Subcloning and characterization of the binding domain of fragment B of diphtheria toxin

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The binding domain (R domain) of diphtheria toxin as defined from the recently published crystal structure [Choe, Bennett, Fujii, Curmi, Kantardjieff, Collier and Eisenberg (1992) *Nature* (London) 357, 216–222] was subcloned. The 17 kDa peptide containing amino acids 378–535 from fragment B of diphtheria

toxin preceded by the tripeptide Met-His-Gly bound specifically and with high affinity to diphtheria-toxin receptors. It efficiently inhibited the toxicity of full-length toxin. The binding domain entered the detergent phase of Triton X-114 at pH values below 6, indicating that it exposed hydrophobic regions at acidic pH.

INTRODUCTION

Diphtheria toxin is an exotoxin produced by pathogenic strains of *Corynebacterium diphtheriae*. The toxin has two disulphide-linked fragments, both of which are required for toxicity to eukaryotic cells (Collier, 1975; Pappenheimer, 1977; Eidels et al., 1983; Middlebrook and Dorland, 1984). Fragment A has ADP-ribosylation activity, inactivating elongation factor 2 and thereby inhibiting protein synthesis, whereas fragment B is responsible for binding to cell-surface receptors on susceptible cells and for facilitating entry of fragment A into the cytoplasm.

To be active, the toxin must be proteolytically cleaved in an exposed region between the A- and B-fragments (DeLange et al., 1976; Collier and Kandel, 1971). However, both nicked and unnicked forms of the toxin bind to cellular receptors (Drazin et al., 1971). Fragment B binds specifically to cells in the absence of fragment A and with higher affinity than the whole toxin (McGill et al., 1989). In attempts to determine which part of fragment B is required for binding, the C-terminal 6 kDa part was prepared and shown to inhibit specific binding of full-length toxin (Rolf et al., 1990). The recently published crystal structure of diphtheria toxin (Choe et al., 1992) revealed three abutting domains connected by interdomain linkers, of which the two C-terminal domains constitute the B-fragment. The C-terminal R domain consists of residues 386–535. The three-dimensional picture of this domain resembles that of immunoglobulin variable domain (Choe et al., 1992). From the crystal structure it was assumed that a peptide consisting of amino acids 386–535 is responsible for receptor recognition. We decided to subclone this R domain and study the characteristics of interaction between this domain and diphtheria-toxin receptors present on sensitive cells.

MATERIALS AND METHODS

Materials

Amiloride was a gift from Merck, Sharp and Dohme, Drammen, Norway. Pronase E (from *Streptomyces griseus*), dithiothreitol and ouabain were purchased from Sigma, St. Louis, MO, U.S.A. [³⁵S]Methionine and ²²NaCl were obtained from Amersham International. Micrococcal-nuclease-treated rabbit reticulocyte lysate and RNasin ribonuclease inhibitor were obtained from Promega, Madison, WI, U.S.A. Crude diphtheria toxin, from Connaught Laboratories, Willowdale, Ont., Canada, was purified as described by Sandvig and Olsnes (1981). Restriction endonucleases, T₄ DNA ligase and T₄ DNA polymerase were

obtained from New England Biolabs, Beverly, MA, U.S.A. T3 RNA polymerase was obtained from Gibco-BRL, Eggenstein, Germany. Triton X-114 was obtained from Fluka A.G., Buchs, Switzerland.

Buffers

Hepes medium comprised bicarbonate-free Eagle's minimal essential medium buffered with 20 mM Hepes and adjusted to the pH indicated. PBS consisted of 140 mM NaCl/10 mM Na₂HPO₄, pH 7.2. The dialysis buffer was 140 mM NaCl/2 mM CaCl₂/20 mM Hepes, pH 7.0. The lysis buffer was 0.1 M NaCl/10 mM Na₂HPO₄/1% Triton X-100/1 mM EDTA/10 mM NaF/1 mM NEM/1 mM phenylmethanesulphonyl fluoride, pH 7.4. Mes/gluconate buffer was 140 mM NaCl/20 mM Mes/5 mM gluconate, adjusted to pH 4.8 with Tris.

Bacterial strain

Escherichia coli strain DH5α was used in the cloning procedures.

Cell culture

African-green-monkey kidney cells (Vero), human osteosarcoma U-2 OS cells and mouse L cells were propagated under 5% CO₂ in Eagle's minimal essential medium containing 5% (v/v) fetal-bovine serum and seeded into 12- or 24-well disposable Costar plates 2 days before the experiments. Cells were used at a density of 10⁵ and 5 × 10⁴ cells/well respectively.

Plasmids

pBD-1 encodes the protein A58 (McGill et al., 1989). pBD-23 encodes B-fragment as described (Stenmark et al., 1992a). pBD-48:pBD-1 was modified without changing the amino acid sequence, but introducing *Sma*I, *Spe*I, *Eag*I, *Bst*BI and *Hind*III sites (Falnes et al., 1992). pBD-87 encodes the C-terminal part of fragment B (here denoted the R domain) starting at Pro³⁷⁸. Pro is preceded by the tripeptide Met-His-Gly. pBD-87 was made in the following way: pBD-48 was cut with *Nco*I and *Eag*I; the overhangs were filled in with T4-polymerase and re-ligated. Then the plasmid was cut with *Nco*I, the overhangs filled in with T₄-polymerase and re-ligated. In the resulting plasmid an *Nsi*I site was generated.

SDS/PAGE

Electrophoresis was carried out in 13.5% (w/v) polyacrylamide gels as described (McGill et al., 1989). After electrophoresis the gels were fixed for 30 min in 4% (v/v) acetic acid/27% (v/v) methanol, and then treated for 30 min with 1 M sodium salicylate, pH 5.8, containing 2% (v/v) glycerol. For fluorography, Kodak XAR 5 films were exposed to the dried gels at -80°C .

In vitro transcription and translation

Plasmid DNA (0.2–0.5 μg), linearized downstream of the insert with *Eco*RI, was transcribed with T3 RNA polymerase in 20 μl reaction mixtures as described (Maniatis et al., 1982). Ethanol-precipitated mRNA was dissolved in 20 μl of water containing 10 mM dithiothreitol and 0.1 unit/ μl RNasin. The mRNA was translated in micrococcal-nuclease-treated rabbit reticulocyte lysate, using 5 μl of the dissolved mRNA per 150 μl of lysate. The lysate contained 25 μM each of 19 amino acids (without methionine) and 1 μM [^{35}S]methionine (1000 Ci/mmol). When non-radioactive proteins were synthesized, the radioactive methionine was replaced by 25 μM unlabelled methionine. The amount of translation product was estimated as previously described (Stenmark et al., 1992b). The typical yield of non-purified protein was in the 10 nM range. After translation, the lysate was dialysed against dialysis buffer to remove free methionine and the reducing agent present in the lysate. The dialysis membrane had an M_r cut-off of 3500.

Measurement of binding to cells

In binding experiments, [^{35}S]methionine-labelled protein (approx. 0.1 nM) with or without excess amounts (20 nM) of unlabelled diphtheria toxin was added to Vero cells, U-2 OS cells or mouse L cells in microtitre plates (10^5 cells/well). The binding was at 20°C for 20 min. The cells were subsequently washed five times with Hepes medium and thereafter lysed in 250 μl lysis buffer. Nuclei were removed by centrifugation in an Eppendorf centrifuge at 14000 rev/min (r_{av} , 7.2 cm) for 2 min, and protein in the supernatant fraction was precipitated by 5% (w/v) trichloroacetic acid. The trichloroacetic acid pellets were washed three times with diethyl ether and analysed by SDS/PAGE under non-reducing conditions.

RESULTS AND DISCUSSION

Binding of the R domain to diphtheria toxin receptors

Fragment B consists of the C-terminal 341 amino acids of diphtheria toxin, starting at amino acid 195 (see Figure 1). The proposed binding domain (Choe et al., 1992) consists of the C-terminal 158 amino acids and will here be referred to as the R domain. This domain was cloned and expressed in a cell-free system as described in the Materials and methods section. Due to cloning procedures the 158 C-terminal amino acids are preceded by the tripeptide Met-His-Gly.

When whole B-fragment is added to Vero cells, it binds to the cells in a saturable manner. The binding is blocked by excess amounts unlabelled diphtheria toxin, indicating binding to specific receptors (McGill et al., 1989). Also the human osteosarcoma cell line U-2 OS did bind diphtheria toxin B-fragment (Figure 2, lane 3), and the binding was strongly reduced in the presence of unlabelled diphtheria toxin (lane 4). Further-

more, the R domain was bound to U-2 OS cells (Figure 2, lane 1), and the binding was strongly reduced in the presence of unlabelled diphtheria toxin (lane 2). The R domain also bound efficiently to Vero cells (results not shown), but not to mouse L cells, known to lack diphtheria toxin receptors (results not shown).

To test by a different method whether the binding was to diphtheria-toxin receptors responsible for mediating toxicity and not to some irrelevant structure, we tested if the R domain made *in vitro* could compete for binding of full-length diphtheria toxin and thereby inhibit cytotoxicity. As demonstrated in Figure 3, the ID_{50} for diphtheria toxin was shifted to a 10-fold higher concentration in the presence of a 5×10^{-11} M concentration of either the R domain or full-length fragment B. This indicates that the R domain binds to the functional toxin receptor and that the affinity of the binding is increased approx. 100-fold compared with full-length toxin, since the saturation of cellular receptors with full-length diphtheria toxin occurs at approx. 5×10^{-9} M.

The present work confirms that the C-terminal part of fragment B, proposed to be responsible for binding (Choe et al., 1992), contains sufficient structural information to recognize specifically the diphtheria-toxin receptor. Our data indicate that the R domain binds with approximately the same affinity as does full-

Figure 1 Schematic representation of recombinant proteins derived from diphtheria toxin

Fragment A is depicted as a hatched box, the N-terminal part of fragment B is depicted as an open box and the C-terminal part of fragment B is depicted as a cross-hatched box. The numbers indicate amino acid position according to the sequence published by Greenfield et al. (1983).

Figure 2 Binding of [^{35}S]methionine-labelled R domain and fragment B to U-2 OS cells

The binding was carried out at 20°C as described in the Materials and methods section. Bound protein was analysed by SDS/PAGE (non-reducing conditions) and fluorography. Lane 1, R domain bound to cells in the absence of unlabelled diphtheria toxin; lane 2, R domain bound to cells in the presence of 10 $\mu\text{g}/\text{ml}$ unlabelled diphtheria toxin; lane 3, B fragment bound to cells in the absence of unlabelled diphtheria toxin; lane 4, B fragment bound to cells in the presence of 10 $\mu\text{g}/\text{ml}$ unlabelled diphtheria toxin.

Figure 3 Inhibition of diphtheria-toxin cytotoxicity by the R domain and by fragment B

To Vero cells in 24-well microtitre plates incubated in growth medium was added (●) 50 pM R domain made *in vitro*, (▲) 50 pM fragment B made *in vitro* or (■) the same amount of reticulocyte lysate where no mRNA had been added. Then increasing concentrations of nicked diphtheria toxin were added. The cells were incubated at 37 °C overnight and subsequently for 30 min at 37 °C in leucine-free Hepes medium containing 4 μ Ci/ml [3 H]leucine. Then the cells were washed twice with 5% trichloroacetic acid and finally dissolved in 0.1 M KOH and the radioactivity associated with the cells was measured.

length B fragment and with approx. 100-fold increased affinity compared with full-length toxin. The C-terminal 5982 Da fragment created by cleavage with hydroxylamine (Rolf et al., 1990), which also inhibited toxicity by full-length diphtheria toxin, was demonstrated to have a dissociation constant of 2.5×10^{-9} M (Rolf et al., 1990). This shows that even if the C-terminal ~ 6 kDa part of fragment B contains sufficient structural information to give specific binding, the N-terminal ~ 11 kDa part of the R domain is required in order to obtain a high-affinity binding.

It has previously been shown that deletions in the B-fragment N-terminal to the R domain strongly reduced specific binding (McGill et al., 1989; Stenmark et al., 1992b). The present data indicate that the decreased binding of the deletion mutants is due to perturbed folding of the protein, and not to direct involvement of N-terminal sequences of fragment B in the binding.

Amphiphilic properties of the R domain

At low pH, fragment B inserts into cellular membranes (Moskaug et al., 1988; McGill et al., 1989). Furthermore, at low pH, fragment B partitions into Triton X-114. The non-ionic detergent Triton X-114, which is soluble in water at 4 °C, forms two phases after incubation at temperatures above 20 °C. Hydrophilic proteins tend to stay in the aqueous phase, whereas amphiphilic proteins are found in the detergent phase (Bordier, 1981). We measured the partitioning of the R domain in Triton X-114 at different pH values. As shown in Figure 4, the R domain disappeared from the aqueous phase at pH values below 6 (lanes 11–15). This pH profile is almost identical with that of full-length fragment B (Figure 4, lanes 1–5), but different from that of

Figure 4 Partitioning of the R domain, fragment A and fragment B in the aqueous phase of Triton X-114 at different pH values

A mixture of reticulocyte lysate (2 μ l, containing radiolabelled R domain, fragment A or fragment B) and Triton X-114 (100 μ l of a stock solution adjusted to the pH values indicated) was incubated for 15 min at 4 °C and then for 30 min at 37 °C. The phases were separated by centrifugation in an Eppendorf centrifuge for 2 min. The aqueous phase was analysed by SDS/PAGE and fluorography.

fragment A, which did not disappear from the aqueous phase even at pH 5 (Figure 4, lanes 6–10). The data indicate that the R domain exposes hydrophobic regions at low pH. However, the R domain did not form proteinase-protected fragments specifically at low pH, nor did the R domain form ion channels in cellular membranes at low pH (results not shown). Formation of cation-selective ion channels has been observed both for fragment B and for full-length diphtheria toxin (Stenmark et al., 1989; Sandvig and Olsnes, 1988).

Our results support the notion that the N-terminal part of fragment B is required for channel formation. This is in accordance with the findings that several point mutations in the N-terminal part of fragment B (the T domain) inhibited channel formation by fragment B *in vivo* (Falnes et al., 1992) and by whole toxin *in vivo* (O'Keefe et al., 1992) and *in vitro* (Mindell et al., 1992). Furthermore, crm45, a deletion mutant lacking the R domain, was found to form channels in lipid bilayers (Kagan et al., 1981), indicating that channel formation is a property of the T domain.

Some point mutations inhibiting binding of diphtheria toxin have been reported. The mutations S525F, L390F, and S508F reduced the toxicity to 1/1000–1/10000 and the binding activity to 1/100–1/8000 of that of the wild-type toxin (Greenfield et al., 1987). The mutant D392K also showed strong reduction in binding (Falnes et al., 1992). It is noteworthy that three of these four point mutations cluster in the portion of the folded structure resembling the immunoglobulin variable-like moiety, facing away from the rest of the diphtheria-toxin monomer and being close to the T domain.

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REFERENCES

- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607
- Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M. G., Kantardjiev, K. A., Collier, R. J. and Eisenberg, D. (1992) *Nature (London)* **357**, 216–222
- Collier, R. J. (1975) *Bacterial. Rev.* **39**, 54–85

- Collier, R. J. and Kandel, J. (1971) *J. Biol. Chem.* **246**, 1496–1503
- DeLange, R. J., Drazin, R. E. and Collier, R. J. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 69–72
- Drazin, R., Kandel, J. and Collier, R. J. (1971) *J. Biol. Chem.* **246**, 1504–1510
- Eidels, L., Proia, R. L. and Hart, D. A. (1983) *Microbiol. Rev.* **47**, 596–620
- Falnes, P. Ø., Madhus, I. H., Sandvig, K. and Olsnes, S. (1992) *J. Biol. Chem.* **267**, 12284–12290
- Greenfield, L., Bjorn, M. J., Horn, G., Fong, D., Buck, G. A., Collier, R. J. and Kaplan, D. A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6853–6857
- Greenfield, L., Gray Johnson, V. and Youle, R. J. (1987) *Science* **238**, 536–539
- Kagan, B. L., Finkelstein, A. and Colombini, M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4950–4954
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- McGill, S., Stenmark, H., Sandvig, K. and Olsnes, S. (1989) *EMBO J.* **8**, 2843–2848
- Middlebrook, J. L. and Dorland, R. B. (1984) *Microbiol. Rev.* **48**, 199–221
- Mindell, J. A., Silverman, J. A., Collier, R. J. and Finkelstein, A. (1992) *Biophys. J.* **62**, 41–44
- Moskaug, J. Ø., Sandvig, K. and Olsnes, S. (1988) *J. Biol. Chem.* **263**, 2518–2525
- O'Keefe, D. O., Cabiliaux, V., Choe, S., Eisenberg, D. and Collier, R. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6202–6206
- Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem.* **46**, 69–94
- Rolf, J. M., Gaudin, H. M. and Eidels, L. (1990) *J. Biol. Chem.* **265**, 7331–7337
- Sandvig, K. and Olsnes, S. (1981) *J. Biol. Chem.* **259**, 9068–9076
- Sandvig, K. and Olsnes, S. (1988) *J. Biol. Chem.* **263**, 12352–12359
- Stenmark, H., McGill, S., Olsnes, S. and Sandvig, K. (1989) *EMBO J.* **8**, 2849–2853
- Stenmark, H., Afanasiev, B. N., Ariansen, S. and Olsnes, S. (1992a) *Biochem. J.* **281**, 619–625
- Stenmark, H., Ariansen, S., Afanasiev, B. N. and Olsnes, S. (1992b) *J. Biol. Chem.* **267**, 8957–8962