C-terminal half of tetanus toxin fragment C is sufficient for neuronal binding and interaction with a putative protein receptor

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Tetanus neurotoxin (TeNT) is a powerful bacterial protein toxin that cleaves VAMP/synaptobrevin, an essential protein of the synaptic vesicle fusion machinery, and consequently blocks neurotransmission. The extreme neurospecificity of TeNT is determined by the binding of its C-terminal domain (fragment C or H_C) to neuronal receptors. Whereas polysialogangliosides are known acceptors for the toxin, the existence of additional protein receptors has also been suggested. We have reported previously on a 15 kDa cell-surface glycoprotein that interacts with TeNT in neuronal cell lines and motoneurons [Herreros, Lalli, Montecucco and Schiavo (2000) J. Neurochem., in the press]. Here, on the basis of the structural information provided by the crystallization of fragment C of TeNT, we have expressed its C- and N-terminal halves as recombinant proteins and analysed their binding abilities to rat phaeochromocytoma (PC12) cells differentiated with nerve growth factor. We found that the C-terminal subdomain of the fragment C of TeNT is necessary and sufficient for cell binding and for the interaction with the 15 kDa putative receptor. In contrast, the N-terminal half showed a very poor interaction with the cell surface. These results restrict the binding domain of TeNT to the C-terminal half of the fragment C and highlight the importance of this domain for the neurospecific interaction of the toxin with the synapse. Furthermore, these findings support the use of this portion of TeNT as a neurospecific targeting device, pointing to an involvement of the N-terminal subdomain in later steps of the intoxication pathway.

Key words: cross-linking, gel filtration, p15, PC12 cells.

INTRODUCTION

Tetanus neurotoxin (TeNT) is a bacterial protein of the family of the clostridial neurotoxins, which includes different serotypes (A to G) of botulinum neurotoxins (BoNTs). In the active form, clostridial neurotoxins consist of a heavy (100 kDa) and a light (50 kDa) chain linked by a disulphide bond (see Figure 1A) [1]. The heavy chains are responsible for neurospecific binding and internalization, whereas the light chains are metalloproteases that selectively cleave synaptic proteins, therefore inhibiting neurotransmitter release [2,3].

TeNT binds with low affinity to polysialogangliosides via the C-terminal fragment of the heavy chain (fragment C or H_C) [4]. This fragment is competent for binding and internalization [5] and it is retrogradely transported *in vivo* similarly to the holotoxin [6–8]. However, TeNT binding sites were shown to be partly sensitive to proteases [9–12], suggesting that protein co-receptor(s) for TeNT may exist. Cross-linking experiments have indicated the presence of a putative 15–20 kDa (p15) receptor protein for TeNT in rat phaeochromocytoma (PC12) cells differentiated with nerve growth factor (NGF) [13], in other neuronal cell lines and in rat motoneurons [14].

The crystal structures of TeNT H_C and of BoNT/A have been resolved [15–17]. The H_C fragments of TeNT and BoNTs are closely related and consist of two subdomains of almost identical size that are predicted to fold independently. The N-terminal subdomain (H_CN) structure is related to plant lectins, whereas the C-terminal subdomain (H_CC) exhibits a β-trefoil motif that is present in proteins involved in recognition and binding [15].

On the basis of the information provided by the crystal structure, we have expressed the H_C fragment of TeNT and its subdomains, H_CC and H_CN, as recombinant proteins. Here we characterize these proteins and the interaction between the two H_C subdomains, which show the ability to heterodimerize *in vitro*. By performing binding and cross-linking experiments in NGF-differentiated PC12 cells, we analyse the structural requirements for the neuronal binding of the H_C fragment of TeNT. We demonstrate that H_CC is necessary and sufficient for the interaction with the neuronal cell surface and with the putative receptor protein p15, indicating that this piece contains all the determinants for achieving full binding.

MATERIALS AND METHODS

Cloning and expression of TeNT H_C and its subdomains

Recombinant TeNT H_C was expressed and purified as described previously [5]. For the cloning of H_C subdomains, suitable primers corresponding to the C-terminal (5′-CATATGTCTATAACCTTTTTAAGAGACTTC-3′ and 5′-GTCGACTTAATCATTTGTCCATCCTTCATC-3′) and N-terminal (5′-CATATGTCAACACCAATTCCATTTTCT-3′ and 5′-GTCGACTTATAAATAATAACTTGTGTATAA-3′) subdomains were designed and the H_CN (residues 856–1110) and H_CC (residues 1111–1315) specific DNA species were amplified by standard PCR. PCR fragments were inserted into the PCR-Script cloning vector (Stratagene, La Jolla, CA, U.S.A.) and then subcloned

Abbreviations used: BoNT, botulinum neurotoxin; GST, glutathione S-transferase; H_C, C-terminal fragment of the heavy chain or fragment C; H_CC and H_CN, C- and N-terminal subdomains of TeNT H_C; NGF, nerve growth factor; PC12, rat phaeochromocytoma; TeNT, tetanus neurotoxin; VSV-G, vesicular stomatitis virus G-protein.

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into pGEX-4T3-VSV-G vector [5] in the *Nde*I/*Sal*I site following the vesicular stomatitis virus G-protein (VSV-G) epitope [18] and the consensus sequence for phosphorylation by protein kinase A [19]. The sequences of the inserts were checked by direct automated sequencing. Vectors containing the two subdomains were transformed into BL21(DE3)pLys (Stratagene). Glutathione S-transferase (GST) fusion proteins were expressed for 4 h at 30 °C in the presence of 0.4 mM isopropyl β-D-thiogalactoside. After cleavage with thrombin, the proteins were concentrated by dialysis against 15 % (w/v) poly(ethylene glycol) (Fluka, Gillingham, Dorset, U.K.) in 20 mM Hepes/NaOH (pH 7.4)/100 mM NaCl/0.1 mM dithiothreitol and then dialysed against 20 mM Hepes/NaOH (pH 7.4)/100 mM NaCl/0.1 mM dithiothreitol/10 % (v/v) glycerol before snap-freezing in liquid nitrogen and storage at −80 °C. In some cases the subdomains were further purified on anion-exchange (MonoQ) column (Amersham Pharmacia Biotech, Amersham, U.K.).

Radiolabelling

For phosphorylation by protein kinase A, 5 μg of protein was incubated (5 min, 30 °C) with 100 μCi of [γ-^{32}P]ATP (10 mCi/ml, 3000 Ci/mmol; Amersham Pharmacia Biotech) in reaction buffer [50 mM Mes/HCl (pH 6.9)/10 mM $MgCl_2$/1 mM dithiothreitol/0.5 mM EDTA/1 mg/ml ovalbumin] with 0.4 m-unit of the catalytic subunit of protein kinase A (4 m-units/μl; Roche Boehringer-Mannheim, Lewis, U.K.) diluted in 5 mM Mes/HCl (pH 6.9)/0.5 mM dithiothreitol/0.2 mM EDTA/0.5 mg/ml ovalbumin. Free [γ-^{32}P]ATP was separated from the labelled protein on PD-10 columns (Amersham Pharmacia Biotech) equilibrated with 0.1 % ovalbumin in PBS. The fraction containing the greatest amount of radiolabelled protein was stored at −80 °C. The specific radioactivity was approx. 1500–1800 Ci/mmol.

Gel filtration

A Superdex 200 HR 10/30 column (Amersham-Pharmacia Biotech) was equilibrated with Hanks solution (see below) free of phosphate and glucose and loaded with ^{32}P-H_C or ^{32}P-H_CC (40 nCi) premixed with unlabelled H_CC, H_CN or BSA (1.8–2.1 nmol). Analysis was performed by following the manufacturer's instructions and 0.4 ml fractions were collected. Elution profiles were determined by both UV absorbance and radioactivity detection in a β-counter (Beckman, Fullerton, CA, U.S.A.). Selected fractions were run on SDS/PAGE gels, stained with Coomassie Blue and exposed to X-ray film.

Cell culture

PC12 cells were cultured as described previously [20]. For cross-linking experiments, cells were seeded in 12-well plates (Costar-Corning, Cambridge, MA, U.S.A.) at a density of 25000 cells per well. After 24 h the medium was supplemented with 75 ng/ml NGF (7S; Alexis, San Diego, CA, U.S.A.). Cells were used after 7–8 days of treatment with NGF.

Binding and cross-linking experiments

For binding experiments, H_C or its subdomains (80 nM) were bound for 1 h on ice in Hanks buffer [1.26 mM $CaCl_2$/5.36 mM KCl/0.44 mM KH_2PO_4/0.81 mM $MgSO_4$/136 mM NaCl/0.42 mM Na_2HPO_4/6.1 mM glucose/20 mM Hepes/NaOH (pH 7.4)] containing 0.1 % BSA. For internalization studies, cells were incubated for 1 h at 37 °C with H_C or the H_CC subdomain in culture medium. After fixation and blocking, cells were immunostained with monoclonal antibodies against the VSV-G epitope, as described previously [5]. To monitor internalization, cells were permeabilized with 0.1 % Triton X-100 [5]. Controls were performed by omitting H_C or its subdomains from the incubation.

For cross-linking experiments, cells were cooled on ice and washed with ice-cold Hanks buffer. Cells were incubated with ^{32}P-H_C subdomain (200–300 pM) in 0.2 % BSA/Hanks for 2 h on ice. In competition assays, cells were preincubated for 20 min with 2 μM unlabelled proteins before addition of the ^{32}P-subdomain. After binding, cells were washed with Hanks buffer and incubated for 10 min at 4 °C with 0.22 mM bis-(2-[succinimido-oxycarbonyloxy]ethyl) sulphone ('BSOCOES'; Pierce, Rockford, IL, U.S.A.) in Hanks buffer. The reaction was stopped by removing the cross-linker and adding 20 mM glycine in the same buffer. Cells were then solubilized in 4 % (w/v) octyl β-D-glucopyranoside (Roche Boehringer-Mannheim) in Hanks buffer supplemented with 1 mM iodoacetamide, 100 μM PMSF (Fluka), 1 mM benzamidine (Fluka), 1 μg/ml aprotinin and 1 μg/ml leupeptin (Sigma, Poole, Dorset, U.K.). After 5 min the detergent was diluted to 0.8 % and proteins were precipitated with 6.5 % (w/v) trichloroacetic acid. Samples were prepared for SDS/PAGE and analysed in 6–12 % or 12–18 % (w/v) polyacrylamide gradient gels [21], followed by autoradiography.

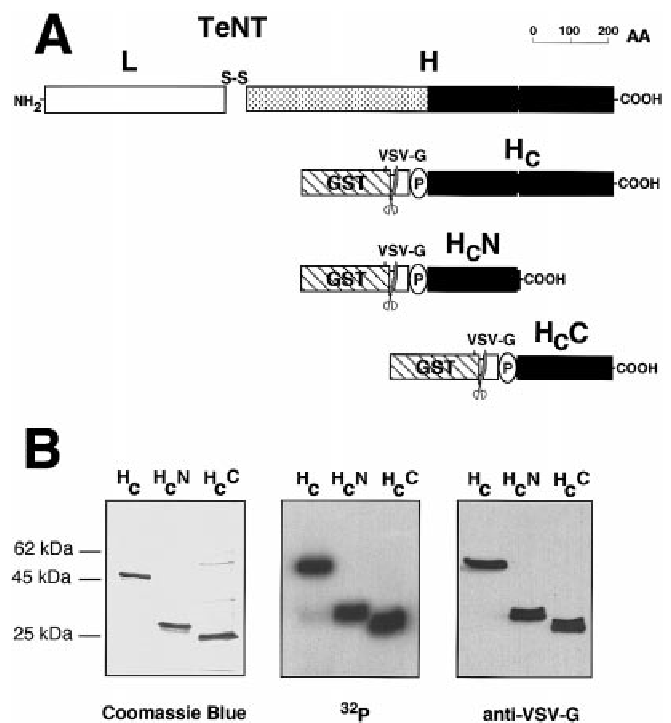

Figure 1 Characterization of TeNT H_C and its subdomains

(**A**) Schematic representation of TeNT and the GST fusion proteins used in this study. A kinase site (P) for radiolabelling and the VSV-G tag have been introduced at the N-terminus of the recombinant proteins. H, heavy chain; L, light chain. (**B**) Coomassie staining (3 μg per lane), ^{32}P autoradiography (100000 c.p.m. per lane, 1.5 ng) and Western blotting (0.3 μg per lane) with a monoclonal antibody against the VSV-G tag of H_C, H_CN and H_CC.

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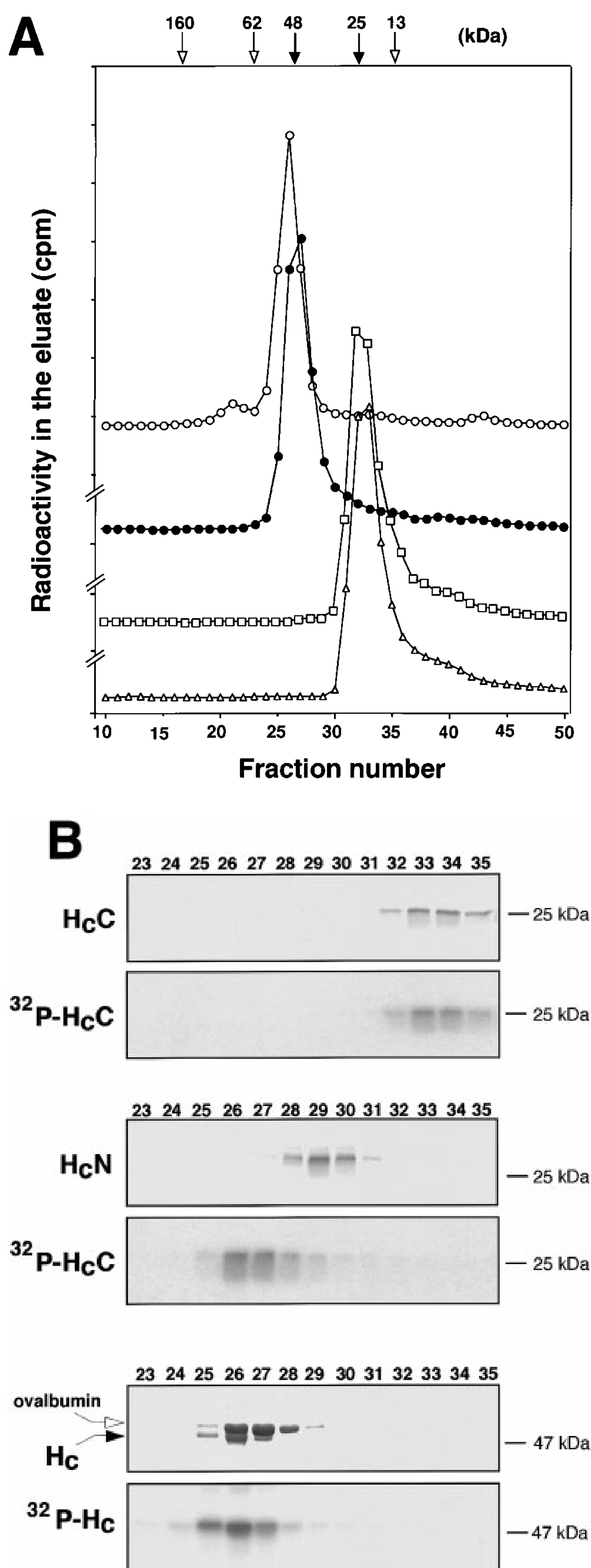

Figure 2 Oligomerization state of TeNT H_C and of its subdomains

(**A**) Gel-filtration analysis, on a Superdex 200 HR 10/30 column, of ^{32}P-H_C and unlabelled H_C (○), ^{32}P-H_CC and unlabelled H_CN (●), ^{32}P-H_CC and unlabelled H_CC (□) or ^{32}P-H_CC together with an unrelated protein (△). The open arrows indicate the positions of protein standards and filled arrows the monomers of H_C and H_CC. Divisions of the *y*-axis represent 1500 c.p.m. (**B**) Coomassie staining (upper panels in each pair) and autoradiography (lower panels in each pair, indicated with ^{32}P-H_CC and ^{32}P-H_C) of the same eluted fractions. Note the shift of ^{32}P-H_CC towards heavier fractions (coinciding with the fractions containing H_C) when H_CN was present. Ovalbumin, which was used as a carrier protein in the radiolabelling reaction, migrated close to H_C.

RESULTS AND DISCUSSION

Production and characterization of recombinant TeNT H_C and its subdomains

Recombinant TeNT H_C and its two subdomains H_CN (residues 856–1110) and H_CC (residues 1111–1315) were efficiently expressed in *Escherichia coli* and purified as soluble GST fusion proteins by using a modified vector containing the VSV-G epitope [18] and the consensus sequence for phosphorylation by protein kinase A [19] (Figure 1A). After cleavage of the GST moiety, H_C presents an apparent mobility of 48 kDa in SDS/PAGE, whereas the two subdomains run as 25 kDa (H_CC) and 29 kDa (H_CN) bands (Figure 1B). The three proteins can be phosphorylated *in vitro* by protein kinase A at the site introduced at the N-terminus (Figure 1B). This method, in contrast with other protocols used in the past, avoids the direct chemical modification of the residues within H_C and minimizes the loss of biological activity. Purified recombinant H_C and its subdomains can be detected by a monoclonal antibody against the VSV-G tag (Figure 1B), thus allowing their detection in cell-interaction experiments (see below).

H_C subdomains of TeNT heterodimerize *in vitro*

The crystal structure of TeNT H_C [15,16] and of the closely related BoNT/A [17] revealed a limited interaction between the two subdomains H_CC and H_CN, suggesting that these two modules fold independently of each other. This information prompted us to dissect the H_C fragment by preserving its minimal folding subunits. Analysis of the crystal structures revealed that the side chains of residues at the interface between H_CC and H_CN are well defined by electron density and have temperature factors that are lower than the average temperature factors of all the protein atoms. These observations, together with the high surface area buried at the interface (2447.9 $Å^2$; T. C. Umland, personal communication), suggest that these two subdomains could interact. To test this possibility, we performed gel-filtration experiments by using radiolabelled TeNT H_C as tracer. TeNT H_C yielded a major peak corresponding to its molecular size (48 kDa) (Figure 2A, ○). ^{32}P-H_CC migrated with a mobility very similar to that expected on the basis of its theoretical molecular mass and it did not homodimerize even in the presence of high concentrations of unlabelled H_CC (Figure 2A, □). In contrast, the addition of unlabelled H_CN promoted the formation of ^{32}P-H_CC and H_CN heterodimers, which migrated as the native H_C (Figure 2A, ●). This change in the mobility of ^{32}P-H_CC was also analysed by running selected fractions in SDS/PAGE gels. A shift of ^{32}P-H_CC to the fractions containing H_C was observed only when H_CN was present (Figure 2B). The ability of the two subdomains to heterodimerize was confirmed by cross-linking experiments in solution (J. Herreros and G. Schiavo, unpublished work) and with neuronally differentiated cells (see below).

H_CC subdomain of TeNT is sufficient for the interaction with p15

Deletion analysis and photolabelling experiments provided evidence that the last 23–34 residues at the C-terminus of the H_CC

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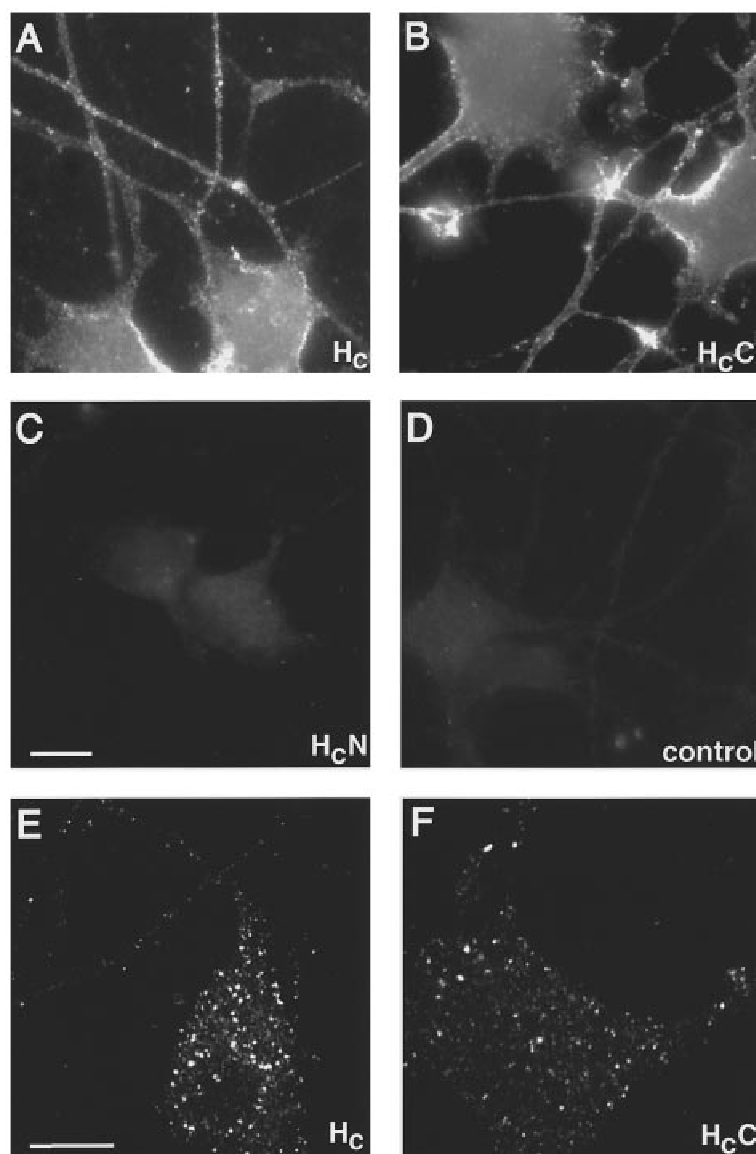

Figure 3 Binding and internalization of TeNT H_C and of its subdomains in NGF-differentiated PC12 cells

(**A–D**) Binding of 80 nM TeNT H_C (**A**), H_CC (**B**), H_CN (**C**) to NGF-differentiated PC12 cells immunodetected with antibodies against the VSV-G tag. The binding patterns of H_C (**A**) and H_CC (**B**) were very similar and gave punctate immunostaining on the plasma membrane of the neurites and cell bodies. The binding of H_CN (**C**) was negligible and was identical with that in the control [(**D**), no toxin]. (**E**, **F**) Internalization of 80 nM TeNT H_C (**E**) and H_CC (**F**) immunodetected with the same antibodies against the VSV-G tag. Images in (**E**) and (**F**) are confocal laser scanning microscope sections. Scale bars, 10 μm.

fragment interact directly with polysialogangliosides and suggested that this region is necessary for the binding and possibly for the correct folding of H_C [22,23]. We therefore used the recombinant H_CN and H_CC subdomains in binding experiments to investigate whether both domains were needed to achieve full binding to the neuronal surface.

Binding of H_C and of H_CC to NGF-differentiated PC12 cells at 4 °C gave very similar punctate patterns on the plasma membrane, along both the cell bodies and the neurites (Figures 3A and 3B), with occasional accumulations of the signal in non-morphologically differentiated areas of the soma and the neurites. In contrast, the binding of H_CN to NGF-differentiated PC12 cells was negligible and comparable to the control (Figures 3C and 3D). The binding of H_CC is functional because this subdomain was internalized, giving an immunostaining pattern similar to that of H_C, although quantitatively less intense (Figures 3E and 3F). This difference could indicate a possible minor contribution of H_CN in the binding affinity of H_C or the involvement of H_CN in intracellular recognition processes (see below). Taken together, these findings show that isolated H_CC contains the determinants for the neurospecific binding and uptake of TeNT.

After binding and cross-linking of ^{32}P-H_C to NGF-differentiated PC12 cells, a typical approx. 65 kDa cross-linking product was observed, which strongly indicated the interaction of H_C with a 15 kDa protein (p15) (Figure 4A). The radioactive band

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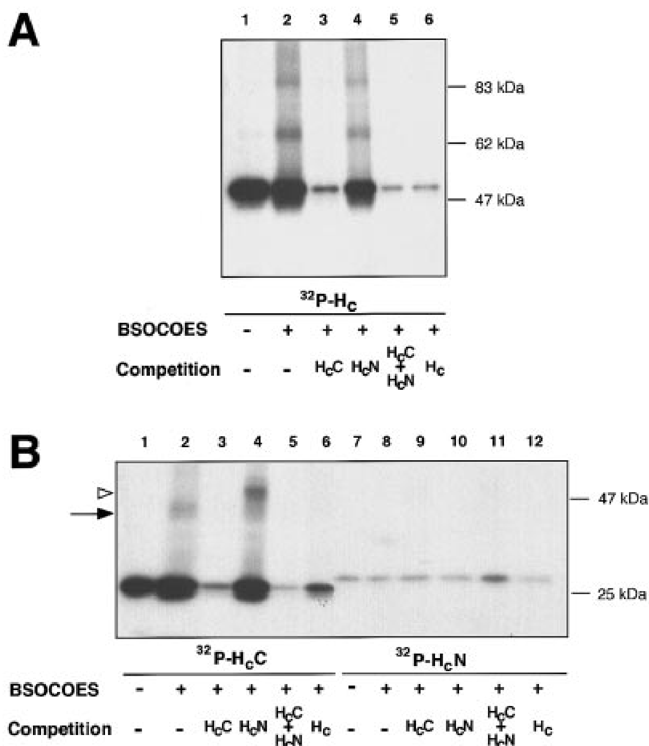

Figure 4 C-terminal subdomain of H_C interacts with the putative 15 kDa TeNT protein receptor

(**A**) Competition of the ^{32}P-H_C binding to NGF-differentiated PC12 cells by preincubation with unlabelled H_CN and H_CC subdomains. The addition of H_CC completely abolished ^{32}P-H_C binding (lanes 3 and 5), similarly to the entire H_C (lane 6), whereas H_CN was ineffective (lane 4). (**B**) ^{32}P-H_CC (lanes 1–6) and ^{32}P-H_CN (lanes 7–12) binding and competition by unlabelled subdomains. ^{32}P-H_CC bound to p15 in NGF-differentiated PC12 cells and formed an approx. 45 kDa cross-linking product (filled arrow, lanes 2 and 4). After pretreatment with unlabelled H_CN (lane 4), an additional cross-linking product of slightly higher molecular mass was formed (open arrowhead) which probably corresponds to the H_CC/H_CN heterodimer (see text). Unlabelled H_CC completely abolished ^{32}P-H_CC binding (lanes 3 and 5), similarly to H_C (lane 6), whereas H_CN had no effect (lane 4). ^{32}P-H_CN bound very poorly and the binding was not altered by the presence of unlabelled H_CC (lanes 9 and 11). Abbreviation: BSOCOES, bis-(2-[succinimido-oxycarbonyloxy]ethyl) sulphone.

running at approx. 83 kDa obtained after cross-linking (Figure 4A, lanes 2 and 4) is likely to represent H_C homodimers [24]. Preincubation of the cells with unlabelled H_CC potently inhibited the binding of radioactive H_C and abolished the appearance of the 65 kDa cross-linking product (Figure 4A, lane 3). In contrast, H_CN altered neither the total binding of H_C nor its interaction with the putative 15 kDa protein receptor (Figure 4A, lane 4). Consistently, the interaction of ^{32}P-H_CN with NGF-differentiated PC12 cells was extremely poor and did not originate any detectable cross-linking product (Figure 4B). In contrast, ^{32}P-H_CC binding to NGF-differentiated PC12 cells was similar to that of ^{32}P-H_C and yielded an approx. 45–47 kDa cross-linking product (Figure 4B, lanes 2 and 4), probably representing the interaction of the ^{32}P-H_CC with p15. These results strongly indicate that the H_CC subdomain has a dual binding specificity by interacting with both polysialogangliosides and the 15 kDa protein receptor.

Pretreatment of NGF-differentiated PC12 cells with H_CN did not inhibit the binding of ^{32}P-H_CC but promoted the additional formation of an approx. 50 kDa product (Figure 4B, lane 4) probably representing H_CC–H_CN heterodimers. An approx. 50 kDa cross-linking product was also observed after binding of ^{32}P-H_CN and preincubation with unlabelled H_CC at longer exposure times (J. Herreros and G. Schiavo, unpublished work). Taken together, these results demonstrate that the H_CC subdomain is necessary and sufficient for the interaction with the putative TeNT receptor p15 and with the neuronal surface. H_CC can therefore be considered the core domain for TeNT neurospecificity, because it contains both the polysialoganglioside-binding site(s) and the portion(s) responsible for the interaction with a putative receptor protein.

In our experimental system, the H_CN subdomain neither interacted significantly with NGF-differentiated PC12 cells nor altered the binding properties of H_CC. The lack of a direct participation of H_CN in the binding does not exclude its involvement in protein–protein interactions in later stages of the intoxication pathway. The presence of a lectin-like domain in the H_CN structure suggests that it might have a role in the recognition of oligosaccharide-containing molecules, although available experimental evidence does not support this conclusion. One attractive possibility is that H_CN is involved in the highly specialized intracellular trafficking of TeNT. H_CN could bind to glycosylated component(s) in the lumen of the endocytic vesicle via its lectin moiety, thus determining the sorting of TeNT to the retrograde transport pathway. This model presents analogies to the mechanism of action proposed for Emp47p and members of the VIP-36 and ERGIC-53 protein families. These animal lectins and their yeast counterpart seem to be involved in the trafficking of glycoproteins and glycolipids at different stages of the secretory pathway by a mechanism that is dependent on their ability to interact with oligosaccharides [25–27]. A functional assay for retrograde transport will be essential to test this hypothesis and to define the importance of the H_C subdomains in TeNT intracellular sorting and transport.

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REFERENCES

1 Montecucco, C. and Schiavo, G. (1995) Quart. Rev. Biophys. **28**, 423–472
2 Niemann, H., Blasi, J. and Jahn, R. (1994) Trends Cell Biol. **4**, 179–185
3 Herreros, J., Lalli, G., Montecucco, C. and Schiavo, G. (1999) in The Comprehensive Sourcebook of Bacterial Protein Toxins (Freer, J. H. and Alouf, J. E., eds.), pp. 202–228, Academic Press, London
4 Halpern, J. L. and Neale, E. A. (1995) Curr. Top. Microbiol. Immunol. **195**, 221–241
5 Lalli, G., Herreros, J., Osborne, S. L., Montecucco, C., Rossetto, O. and Schiavo, G. (1999) J. Cell Sci. **112**, 2715–2724
6 Bizzini, B., Stoeckel, K. and Schwab, M. (1977) J. Neurochem. **28**, 529–542
7 Schwab, M., Agid, Y., Glowinski, J. and Thoenen, H. (1977) Brain Res. **126**, 211–224
8 Evinger, C. and Erichsen, J. T. (1986) Brain Res. **380**, 383–388
9 Pierce, E. J., Davison, M. D., Parton, R. G., Habig, W. H. and Critchley, D. R. (1986) Biochem. J. **236**, 845–852
10 Yavin, E. and Nathan, A. (1986) Eur. J. Biochem. **154**, 403–407
11 Parton, R. G., Ockleford, C. D. and Critchley, D. R. (1988) Brain Res. **475**, 118–127
12 Bakry, N., Kamata, Y., Sorensen, R. and Simpson, L. L. (1991) J. Pharmacol. Exp. Ther. **258**, 613–619
13 Schiavo, G., Ferrari, G., Rossetto, O. and Montecucco, C. (1991) FEBS Lett. **290**, 227–230
14 Herreros, J., Lalli, G., Montecucco, C. and Schiavo, G. (2000) J. Neurochem., in the press
15 Umland, T. C., Wingert, L. M., Swaminathan, S., Furey, W. F., Schmidt, J. J. and Sax, M. (1997) Nat. Struct. Biol. **4**, 788–792

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16 Knapp, M., Segelke, B. and Rupp, B. (1998) Am. Cryst. Ass., Abstr. Papers **25**, 90
17 Lacy, D. B., Tepp, W., Cohen, A. C., DasGupta, B. R. and Stevens, R. C. (1998) Nat. Struct. Biol. **5**, 898–902
18 Soldati, T. and Perriard, J.-C. (1991) Cell **66**, 277–289
19 Kaelin, Jr, W. G., Krek, W., Sellers, W. R., DeCaprio, J. A., Ajchenbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J. and Blanar, M. A. (1992) Cell **70**, 351–364
20 Sandberg, K., Berry, C. J. and Rogers, T. B. (1989) J. Biol. Chem. **264**, 5679–5686
21 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
22 Halpern, J. L. and Loftus, A. (1993) J. Biol. Chem. **268**, 11188–11192
23 Shapiro, R. E., Specht, C. D., Collins, B. E., Woods, A. S., Cotter, R. J. and Schnaar, R. L. (1997) J. Biol. Chem. **272**, 30380–30386
24 Ledoux, D. N., Be, X. H. and Singh, B. R. (1994) Toxicon **32**, 1095–1104
25 Schroder, S., Schimmoller, F., Singer-Kruger, B. and Riezman, H. (1995) J. Cell Biol. **131**, 895–912
26 Fiedler, K. and Simons, K. (1996) J. Cell Sci. **109**, 271–276
27 Itin, C., Roche, A. C., Monsigny, M. and Hauri, H. P. (1996) Mol. Biol. Cell **7**, 483–493

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