Tyrosine-1290 of tetanus neurotoxin plays a key role in its binding to gangliosides and functional binding to neurones

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Abstract Tetanus toxin acts by blocking the release of glycine from inhibitory neurones within the spinal cord. An initial stage in the toxin's action is binding to acceptors on the nerve surface and polysialogangliosides are a component of these acceptor moieties. Using site-directed mutagenesis, we identify tyrosine-1290 of tetanus toxin as a key residue that is involved in ganglioside binding. This residue, which is located at the centre of a shallow pocket on the β-trefoil domain of the tetanus H_c fragment, is also shown to play a key role in the functional binding of tetanus toxin to spinal cord neurones leading to the inhibition of neurotransmitter release. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Tetanus; Botulinum; Ganglioside; Neurotoxin; Receptor; Motoneuron

1. Introduction

Tetanus toxin (TeNT) and the seven botulinum toxin serotypes (BoNTs) make up the clostridial neurotoxin family which act by blocking neurotransmitter release [1,2]. The BoNTs act primarily at the neuromuscular junction which results in the widespread flaccid paralysis characteristic of botulism. TeNT, in contrast, while initially binding to the peripheral nervous system, is retrogradely transported to the central nervous system where it blocks glycine release from inhibitory interneurones. It is this different trafficking of TeNT that give rise to the spastic paralysis observed with tetanus intoxication and distinguishes it from the BoNTs.

TeNT and the BoNTs are structurally similar, they have 30–40% sequence homology and each neurotoxin consists of a heavy chain (100 kDa) and a light chain (50 kDa) linked by a disulphide bridge [1]. The neurotoxins exert their biological effects on their target nerve cell via similar multi-step mechanisms [1,2]. Following a binding step, in which the neurotoxins bind to ecto-acceptors on the presynaptic nerve surface [3], the toxins are internalised and translocated within the nerve ending. Once inside the nerve terminal, each toxin mediates its effect via a highly specific zinc-dependent endopeptidase activity directed at small proteins (either VAMP/synaptobrevin, SNAP-25 or syntaxin) involved in the fusion and release of synaptic vesicles [2]. Domains within the C-terminal 50 kDa fragment (H_c fragment) of each neurotoxin heavy chain play the primary role in acceptor binding [3,4] while the N-terminal 50 kDa of the heavy chain is involved in translocation of the enzymically active light chain to within the nerve terminal [5].

The isolated H_c fragment of TeNT retains the high affinity ($K_d < 10^{-9}$ M) neuronal binding properties of the holotoxin and has been used in a variety of studies aimed at characterising the nature of the acceptor [6–8]. A number of reports have demonstrated that TeNT binds to the polysialic gangliosides, GD_{1b} and GT_{1b}, but not the monosialic acid ganglioside GM_1 (reviewed in [1]). Recently it has been demonstrated that ganglioside biosynthesis in primary spinal cord neurones is essential for their intoxication with TeNT [9]. While polysialogangliosides are clearly implicated as a component of the neurotoxin acceptor, the protease sensitivity of TeNT binding to both synaptosomes and neuronal cells also suggests the involvement of a protein component [10] and a two-component acceptor model has been proposed [1,11]. A 15 kDa glycoprotein, which is present in both toxin-sensitive PC12 cells and spinal cord neurones, has been shown to interact with the C-terminal domain of TeNT H_c fragment by cross-linking experiments and has been proposed as the protein acceptor component for TeNT [12,13].

Crystal structures of TeNT H_c [14], BoNT/A [15] and BoNT/B [16] reveal two distinct domains within the H_c fragment of each toxin: a lectin-like jelly-roll domain (residues 865–1110 of TeNT) and a β-trefoil domain (residues 1125–1315) (Fig. 1). Previous studies on TeNT have demonstrated that only the β-trefoil domain is required for cell and ganglioside binding and photoaffinity labelling studies with ganglioside GT_{1b} have implicated H1293 in the vicinity of the ganglioside binding site [17]. In a recent study which characterised the binding properties of TeNT H_c mutants, it was shown that a H1293 mutant in which the ganglioside binding was greatly reduced still demonstrated retrograde transport within motor neurones [18]. These authors concluded that other regions within the C-terminal domain are involved in cell binding leading to retrograde transport.

In the present study, we have sought to clarify the regions of TeNT H_c involved in ganglioside activity with respect to the biological action of the neurotoxin. Site-directed mutants have been constructed within the C-terminal region of TeNT H_c and characterised with respect to their binding to synaptic membranes, gangliosides and spinal cord neurones. We iden-

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Abbreviations: TeNT, tetanus neurotoxin; BoNT, botulinum neurotoxin; TeNT H_c, C-terminal 50 kDa fragment of tetanus neurotoxin

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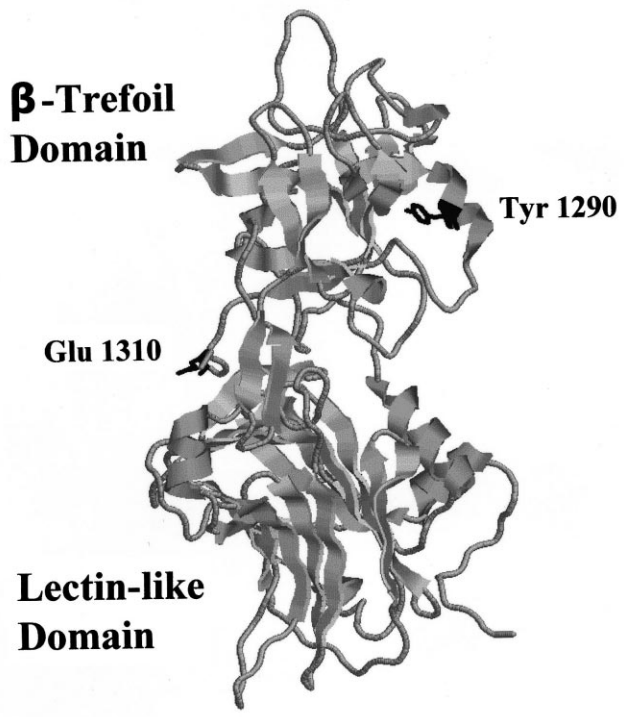

Fig. 1. TeNT H_c structure. Three-dimensional structure of TeNT H_c fragment showing the β-trefoil and lectin-like domains. The positions of tyrosine-1290, located within helix-5 [20], and glutamate-1310, located within the C-terminal domain, are also shown.

tify a tyrosine residue which plays a critical role in both the binding of TeNT to gangliosides and its functional binding to spinal cord neurones leading to the inhibition of calcium-dependent glycine release.

2. Materials and methods

2.1. Production and purification of TeNT H_c fragment mutants

A fully synthetic TeNT H_c gene was synthesised using a codon bias optimised for expression in *Escherichia coli*. Restriction sites *Bam*HI and *Xba*I were incorporated at the 5′ and 3′ ends, respectively, to allow subcloning into expression vectors. Site-directed mutants were constructed by PCR or splice overlap PCR using mutagenic primers [19] and their sequences confirmed. H_c-encoding fragments were subcloned into the vector pMAL-c2x (New England Biolabs) and expressed as maltose binding protein (MBP) fusions in the host strain BL21 (Novagen). A single colony was inoculated into 200 ml of Terrific Broth (24 g/l yeast extract, 12 g/l tryptone, 9.4 g/l KH_2PO_4, 2.2 g/l K_2HPO_4, pH 7.2) supplemented with 100 μg/ml ampicillin and 0.5% (w/v) glucose and grown overnight at 30°C in a shaking incubator. Cultures were diluted 1:6 with fresh medium and grown to an OD_{600} of approximately 2.0 at 30°C. IPTG was added (500 μM) and cultures grown for 2 h at 25°C. Cell pellets were resuspended in 20 mM Tris–HCl pH 7.4, 500 mM NaCl, 1 mM EDTA (binding buffer) and lysed by sonication. Cell debris was removed by centrifugation at $27\,000 \times g$ and the supernatant fluid applied to a 5 ml amylose resin (NEB) column equilibrated in the binding buffer. After washing with binding buffer, the fusion protein was eluted with binding buffer supplemented with 10 mM maltose. MBP was removed from fusion proteins by treatment with factor Xa protease (1 U/mg) at 20°C for 18 h in the elution buffer. TeNT H_c fragments were purified by anion exchange (Mono-Q) chromatography and then dialysed against 20 mM HEPES pH 7.4 containing 200 mM NaCl.

2.2. Radiolabelling of TeNT

TeNT, purified by ion exchange chromatography [20], was dialysed against 0.1 M borate buffer pH 8.5 and then 100 μl containing 300 μg TeNT was reacted with dried Bolton and Hunter reagent (37 MBq; Amersham Pharmacia Biotech) for 15 min on ice with gentle agitation. Unreacted reagent was separated from the ^{125}I-labelled TeNT by gel filtration on Sephadex G25 (PD10 column, Pharmacia) equilibrated in 0.05 M HEPES, 0.2 M NaCl pH 7.4.

2.3. Binding to rat brain synaptic membranes

Rat cerebrocortical synaptosomes were purified as described previously [4] and diluted in 0.05 M HEPES pH 7.4 buffer to a final concentration of 2 mg/ml synaptosomal protein. Binding assays were performed in either 0.05 M HEPES pH 7.4 buffer containing 0.6% bovine serum albumin (BSA) (HEPES binding buffer) or Hanks' pH 7.4 buffer containing 0.6% BSA (Hanks' binding buffer). Binding assay incubations (0.2 ml) contained a final concentration of 2 nM ^{125}I-labelled TeNT and varying concentrations of TeNT H_c fragments. Assays performed in HEPES binding buffer were initiated by the addition of synaptosomal membranes to a final concentration of 1 μg/ml synaptosomal protein and those in Hanks' buffer by the addition of 100 μg/ml synaptosomal protein. Assays were incubated for 2 h at 2°C and then washed twice with 1 ml aliquots of the respective binding buffer and radioactivity in the synaptosome pellet determined [4].

2.4. Binding to gangliosides

Gangliosides GT_{1b} or GM_1 (>98% pure; Research Biochemicals International) were diluted to 20 μg/ml in methanol, added to microtitre plates (50 μl/well) and allowed to dry for 16 h at 22°C. Plates were then blocked with HEPES binding buffer for 1 h at 37°C before being washed six times with phosphate-buffered saline, pH 7.4. Binding assay mixtures containing 2 nM ^{125}I-labelled TeNT and various concentrations of unlabelled TeNT H_c fragments in HEPES binding buffer were added (50 μl/well) and incubated for 1 h at 22°C. Plates were then washed with 2×150 μl of HEPES binding buffer and the radioactivity bound to plate wells determined.

2.5. Measurement of glycine release from embryonic spinal cord neurones

Embryonic spinal cord (eSC) neurones were prepared and maintained as described previously [21]. For competition experiments, 21 day old eSC neurones were incubated with 150 pM TeNT with or without TeNT H_c fragments for 30 min at 37°C in medium containing 5% donor horse serum. The toxin was removed and the cells washed twice with medium and incubated for a further 20 h at 37°C. The eSC neurones were then loaded with [^{3}H]glycine prior to the determination of basal and potassium-stimulated release of the transmitter as described [21].

3. Results and discussion

3.1. Effect of TeNT H_c mutants on binding to brain synaptosomal membranes

The acceptor binding efficiencies of mutants were evaluated initially in competition experiments with ^{125}I-labelled TeNT to rat brain synaptosomes. Previous studies have shown that at low ionic strength, TeNT binds to a neuraminidase-sensitive, ganglioside-like acceptor on synaptic membranes which is largely insensitive to proteases, while in physiological buffers, TeNT binds at lower density to acceptors which are sensitive to both neuraminidase and proteases which suggests the involvement of a protein acceptor component [10]. Binding of TeNT H_c mutants to synaptosomes was therefore evaluated under both buffer conditions. Competition experiments were performed using native and mutant H_c fragments both as MBP fusions and after removal of MBP. No differences in their binding characteristics were observed.

A number of studies have illustrated the importance of the C-terminal region of the β-trefoil domain of the TeNT H_c fragment, so site-directed mutants were initially conducted in this region. Table 1 summarises the effects of various site-directed mutants on acceptor binding properties of TeNT H_c

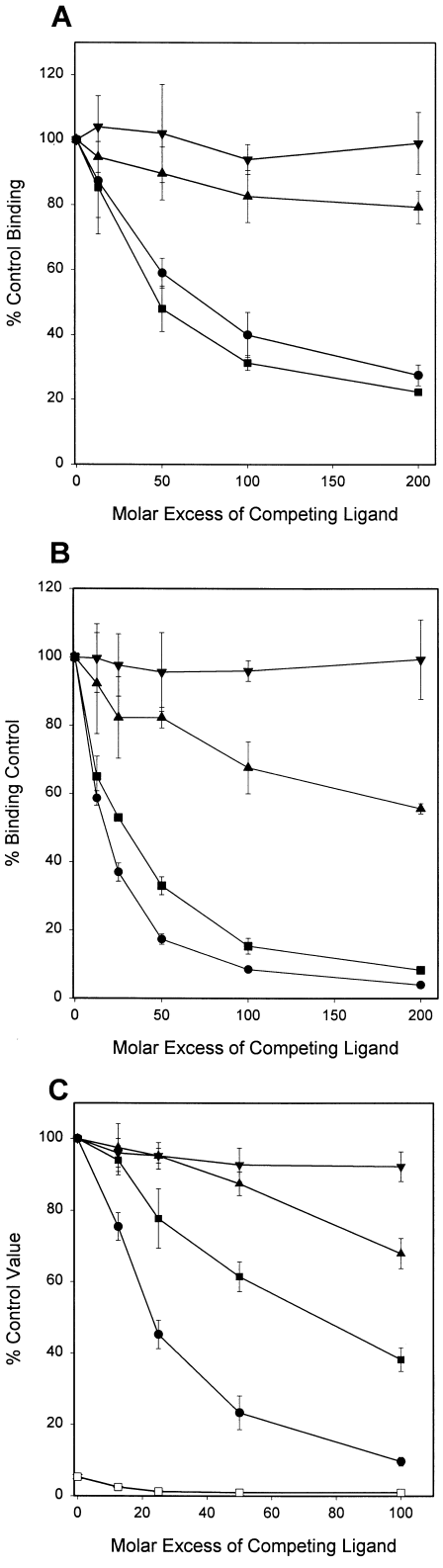

Fig. 2. Binding of TeNT H_c mutants to synaptic membranes and gangliosides. Competitive binding experiments were performed using a fixed concentration of ^{125}I-labelled TeNT (2 nM) and various concentrations of competing ligands. A: Binding was carried out in Hanks' buffer pH 7.4 containing 0.6% BSA to rat brain synaptic membranes. Competing ligands were: native TeNT H_c (●), K1295A (■), E1310A (▲), Y1290A (▼). B: Binding was carried out in 0.05 M HEPES buffer pH 7.4 containing 0.6% BSA to rat brain synaptic membranes. Competing ligands were: native TeNT H_c (●), Y1290F (■), Y1290A (▲), Y1290S (▼). C: Binding to ganglioside GT_{1b} in 0.05 M HEPES buffer pH 7.4 containing 0.6% BSA. Competing ligands were: native TeNT H_c (●), Y1290F (■), Y1290A (▲), Y1290S (▼). The relative binding to GM1 ganglioside using TeNT H_c as competing ligand is also shown (□). Each graph represents data from one experiment in quadruplicate with S.D. In each case, quantitatively similar results were obtained in two further experiments.

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mutants to brain synaptic membranes. Several alanine substitutions were made in the region 1305–1310, close to the C-terminus, which has been reported to be critical to acceptor binding [3]. Of these, E1310A was found to significantly reduce the binding. This was more marked when binding assays were performed under physiological conditions in Hanks' buffer (Fig. 2A). Other mutants made in this C-terminal region had little or no effect on binding. Inspection of the three-dimensional structure of TeNT H_c fragment reveals that the C-terminal eight residues border one side of a shallow surface pocket which is defined on other sides by strands β17, β22 and loops preceding these strands [14]. Alanine mutants made to these regions were found to have little of no effect on synaptosome binding. Since, of the mutants analysed, only E1310A significantly reduced the binding of TeNT H_c to synaptic membranes, more conservative mutations were made to this and the adjacent charged residue (D1309) to further analyse their role in binding. Neither the E1310Q nor the D1309N mutation was found to have any effect on synaptosome binding (Table 1a). Collectively, these data argue against a direct role for C-terminal residues 1305–1310 in acceptor binding.

Previous studies employing photoaffinity labelling have implicated H1293 in ganglioside binding, and mutagenesis of this residue significantly reduced binding to gangliosides in solid-

Table 1
Binding of TeNT H_c mutants to synaptosomal membranes

H_c mutant	Binding relative to native TeNT H_c (mean values ± S.D.; $n = 4$)	
	HEPES buffer	Hanks' buffer
(a) C-terminal pocket mutants		
(i) Alanine mutations		
E1310→A	35 ± 13%	9.5 ± 2.9%
D1309→A	as native	as native
F1305→A	as native	as native
W1303→A	as native	35 ± 8%
R1168→A	as native	32 ± 4%
Y1170→A	as native	as native
(ii) Conservative mutations		
E1310→Q	as native	as native
D1309→N	as native	as native
E1310→Q; D1309→N	as native	as native
R1168→K	as native	as native
(b) Helix-5 region mutants		
(i) Alanine mutations		
Y1290→A	7.7 ± 1.5%	< 1%
N1292→A	as native	as native
K1295→A	as native	as native
K1297→A	as native	as native
(ii) Conservative mutations of Y1290		
Y1290→F	42 ± 11%	54 ± 3.5%
Y1290→S	< 3%	< 1%

phase assays [17,18]. To investigate the role in acceptor binding of this region around helix-5 [14], which is located on the opposite side of the β-trefoil domain to the C-terminus, further point mutants were constructed in the vicinity of H1293 (Table 1b). Mutant Y1290A significantly reduced the binding to synaptosome membranes in assays performed using low ionic strength buffer at pH 7.4 (Fig. 2B) and in similar assays performed in Hanks' buffer, the binding of mutant Y1290A was abolished (Fig. 2A). Further substitutions of Y1290 were made to determine their effects on the acceptor binding. Mutant Y1290F showed approximately twofold reduced binding to synaptic membranes compared to control TeNT H_c, while in the case of Y1290S, binding was further reduced compared to the Y1290A mutant (Fig. 2B). In some competition experiments with Y1290S, the mutant appeared to increase the binding of ^{125}I-labelled TeNT compared to the control. This effect, however, was not statistically significant.

To assess the structural integrity of the Y1290 TeNT H_c mutants, circular dichroism (CD) spectroscopic analysis was performed in the far UV region. No significant differences in the CD spectra of the three Y1290 mutants were observed when compared to native TeNT H_c suggesting that little or no perturbation of the three-dimensional structure had been induced.

3.2. Ganglioside binding of the TeNT H_c Y1290 mutants

The reduction in synaptosome acceptor binding activity of the Y1290 mutants in HEPES (0.05 M, pH 7.4) binding buffer suggests a loss of ganglioside binding activity. To confirm this, mutants were assessed in competitive binding assays with ^{125}I-labelled TeNT to solid-phase GT_{1b} gangliosides and their binding compared with native TeNT H_c (Fig. 2C). The binding of Y1290 mutants to ganglioside was similar to that observed to synaptic membranes in the HEPES binding buffer. The binding of Y1290A and Y1290F was reduced to 16.1 ± 2.9% and 38 ± 2% (S.D.; $n = 4$) of the control value respectively. The binding of Y1290S was greatly reduced (< 2% of the control value). No specific binding of ^{125}I-labelled TeNT was observed in control experiments when GM_1 ganglioside was substituted for GT_{1b} ganglioside.

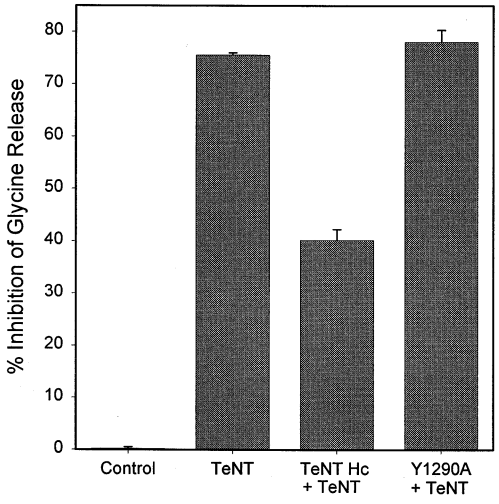

Fig. 3. Competition of TeNT H_c mutants with acceptors on spinal cord neurones. Inhibition of potassium-stimulated glycine release from eSC neurones was determined in the presence of TeNT and competing H_c fragments: no TeNT (control); 150 pM TeNT (TeNT); 150 pM TeNT+150 nM TeNT H_c (TeNT H_c+TeNT); 150 pM TeNT+150 nM Y1290A mutant (Y1290A+TeNT). The bar chart represents data from one experiment in quadruplicate with S.D. Quantitatively similar results were obtained in two further experiments.

The significant reductions in both GT_{1b} ganglioside and synaptosome binding observed with all Y1290 mutants suggest a key role for this residue in binding to the ganglioside. This residue is also highly conserved within the BoNTs of the clostridial neurotoxin family which also appear to bind a ganglioside as a component of the cellular acceptor [1]. Recently, a putative ganglioside binding site on the clostridial neurotoxins has be proposed by Ginalski et al. [22] using structure-based sequence alignment. Inspection of the TeNT H_c structure shows that Y1290 is orientated towards the centre of this shallow pocket which is located on the β-trefoil domain. In addition, H1293, which is also implicated in ganglioside binding [23], is located on the rim of this pocket. In a study in which TeNT H_c structure was determined in the presence of various sugar residues, lactose was found to bind to the proposed ganglioside binding pocket and form a hydrogen bond with the OH of Y1290 [23]. In summary, the data presented provide direct evidence for the location of the ganglioside binding pocket as proposed by Ginalski et al. [22] and suggest that Y1290 plays a key role in binding to the sugar.

3.3. Effect of mutants on the functional binding of TeNT to spinal cord neurones

TeNT binds to a diverse population of acceptors on neuronal tissue of which only a small proportion lead to the functional internalisation of the neurotoxin and subsequent blockade of neurotransmitter release. Studies were therefore undertaken using spinal cord neurones to determine whether or not Y1290 plays a role in the functional acceptor binding of TeNT as measured by its ability to block the calcium-mediated release of glycine. Incubation of spinal cord neurones with 150 pM TeNT inhibited 60–80% of the calcium-mediated glycine release. In the presence of an excess of native TeNT H_c as a competing ligand, the inhibitory effects of TeNT are reduced (Fig. 3). In similar experiments performed with the Y1290A mutant no competition with TeNT was observed which suggests that this mutation results in a loss of binding to the functional acceptor for TeNT.

In overall conclusion, data presented in the present study suggest a key role of Y1290 in the binding of TeNT to its neuronal acceptor. This residue lies at the heart of a shallow pocket on the β-trefoil domain of TeNT H_c proposed as a putative acceptor binding site [22] and the data presented here provide the first direct evidence for a role of this pocket in ganglioside binding. Furthermore, Y1290 is also shown to play a critical role in the binding of TeNT to its functional acceptors on spinal cord neurones.

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