminal moiety of the HC, termed HC1, has been employed to traffic enzymes into the central nervous system in vivo (15, 16) but the transport efficiency was very poor (17, 18). Possible reasons for this low efficacy of HC1 include: (i) reduced binding affinity for central neuronal membranes in vitro compared with HC or TeTx (19, 20); (ii) its preferential or predominant interaction with non-productive acceptors on peripheral neurons that do not mediate optimal retrograde traffic; (iii) the absence of the contributions of the N-terminal half of the HC, as well as the inter-chain disulfide, to the internalization of TeTx that results in toxicity (21). In this context, it is notable that motor nerve terminals appear to possess two distinct pools of TeTx acceptor. In addition to the aforementioned productive acceptor that underlies retrograde trafficking leading to the spastic paralysis typical of tetanus, low affinity binding sites exist that apparently release the neurotoxin proximal to its synaptic site of internalization; hence, at high doses TeTx induces flaccid paralysis at neuromuscular junctions in vitro (22) and in vivo (23). These various findings highlight that TeTx, devoid of toxicity, will provide the most effective vehicle for targeted delivery to the central nervous system, being functional at very low concentrations by...
targeting the high affinity acceptor, which enters the retrograde trafficking pathway and culminates in translocation of LC into the neuronal cytosol. Hence, a new strategy to achieve this goal is described herein.

To circumvent the difficulties of preparing the individual chains of TeTx and achieving adequate reconstitution, TeTx was expressed as a single chain (SC) in *Escherichia coli* to yield a high level of correctly folded and soluble toxin, thereby, mimicking *Clostridium tetani*. Attachment of a tag afforded complete purification while incorporation of an enterokinase (EK)-susceptible linker allowed controlled nicking to create the fully active, disulfide-linked dichain. A similarly created pro-enzyme, mimicking a high level of correctly folded and soluble toxin, thereby, antagonized the toxin.

### MATERIALS AND METHODS

Restriction endonucleases, DNA purification kits, and *E. coli* were from Promega. Talon resin and a site-directed mutagenesis kit were bought from CLONTECH and Stratagene, respectively. pTrcHisA vector was purchased from Invitrogen. A monoclonal antibody specific for the poly-histidine tag was obtained from Qiagen. Cell culture media, reagents, and glass beads were purchased from Sigma-Aldrich. Horse serum was supplied by Life Technologies, Inc. and primers by MWG-Biotech.

**Preparation of SC TeTx Constructs—** Every construct had unique restriction sites inserted for the purpose of validation. The pTrcHisA vector was modified using the QuikChange site-directed mutagenesis kit by the insertion of two restriction sites (SalI and HindIII) into its multiple cloning site (MCS), ahead of the EK dIII site was replaced, by *Hin* dIII site was replaced, by *Hin* SalI, SmaI, or HindIII enzymes. The modified pTrcHisA was confirmed by agarose electrophoresis after digestion with SalI, SmaI, or HindIII enzymes.

### Expression and Purification of SC TeTx and HC

**Expression and Purification of SC TeTx and HC—** All the work with the SC TeTx WT was carried out under containment level 3, according to an approved strict safety protocol. *E. coli* transformed with pTrcHisA-SC TeTx WT or E234A were used to inoculate 10 ml of LB medium, and grown overnight with agitation (200 rpm) at 37 °C. An aliquot (2 ml) of each culture was transferred to 200 ml of fresh LB medium with the addition of ampicillin (100 μg/ml); the cells were grown further at 37 °C with agitation to reach A600 nm = 0.5.

### Determination of the Proteolysis of Synthetic HV62 Polypeptide by Nativity TeTx and SC Preparations—** An appropriate concentration of each sample was incubated at 37 °C with 15 μM HV62, a synthetic peptide corresponding to residues 33–94 of human synaptobrevin/VAMP-2 (HV62) in 60 μl of buffer (50 mM HEPES-NaOH (pH 7.4), 0.2 mM dithiothreitol (DTT), 0.2 mg/ml bovine serum albumin (BSA), and 50 μM ZnCl2). Bovine pancreatic trypsin inhibitor (500 nt) was included in all incubations to prevent EK cleavage of HV62. The reactions were stopped after 30 min by addition of 60 μl of 5 mM EDTA and 1% (v/v) trifluoroacetic acid (pH 2). Reverse-phase high pressure liquid chromatography (RP-HPLC) on a Micropax C18 column equilibrated in 0.05% trifluoroacetic acid, and elution with a 0–90% acetonitrile gradient separated the products; the peak height of one (residues 77–94) was used to calculate the percentage of HV62 hydrolyzed (for details see Ref. 26).

**Assessment of the Neuromuscular Paralytic Activity and Lethality of Native TeTx and Recombinant Variants—** Mouse phrenic-nerve hemidiaphragm preparations were dissected, bathed in Krebs-Ringer (KR) buffer (in mM; NaCl 118, KCl 4.7, MgSO4 1.2, NaHCO3 23.8, KH2PO4 5.3, KHPO4 1.2, CaCl2 2.5, and glucose 11.7, pH 7.4) containing 0.1% (v/v) BSA and gassed with 5% CO2/95% O2. After equilibration, TeTx, a recombinant sample (or buffer as a control) was added and toxinduced neuromuscular paralysis was determined as the time taken for nerve-evoked muscle contraction to decrease to 10% of original value, as described elsewhere (27). For continued paralysis studies for a period of 30 min, C. The tissues were thus prepared and incubated for 4 h in Krebs-Ringer buffer (KR; equivalent to 28). In some experiments, native TeTx was mixed with mutated TeTx SC or nicked, or HC before co-injection into mice; these animals were continuously monitored for the appearance of tetanus symptoms over the following 96 h.

**Identification and Site-directed Mutagenesis of a Protease-susceptible Bond in SC TeTx—** In view of the appreciable toxicity of the un-nicked toxin in mice, its susceptibility to nicking was evaluated. SC TeTx (50 μg) was treated at 37 °C for 0.5 h with trypsin (final concentration, 1 μg/ml), before the addition of SDS sample buffer and boiling for 5 min; 2 μg of the same toxin (treated similarly except not exposed to protease) was used as a control. All the samples were subjected to SDS-PAGE, in the presence of reducing agents, and electrophoresed onto an Immunoblot-P membrane, and stained with Ponceau red to reveal the HC, which was cut out for N-terminal sequencing. Because this showed that the peptide bond between Arg296 and Ser297 was cleaved (see "Results"), this Arg was replaced with Gly using the QuikChange site-directed mutagenesis kit with the following two primers: a, 5'-AGG GAT CGA TGG GGA TTC CTT GCA TTC AGT CAT GTT GC-3'; b, 5'-TCT
and Methods

of the TeTx construct are shown in 5 min at 37 °C in KRH containing either 1.4 mM Ca\(^{2+}\) or 0.5 mM EGTA was added for a 5-min stimulation period. The neurons were washed four times as before and incubated for 5 min at 37 °C in KRH containing either 1.4 mM Ca\(^{2+}\) or 0.5 mM EGTA to assess Ca\(^{2+}\)\textsubscript{i}.

Assay of Glutamate Release

The amounts of [\(^{14}\)C]glutamate in basal and stimulated samples were measured as above and expressed as a percentage of the total cell content. The quantities of KCl-induced [\(^{14}\)C]glutamate were measured as above and expressed as a percentage of the total cell content. The quantities of KCl-induced [\(^{14}\)C]glutamate were measured as above and expressed as a percentage of the total cell content.

Preparation and Maintenance of Cerebellar Granule Neurons and Assay of Glutamate Release—These cells were dissociated from the cerebella of 7- to 8-day-old rats, using established methodology (29). These cells were dissociated from the cerebella of 7- to 8-day-old rats, using established methodology (29). These cells were dissociated from the cerebella of 7- to 8-day-old rats, using established methodology (29).

The two constructs gave the expected DNA fragments on agarose gel electrophoresis when the requisite restriction enzymes were used (cf., Fig. 1); for example, digestion of either with a combination of SalI and HindIII released LC DNA, whereas BamHI gave HC DNA (data not shown). This subcloning of the SC TeTx gene resulted in 66 extra nucleotides at the 5′-end; also, a 51-nucleotide linker encoding an EK substrate consensus motif was present (Fig. 1). The DNA sequences obtained for the two constructs confirmed that they were in the correct reading frame. As illustrated in Fig. 1, the predicted SC protein was obtained in the bacterial lysate but only after induction, nicking by EK (between Lys\(^{489}\) and Asp\(^{490}\)) should create the dichain composed of a LC, with 22 and 10 extra amino acids at its N and C termini, and HC having a 7-residue N-terminal extension.

Expression and Purification of SC TeTx and E234A—After transformation of E. coli with either of the above-noted constructs, expression of the proteins was induced by IPTG; in each case, a SC protein was obtained in the bacterial lysate but only after induction, as demonstrated by Western blotting with an anti-His tag antibody (Fig. 2A). Moreover, the same single band was also recognized by polyclonal antibodies specific for TeTx LC or HC (Fig. 2A). This evidence together with the expected size (~150 kDa) on SDS-PAGE under reducing conditions confirmed the identity of SC TeTx; an absence of smaller components excluded any nicking, degradation, or premature translation. SC TeTx was purified by nickel-affinity chromatography, as judged from the protein pattern on a Coo-

TCC TAA ATC TGT TAA TGG TCC TCA TCG ATC CTT-3′. The construct was analyzed, and the protein (R496G) was expressed and purified as detailed above for WT; its susceptibility to various trypsin concentrations was monitored by SDS-PAGE and protein staining of the products.

**Fig. 1. Features of the recombinant SC TeTx gene. A.** schematic representation of the plasmid carrying the neurotoxin open-reading frame, which encodes an N-terminal His\(_6\) tag (gray box) and inter-chain linker (hatched box). **B.** sequences of regions 1, 2, and 3 that encompass the ligation sites used during construction of the SC TeTx gene, adjacent to the LC and HC. DNA coding regions are in capital letters while parts of the TeTx construct are shown in bold letters. **Numbers** refer to the amino acids of the recombinant SC TeTx. DNA restriction sites (see "Materials and Methods") and the EK protease site (cleavage occurs between Asp\(^{489}\)-Arg\(^{490}\)) are underlined. Arrows depict the primers used for preparation of the construct (see text).
Properties of Single-chain Activable TeTx and an Atoxic Mutant

Fig. 2. Expression and purification of TeTx exclusively in SC form from E. coli: controlled nicking in vitro. E. coli were transformed with pTrcHisA-SC TeTx WT or E234A, incubated for 16 h at 37 °C in the absence or presence of 1 mM IPTG and aliquots of their lysates subjected to SDS-PAGE and Western blotting (A) with antibodies reactive toward His6, (lanes 1–4). TeTx LC (lane 5), or HC (lane 6). After purification of SC TeTx on Talon resin and incubation with and without EK (see “Materials and Methods” and for details), SDS-PAGE was carried out, followed by protein staining of the gel with Coomassie Blue (B) or Western blotting with an antibody recognizing TeTx HC (C). A sample of native TeTx was also run as a control.

massie Blue-stained SDS-PAGE gel run under non-reducing conditions (Fig. 2B). A doublet was seen for SC TeTx E234A (Fig. 2B) and WT (not shown), migrating slightly behind native TeTx as expected due to the extra residues; only a single band (molecular mass = 150 kDa) was observed under reducing conditions, indicative of the doublet seen in the absence of DTT being attributable to the disulfide in the HC being present in only a fraction of both the native and recombinant molecules (see below). In contrast to the documented SC toxin, native TeTx converted to HC and LC after reduction (Fig. 2B).

Having shown that the recombinant TeTx existed exclusively as an SC protein both in the E. coli lysate and in the purified state, its complete conversion to the dichain was achieved in vitro using EK to cleave the engineered site in the inter-chain linker (Fig. 1). After SC TeTx was incubated with EK, it still migrated in non-reducing SDS-PAGE as the 150-kDa doublet but was totally converted to HC and LC after reduction (Fig. 2, B and C). This established that the inter-chain disulfide had formed in all of the recombinant toxin. After purification, ~4.5 mg of either SC TeTx WT or E234A was generally obtained from 500 ml of culture.

SC TeTx WT Gives a Near-maximal Rate of Cleavage of a Synaptobrevin Peptide: Nicking Enhances Its Protease Activity to Match That of Native TeTx—An established RP-HPLC method, based on the proteolysis of HV62, a 62-mer polypeptide corresponding to residues 33–94 of human synaptobrevin-2, was used to measure the protease activities of the recombinant toxin preparations and investigate the influence of nicking. The initial rates of cleavage (Table I) were determined by quantifying one of the separated products (residues 77–94). Reduced SC TeTx gave a value of 8 nmol min⁻¹mg⁻¹, which represents 39% of that obtained for native dichain TeTx; this demonstrates, for the first time, that nicking is not an absolute prerequisite for the toxin’s catalytic activity. Recombinant nicked WT toxin displayed comparable proteolytic activity to its native counterpart (Table I), establishing that its LC moiety is folded correctly when expressed as an SC in E. coli. Thus, the additional linker and N- and C-terminal residues present in the recombinant TeTx are not detrimental to its proteolytic activity.

Nicked Recombinant TeTx Displays the Same Neuromuscular Paralytic Activity and Mouse Lethality as the Native Toxin: SC TeTx WT Displayed Somewhat Lower Potencies—To assess the abilities of un-nicked and nicked TeTx to bind motor nerve terminals, undergo local internalization/translocation, and block acetylcholine release, they were tested in vitro on the mouse nerve-diaphragm. The nicked WT and TeTx caused neuromuscular paralysis in the same time (Table I), demonstrating that the bacterially expressed TeTx is equipotent to its native counterpart in this multistep intoxication. Likewise, subcutaneous injection into mice of equal quantities of either recombinant nicked or native TeTx caused an indistinguishable pattern of spastic paralysis, due to the blockade of transmitter release at inhibitory synapses in the spinal cord, and showed the same specific neurotoxicity, ~10⁵ LD₅₀/mg (Table I). Notably, the un-nicked TeTx showed lower lethality and neuromuscular potency than the recombinant un-nicked or native toxin, the longer time observed for neuromuscular paralysis (Table I) being equivalent to 2- to 2.5-fold less activity (i.e. un-nicked toxin must be present at 2–2.5 × the concentration of nicked toxin to induce paralysis in an equivalent time; data not shown). Although activity of SC cannot be ruled out, partial nicking of SC TeTx was observed after its application to neurons in culture (see below). Therefore, these findings are indicative of nicking being required for toxicity and that this occurs to a large extent in the diaphragm and the whole animal. Thus, despite heterologous expression in E. coli and modifications to its primary sequence, the recombinant TeTx retains maximal biological activities.

Limited Trypsinolysis of SC TeTx WT Reveals Multiple Nicking Sites between LC and HC: Mutating R496 to G Ablates One Scissile Bond but Does Not Reduce Its Biological Activities—With the aim of engineering a toxin that would be more resistant to nicking in vitro, and yet whose neurotoxicity could be controlled by EK treatment in vitro, the peptide bonds of the SC susceptible to trypsin were first examined. A fixed time of incubation with various trypsin concentrations, followed by SDS-PAGE and protein staining, revealed that several proteolytic sites are present in the LC-HC junction (see Fig. 3A, also reported in Ref. 31). Higher trypsin concentrations yielded single forms of both LC and HC (Fig. 3A), and Edman analysis of the resultant HC gave the partial N-terminal amino acid sequence SLTXX, which only corresponds to residues 497–501 of recombinant TeTx WT (Fig. 3B, denoted by asterisks). Therefore, the Arg⁴⁹⁶–Ser⁴⁹⁷ bond is cleaved by trypsin (Fig. 3B). When Arg⁴⁹⁶ was mutated to Gly, expressed, and purified (Fig. 3C), again a single protein (SC TeTx R496G) of 150-kDa molecular mass was obtained. Notably, its trypsin fragmentation pattern was altered giving one fewer HC and LC precursor than WT at the lower enzyme concentrations, but higher amounts yielded the fully nicked chains; thus, one of three cleavage sites observed with the WT was removed by this mutation. The un-nicked R496G mutant showed no decrease in toxicity (relative to un-nicked WT) or the extent of its activation by trypsin, as determined by the neuromuscular and...
mouse lethality assays (Table I); also, it was as active as WT in blocking neuroexocytosis from cerebellar neurons (Fig. 4). Surprisingly, SC R496G gave a significantly higher rate of proteolysis of HV62 than WT (Table I) and a larger enhancement upon nicking than that seen with the non-mutated toxin; the basis of this difference remains unclear.

**FIG. 3.** Characterization of a trypsin-sensitive bond in SC TeTx: mutation of Arg⁴⁹⁶ to Gly alters the nicking pattern. SC TeTx (6 μg at 0.2 mg/ml) was incubated for 30 min at 37 °C (in 10 mM HEPES-NaOH, pH 7.4, and 145 mM NaCl) with various trypsin concentrations; control samples, lacking the protease, were treated identically. After boiling for 5 min and addition of DTT (50 mM final concentration), the reaction mixtures were subjected to SDS-PAGE and Coomassie Blue staining (A). B, following exposure of SC TeTx WT to 1 μg ml⁻¹ trypsin for 30 min at 37 °C, SDS-PAGE under reducing conditions was performed followed by transfer onto Immobilon-P membrane and protein staining with Ponceau red. The 100-kDa HC band was excised, and its N terminus was sequenced. After five rounds of Edman analysis, four amino acids were identified (marked with asterisks) giving a sequence SLTX (where X is an unidentified residue; D occurs at this position in the native toxin), which corresponds to residues 497–501 of TeTx, indicating that the Arg⁴⁹⁶-Ser⁴⁹⁷ bond is sensitive to trypsin. After mutagenesis to preclude cleavage at this position, tryptic digestion of the R496G mutant was performed (C), exactly as for TeTx WT in A.

**SC TeTx (6 μg/ml) was incubated for 30 min at 37 °C (in 10 mM HEPES-NaOH, pH 7.4, and 145 mM NaCl) with various trypsin concentrations; control samples, lacking the protease, were treated identically. After boiling for 5 min and addition of DTT (50 mM final concentration), the reaction mixtures were subjected to SDS-PAGE and Coomassie Blue staining (A). B, following exposure of SC TeTx WT to 1 μg ml⁻¹ trypsin for 30 min at 37 °C, SDS-PAGE under reducing conditions was performed followed by transfer onto Immobilon-P membrane and protein staining with Ponceau red. The 100-kDa HC band was excised, and its N terminus was sequenced. After five rounds of Edman analysis, four amino acids were identified (marked with asterisks) giving a sequence SLTX (where X is an unidentified residue; D occurs at this position in the native toxin), which corresponds to residues 497–501 of TeTx, indicating that the Arg⁴⁹⁶-Ser⁴⁹⁷ bond is sensitive to trypsin. After mutagenesis to preclude cleavage at this position, tryptic digestion of the R496G mutant was performed (C), exactly as for TeTx WT in A.

**FIG. 4.** Nicked R496G and native TeTx are equipotent in blocking transmitter release from rat cerebellar neurons: nicking enhances the inhibitory activity, which is absent from the E234A mutant. Cerebellar neurons, prepared as outlined under “Materials and Methods” and maintained for 10 days in vitro, were washed using KRH and exposed to the specified concentrations of native TeTx (○), EK-nicked TeTx R496G (□), SC TeTx R496G (■), or EK-nicked TeTx E234A (◇). After 30 min at 37 °C, the wells were washed twice as above and incubated for a further 30 min before replacement of the buffer with KRH containing a glutamate precursor, ¹⁴C-labeled glutamine, which was used for the measurement of glutamate release, as detailed under “Materials and Methods.” Data presented are means ± S.D. (n = 3 or 6).

**FIG. 5.** Innocuous, protease-inactive TeTx E234A antagonizes neuromuscular paralysis caused by native toxin as effectively as HC. For assessing competition, mouse hemi-diaphragms were incubated for 1 h at 4 °C in MKR containing 0.1% BSA only, or the latter plus 100 nM nicked E234A (closed bar) or 100 nM HC (open bar), before the addition of 1 nM native TeTx. Following 30-min exposure to the latter, the tissues were washed three times with MKR and then twice with KR. The temperature was raised to 37 °C, the nerve was stimulated (0.2 Hz, 1.5–2.5 V), and the evoked muscle twitch was recorded, as outlined under “Materials and Methods.” The times taken for each nerve-muscle to be reduced to 10% of the original tension were recorded, and the values for the antagonists were plotted relative to that for TeTx alone.

ingly, SC- and EK-nicked E234A proved to be devoid of toxicity in mice (Table I) and unable to inhibit transmitter release at the neuromuscular junction (Table I) or from cerebellar neurons (Fig. 4). In view of the intended use of E234A as a central nervous system-targeted drug vehicle, we measured the abilities of SC or nicked E234A to antagonize lethal challenges of native TeTx (either 3 or 10 LD₅₀) in mice. Importantly, SC-E234A completely prevented tetanus poisoning caused by 3 LD₅₀ units of native toxin when the largest dose was used, and 300- to 900-fold molar excess significantly delayed the onset of symptoms (Table II). The observed concentration dependence of the ability of E234A to delay/prevent onset of tetanus would be expected for an antagonist competing with TeTx for binding
to its neuronal acceptors. EK nicking of E234A prior to injection gave improved antagonism; even with a challenge of 10 LD50 units of toxin that killed 50% of the injected mice within 4 days.

* This is the detection limit of the RP-HPLC assay; no proteolysis of HV62 was observed using prolonged incubations.

# Properties of Single-chain Activable TeTx and an Atoxic Mutant

## Table I

| Purified TeTx preparations | Initial rate of cleavage of HV62 | Mouse lethality (LD50) | Time for 10 min to cause 90% neuromuscular paralysis |
|----------------------------|---------------------------------|-----------------------|-----------------------------------------------------|
|                            | [relative rate (%)]             | [units/mg]            | [min]                                               |
| Native TeTx                | 20.3 ± 0.9 [100]                | 1 × 10^6              | 145                                                 |
| Un-nicked-SC WT            | 8.0 ± 0.0 [39]                  | 0.5 × 10^6            | 260                                                 |
| Nicked-SC WT R496G         | 22.7 ± 3.4 [112]                | 1 × 10^6              | 150                                                 |
| Un-nicked-SC E234A         | 11.7 ± 0.6 [58]                 | 0.5 × 10^6            | 250 ± 15                                            |
| Nicked-SC E234A            | 52.3 ± 4.9 [258]                | 1 × 10^6              | 135 ± 10                                            |
| Un-nicked-SC E234A         | ≤0.01 [≤0.05]                   | Not tested            | No activity                                          |
| Nicked-SC E234A            | ≤0.01 [≤0.05]                   | ≤50                   | No activity                                          |

*—Un-nicked and nicked samples were shown to be completely in the SC and dichain form, respectively, both before and after incubation with substrate, by SDS-PAGE and protein staining.

## Table II

| TeTx | Competitor | Molar excess of competitor | Hours before symptoms appeared |
|------|------------|----------------------------|-------------------------------|
| SC E234A | HC | 9000 | 22 ± 2 |
| SC E234A | Nic E234A | 300 | 36 |
| SC E234A | SC E234A | 300 | 41 ± 1 |
| SC E234A | Nic E234A | 9000 | 52 ± 4 |
| SC E234A | SC E234A | 9000 | No symptoms |
| Nic E234A | Nic E234A | 9000 | No symptoms |
| SC E234A | HC | 9000 | 16 |
| SC E234A | SC E234A | 9000 | 45 ± 5 |
| SC E234A | Nic E234A | 9000 | 81 ± 14 |

*—Competitor was mixed with native TeTx in 0.9% (w/v) sterile NaCl solution containing 0.25% human serum albumin, prior to subcutaneous injection into mice (T/O, ~20 g) at the base of the neck.

*—Values given are the averages from two experiments (± difference, not shown when values were equivalent) except for * (n = 4; ± S.D.); * animals monitored continuously for 96-h post-injection.

## DISCUSSION

Induction of spastic paralysis by TeTx is mediated by a variety of functionally important, neuronal components and poorly understood protein trafficking processes (4, 5); all these can be investigated by employing TeTx or derivatives as unique probes. A pre-requisite for such studies is an efficient means of producing adequate quantities of recombinant fully active TeTx, as well as variants lacking one or more of its above-noted activities. Thus, a new strategy was devised herein for fast and efficient expression of soluble TeTx as a tagged single chain in E. coli, which not only obviates the time-consuming and costly alternative of anaerobically culturing C. tetani but also facilitates a genetic engineering approach to preparing novel TeTx forms.

Recombinant TeTx was produced in high yield (typically ~9 mg/liter) and easily isolated as a soluble concentrated protein (~4 mg/ml) devoid of contaminants, degradation, or premature-translation products, as determined by SDS-PAGE followed by protein staining and Western blotting. The purified toxin was exclusively in the SC form, and its controlled nicking with EK in vitro gave quantitative conversion to dichain with its disulfide link intact. Most importantly, the nicked toxin matched or exceeded the biological properties of native TeTx in terms of specific activities for its proteolytic and neuroparalytic actions, as well as lethality due to spastic paralysis in mice. Thus, this is the first recombinant preparation of TeTx with abilities to undergo all of the above-mentioned steps required to elicit the symptoms of tetanus, with the same potency as the natural toxin. These much needed advances avoid all the reported difficulties of minimal solubility, aggregation, and low yields when expressing or handling individual HC and LC in the absence of fusion tag (9, 14, 32) and the additional losses during their reconstitution as well as the reduced biological activity of the resultant dichain (9, 14, 19). The much lower specific neurotoxicity of the latter suggests that suboptimal, independent folding of the separate domains occurs and that
Properties of Single-chain Activable TeTx and an Atoxic Mutant

Fig. 6. Protease inactive E234A antagonizes the inhibition of neurotransmitter release by native TeTx from cerebellar neurons without being detrimental to the cells like HC. To measure competition, cerebellar neurons (cultured as in Fig. 4) were washed twice with ice-cold KRH buffer containing 5 mM Mg²⁺ and 0.5 mM Ca²⁺ (i.e. conditions that minimize endocytosis and, thereby, reduce internalization of bound toxin) and exposed in the same medium to the specified concentrations of potential TeTx antagonists for 1 h at 37 °C. A, neurons pre-treated with SC- (○) or EK-nicked (●) E234A were incubated with 0.2 nM native TeTx for 30 min at 4 °C prior to quantifying transmitter release. B, basal (open bars) and 50 mM KCl stimulated (hatched bars) exocytosis were determined in the presence or absence of the E234A or HC. C, cells pre-equilibrated with or without (Control) competitor were incubated for 30 min at 4 °C in the absence (closed bars) or presence (open bars) of 0.2 nM native TeTx. Next, the neurons were washed three times with ice-cold KRH and incubated for 30 min at 37 °C in normal KRH prior to determination of the K⁺-evoked Ca²⁺-dependent component of glutamate release (means ± S.D.) for two separate experiments (n = 6 or 12). D, the concentration dependences for antagonism of TeTx-induced inhibition of transmitter release by SC- (○) or EK-nicked (●) E234A compared with free HC (●). Data in panels A and C were used to calculate the percent inhibition values as follows: [(competitor only − 0.2 nM TeTx only) − (competitor + 0.2 nM TeTx)]/(competitor only − 0.2 nM TeTx only).

the optimal surfaces for interaction between HC and LC of TeTx are only created upon folding of the intact SC, as occurs in C. tetani. Thus, this first high level production of soluble, intact TeTx by recombinant means represents a major advance.

Availability of SC TeTx that could be readily converted to the dichain allowed an investigation of the effects of nicking on each stage of the intoxication process. In terms of the intracellular phases of action, dichain TeTx proved 2-fold more effective than SC in the proteolytic cleavage of synaptobrevin; this finding dismisses the widely held notion that nicking is essential for the enzymic activity (reviewed in Ref. 33). Such a minimal increase in the protease activity upon nicking contrasts markedly with the absolute requirement for reduction of the inter-chain disulfide (Table I (11)). The crystal structure of BoNT/A reveals that its active site is occluded by a loop between the LC and the translocation domain of HC (34); elucidation of the three-dimensional structure of TeTx should, likewise, give a basis for the need to reduce the interchain disulfide, as well as for the slight enhancement brought about by nicking. The relative affinity of SC- and EK-nicked TeTx for acceptor binding was tested by using the recombinant protein to antagonize the productive interaction of native TeTx with acceptors. This protocol required the development of a recombinant TeTx isoform that retains the binding properties of the WT while being devoid of toxicity, an achievement accomplished by site-directed mutagenesis, expression, and purification of SC TeTx E234A. The resultant mutant proved to be unable to cleave synaptobrevin, inhibit transmitter release, or cause neuromuscular paralysis and lacked neurotoxicity in mice, consistent with the known absence of these activities from dichain reconstituted from mutated LC E234A and purified native HC (9, 14). Clearly, TeTx E234A retains high affinity for binding to the ecto-acceptors on both peripheral and central neurons because of its potent antagonism of TeTx-induced neuromuscular paralysis and inhibition of transmitter release from cerebellar granule cells. Importantly, both SC and dichain TeTx E234A attenuated the induction by native toxin of spastic paralysis in mice. Although nicking only slightly improved this antagonism, the importance of this remains unclear, because nicking has been shown to occur in vivo (35). SC TeTx is clearly susceptible to nicking in vitro, and such cleavage was observed with cultured neurons (data not shown); thus, it seems likely that the reduced paralytic activity recorded for SC TeTx WT result from partial nicking by cellular and/or tissue proteases. In support of the latter, native TeTx is readily nicked following exposure to brain tissue, by enzymes released following tissue damage, and during or after its retrograde axonal transport to the spinal cord (35). Thus, to elucidate the importance of nicking in the internalization and intracellular poisoning steps, it is desirable to create a variant resistant to proteases in vivo. Identification of a scissile bond in SC TeTx and its mutation (Arg⁴⁹⁶→Gly) removed one cleavage site but, at least, two other trypsin nicking sites remained, and TeTx R496G was as toxic as WT in vitro and in vivo. Moreover,
Krieglstein and co-workers (31) revealed numerous peptide bonds in the LC-HC linker of native TeTx susceptible to other proteases. Nevertheless, by the gradual replacement of amino acids in protease-labile bonds with other less sensitive pairings, this protein engineering approach may eventually generate a SC with significantly increased resistance to activation in vivo. Such an achievement in the future would attenuate the safety hazard of preparing TeTx.

Exclusive removal of the protease activity leaves intact the domains responsible for all the internalization and retrograde trafficking, thus affording potential application of TeTx E234A as a neuronally targeted transporter for the delivery of research or therapeutic agents into the central nervous system. Importantly, the observed striking superiority of E234A over HC in protecting mice from TeTx demonstrates that the latter fragment cannot efficiently target the receptor responsible for HC in protecting mice from TeTx. This demonstrates that the latter fragment cannot efficiently target the receptor responsible for the retrograde trafficking pathway in vivo. In contrast, the binding component, which leads to local release of TeTx into the cytosol and possesses the N-terminal moiety of HC that is cytosol and possesses the N-terminal moiety of HC that is easily metabolized (41). An additional advantage of the SC TeTx E234A is that it retains the capability to translocate its LC across the membrane into the neuronal cytosol and possesses the N-terminal moiety of HC that is essential for cytosolic delivery of the LC (at least) via a putative low pH-induced membrane penetration event (42); also, the disulfide linking LC to HC (in both TeTx and BoNT/A) is crucial for internalization (36, 37, 43). Furthermore, the SC TeTx expression strategy allows ligation of the DNA encoding a therapeutic protein to the 5’-end of the SC TeTx E234A transporter gene, prior to high yield bacterial expression; the protein adduct attached to the LC would not only be transported to central neurons, but could “piggy-back” into the cytosol of its target cell. Finally, the transporter can be optimized by downsizing the LC to afford the largest cargo while retaining the stability, longevity, neuronal targeting, and delivery properties of TeTx in vivo.

REFERENCES
1. Stockel, K., Schwab, M., and Thoenen, H. (1975) Brain Res. 99, 1–16
2. Ugolini, G. (1995) in Viral Vectors: Gene Therapy and Neuroscience Applications (Loewy, A. D., and Kaplioti, M. G., eds) pp. 293–317, Academic Press, New York
3. Dolly, J. O. (1990) in Neurornuscular Transmission Basic and Applied Aspects (Vincent, A., and Wray, D. W., eds) pp. 107–131, Manchester University Press, Manchester
4. Price, D. L., Griffin, J., Young, A., Peck, K., and Stocks, A. (1975) Science 188, 945–947
5. Wellhuner, H. H. (1992) in Handbook of Experimental Pharmacology (Herken, H., and Hacho, F., eds), Vol 102: Selective Neurotoxicity, pp. 357–417, Springer-Verlag, Berlin
6. Herrero, J., Lalli, G., Montecucco, C., and Schiavo, G. (2000) J. Neurochem. 74, 1941–1950
7. Lalli, G., Herrero, J., Osborne, S. L., Montecucco, C., Rossetto, O., and Schiavo, G. (1999) J. Cell Sci. 112, 2715–2724
8. Montecucco, C. (1986) Trends Biochem. Sci. 11, 314–317
9. Li, Y., Aoki, R., and Dolly, J. O. (1999) J. Biochem. 125, 1200–1208
10. Link, E., Edelmann, L., Chou, J. H., Bina, T., Yamazaki, S., Eisel, U., Baumert, M., Stidhof, T. C., Niemann, H., and Jahn, R. (1992) Biochem. Biophys. Res. Commun. 189, 1017–1023
11. Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., De Laurenti, P. P., Dicie, G. (1986) J. Neurochem. 45, 1040–1047
12. Habig, W. H., Keinumen, J. G., and Hadegree, M. C. (1983) in Biochemical and Biophysical Studies of Proteins and Membranes (Lui, T. Y., ed) pp. 463–473, Elsevier, New York
13. Schwab, M. E., Suda, K., and Thoenen, H. (1979) J. Cell Biol. 82, 788–810
14. Li, Y., Foran, P., Fairweather, N. F., de Paiva, A., Weller, U., Dougan, G., and Dolly, J. O. (1994) Biochemistry 33, 7214–7220
15. Bizzini, B., Grob, F., and Arti, K. (1981) Brain Res. 210, 291–309
16. Coen, L., Osta, R., Maury, M., and Brulet, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9400–9405
17. Figueiredo, D. M., Hallewell, R. A., Chen, L. L., Fairweather, N. F., Dougan, G., Savitt, J. M., Parks, D. A., and Fishman, P. S. (1997) Exp. Neurol. 145, 546–554
18. Fishman, P. S., Savitt, J. M., and Farrant, D. A. (1990) J. Neurosci. 9, 311–315
19. Weller, U., Dauenroth, M.-E., Meyer zu Heringdorf, D., and Habermann, E. (1989) Eur. J. Biochem. 182, 649–656
20. Fishman, P. S., Parks, D. A., Patwardhan, A. J., and Matthews, C. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10156–10161
21. Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
22. Figueiredo, D., Turcotte, C., Frankel, G., Li, Y., Dolly, O., Wilkin, G., Marriot, D., Fairweather, N., and Dougan, G. (1995) Infect. Immun. 63, 3218–3221
23. Matsuda, M., Sugimoto, N., Otsutumi, K., and Hirio, T. (1982) Biochem. Biophys. Res. Commun. 104, 799–805
24. Figueiredo, D., Hallewell, R. A., Chen, L. L., Fairweather, N. F., and Dougan, G. (1990) J. Pharmacol. Exp. Ther. 252, 218–222
25. Montecucco, C., and Schiavo, G. (1993) Trends Biochem. Sci. 18, 324–327
26. de Paiva, A., Ashton, A. C., Foran, P., Schiavo, G., Montecucco, C., and Dolly, J. O. (1993) J. Neurochem. 61, 2338–2341
27. Maisey, E. A., Wadsworth, J. D. F., Poulain, B., Shone, C. C., Melting, J., Gibbs, P., Tauc, L., and Dolly, J. O. (1988) Eur. J. Biochem. 177, 663–691
28. Cambray-Deakin, M. A. (1995) in Neural Cell Culture: A Practical Approach (Cohen, J., and Wilkin, G., eds) pp. 3–13, IRL Press, Oxford, UK
29. Gallo, V., Ciotti, M., Torelli, A., Aloisi, F., and Levi, G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7919–7923
30. Krieglstein, K. G., Hensch, A. H., Weller, U., and Habermann, E. (1991) Eur. J. Biochem. 202, 41–51
31. Fairweather, N. F., Sanders, D., Slater, D., Hude, M., Habermann, E., and Weller, U. (1993) FEBS Lett. 323, 218–222
32. Montecucco, C., and Schiavo, G. (1993) Trends Biochem. Sci. 18, 324–327
33. Lacy, D. B., Tepp, W., Cohen, A. C., Das Gupta, B. R., and Stevens, R. C. (1998) Nat. Struct. Biol. 5, 890–896
34. Habermann, E., Weller, U., and Hude, M. (1991) Naunyn-Schmiedebergs Arch. Exp. Pharmacol. 343, 323–329
35. de Paiva, A., Poulain, B., Lawrence, G. W., Shone, C. C., Tauc, L., and Dolly, J. O. (1995) J. Biol. Chem. 269, 20583–20584
36. Dolly, J. O., de Paiva, A., Foran, P., Lawrence, G., Daniels-Hogke, P., and Ashton, A. C. (1994) Semin. Neurosci. 6, 149–158
37. Daniels-Hogke, P. U., and Dolly, J. O. (1996) J. Neurosci. Res. 44, 263–271
38. Hogy, B., Dauenroth, M. E., Hude, M., Weller, U., and Habermann, E. (1992) Toxicon 30, 63–76
39. Bizzini, B., Stoeckel, K., and Schiavo, M. (1975) J. Neurochem. 28, 529–542
40. Francis, J. W., Huether, B. A., Brown, R. H., Jr., and Fishman, P. S. (1995) J. Biol. Chem. 270, 15434–15442
41. Schiavo, G., Demel, R., and Montecucco, C. (1991) Eur. J. Biochem. 199, 905–911
42. Schiavo, G., Papini, E., Genna, G., and Montecucco, C. (1990) Infect. Immun. 58, 4136–4141
Recombinant Forms of Tetanus Toxin Engineered for Examining and Exploiting Neuronal Trafficking Pathways

Yan Li, Patrick Foran, Gary Lawrence, Nadiem Mohammed, Chan-Ka-Ning Chan-Kwo-Chion, Godfrey Lisk, Roger Aoki and Oliver Dolly

J. Biol. Chem. 2001, 276:31394-31401.
doi: 10.1074/jbc.M103517200 originally published online June 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103517200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 9 of which can be accessed free at http://www.jbc.org/content/276/33/31394.full.html#ref-list-1