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Diphtheria Toxin-Receptor Interaction: A POLYPHOSPHATE-INSENSITIVE DIPHTHERIA TOXIN-BINDING DOMAIN

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SUMMARY: Inositol hexaphosphate, and other polyphosphates, inhibit diphtheria toxin-mediated cytotoxicity by binding to the toxin at a highly cationic site called the P site and preventing toxin binding to cell surface receptors. The binding of diphtheria toxin to a solubilized cell surface glycoprotein (150,000 daltons) is also inhibited by these polyphosphates. Treatment of this 150,000 dalton diphtheria toxin-binding cell surface glycoprotein with papain yielded an 88,000 dalton and a 74,000 dalton diphtheria toxin-binding glycoprotein whose binding to toxin was no longer inhibited by inositol hexaphosphate. This result suggests a model of diphtheria toxin-receptor interaction in which the toxin receptor possesses one binding site which interacts with the P site of the toxin in a polyphosphate-sensitive fashion, and another binding site (located within the papain-derived 74,000-88,000 dalton glycoproteins) which can interact with the toxin at a site distinct from the P site (the X site) in a polyphosphate-insensitive fashion. This X site-receptor interaction may be involved in the binding of CRM proteins that bind to the toxin receptor but that do not bind polyphosphates, or it may be involved in the entry process of the toxin.

Diphtheria toxin (DT) consists of two disulfide-linked fragments, the A fragment (23,000 daltons) and the B fragment (40,000 daltons), both of which are required for intoxication. The A fragment catalyzes the ADP-ribosylation of elongation factor 2 which results in cessation of protein synthesis; the B fragment is involved in the binding of the toxin to specific cell surface receptors (1,2). A number of polyphosphorylated compounds (e.g. ATP and inositol hexaphosphate) inhibit toxin-mediated cytotoxicity by interfering with toxin binding to cell surface receptors (3-7). We have demonstrated that these same polyphosphorylated compounds also inhibit the interaction of the toxin with a detergent solubilized 150,000 dalton DT-

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Abbreviations: DT, diphtheria toxin; PMSF, phenylmethylsulfonyl fluoride; NP-40, Nonidet P-40; TBS, Tris-buffered saline.

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binding cell surface glycoprotein (5,7). The polyphosphates exert their effect by binding to the toxin rather than to the receptor (5,6,8,9). Lory and Collier (6) have named this polyphosphate-binding site on DT the P site, and we have shown that this P site is a highly cationic region located within the carboxy-terminal 8,000 dalton CNBr peptide of the B fragment (8). This 8,000 dalton highly cationic region is itself part of the 17,000 dalton receptor-binding carboxy-terminal portion of the B fragment that is missing in CRM 45, a nontoxic protein that neither binds polyphosphates (6,9) nor binds to cell surface receptors (10,11). Based on the fact that the polyphosphates bind to the P site and prevent subsequent toxin binding to the receptor, and that the P site is within the 17,000 dalton receptor-binding region of the B fragment, we have suggested that the P site is, or is part of, the receptor-binding domain of the toxin (8). We have also postulated that the receptor itself possesses a polyanionic site (P' site) which also binds to the highly cationic P site on the toxin (5,7,8).

As part of our ongoing studies on the toxin-binding domain of the previously described detergent-solubilized DT-binding cell surface glycoprotein (5,7,12,13), we have investigated the effect of proteolytic treatment on this solubilized toxin receptor system. We found that papain treatment of this 150,000 dalton glycoprotein results in the formation of an 88,000 dalton glycoprotein and a 74,000 dalton glycoprotein that bind DT; however, this binding is no longer inhibited by polyphosphates (e.g. inositol hexaphosphate). This result suggests that the receptor possesses one domain (P' site) that interacts with the P site of the toxin in a polyphosphate-sensitive fashion, and that it also possesses another domain (X' site located within the 74,000-88,000 dalton fragments) that can interact with the toxin at a site other than the P site (X site) in a polyphosphate-insensitive fashion.

MATERIALS AND METHODS

Partially purified DT was purchased from Connaught Laboratories (Ontario, Canada) and further purified as previously described (5). Antiserum to DT was produced in rabbits as previously described (5). Inositol hexaphosphate,

phenylmethylsulfonyl fluoride (PMSF), and papain (type III, 2X crystallized 17 units/mg) were from Sigma Chemical Co., St. Louis, MO. Inositol hexaphosphate solutions were prepared in 0.1 M Tris-HCl (pH 7.4) as described (5). Molecular weight protein standards were purchased from Biorad Laboratories, Richmond, CA.

Hamster thymus cells (20×10^7) were prepared and radioiodinated by the lactoperoxidase-catalyzed method as previously described (12,13). These cells were lysed in 0.5% Nonidet P-40 (NP-40) in Tris-buffered saline (TBS) (pH 7.4) at a ratio of 2.5×10^7 cells per ml of 0.5% NP-40. After removal of nuclei by centrifugation fetal calf serum was added to the lysate to a final concentration of 10%. Then one half of the ^{125}I-labeled lysate (4 ml) was treated (20 min, 20°C) with a preparation of papain (2.24 mg) which had been preactivated with an equal volume of 10 mM L-cysteine. The other half of the lysate (control) was treated wth a solution containing only L-cysteine. Both lysates were then treated with iodoacetamide (10 mM) and PMSF (1 mM) and dialyzed against TBS (12). The ^{125}I-labeled glycoprotein-enriched fraction, employed for the detection of the DT-binding glycoproteins, was prepared from each of the lysates by affinity chromatography on a lentil lectin-Sepharose column as previously reported (12,13). DT-binding cell surface glycoproteins were detected by the previously described (12) immunoprecipitation system employing DT (in the presence or absence of 10 mM inositol hexaphosphate), followed by antiserum to DT and by adsorption of the immune complexes onto fixed protein A-bearing Staphylococcus aureus (5,12); and the immune precipitates were then analyzed by SDS-polyacrylamide gel electrophoresis (7,12).

RESULTS AND DISCUSSION

In our laboratory we have characterized a 150,000 dalton DT-binding cell surface glycoprotein that binds to lentil lectin and to DT and have suggested it as a candidate for the toxin receptor (5,7,12,13). The binding of DT to this detergent solubilized DT receptor is specific for the B fragment of DT (7,12,13) and is inhibited by polyanions (5,7) as well as by polycations (7). Recently, we have been interested in characterizing the different domains of this solubilized receptor molecule, e.g. the membrane-binding domain, the lentil lectin-binding domain, and the DT-binding domain. For this purpose we have employed proteolytic modification of the receptor. The detergent-solubilized cell lysate from ^{125}I-labeled hamster thymocytes was treated with papain (under the conditions described in Materials and Methods) and the glycoprotein adherent fraction of this papain-treated lysate was then isolated by lentil lectin affinity chromatography. Assay of this fraction for DT-binding components by the immunoprecipitation system and SDS-polyacrylamide gel electrophoresis (12) revealed the presence of three major components (Fig. 1 lower panel, + papain): One glycoprotein with an apparent size of 150,000 daltons, which probably represents unproteo-

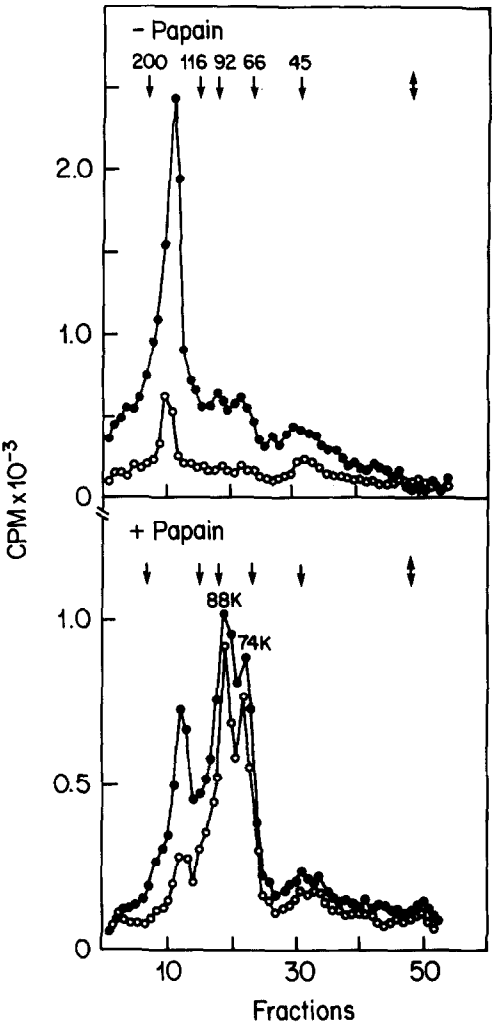

Fig. 1. SDS-polyacrylamide gel electrophoresis of the immunoprecipitated diphtheria-toxin binding glycoproteins. Samples of the radioiodinated glycoprotein fraction obtained from the papain-treated lysate (lower panel) or from the no-papain control lysate (upper panel) were reacted with DT (●) or with DT in the presence of 10 mM inositol hexaphosphate (○) followed by antiserum to DT and S. aureus as described in Materials and Methods. The arrows indicate the positions of the stained molecular weight protein standards, which were electrophoresed on a parallel gel and which consisted of myosin (200,000 daltons), β-galactosidase (116,000 daltons), phosphorylase b (92,000 daltons), bovine serum albumin (66,000 daltons), and ovalbumin (45,000 daltons). The double headed arrow indicates the position of the bromophenol blue dye front. The gels were electrophoresed for 15 h at 4 mA/gel.

lyzed receptor since it has the same mobility as the intact receptor (see Fig. 1 upper panel, - papain control), and two smaller glycoproteins with apparent sizes of 88,000 daltons and 74,000 daltons. Thus, even after proteolytic removal of approximately one-half of the peptide portion from

the 150,000 dalton glycoprotein the resultant smaller glycoproteins still possess the lentil lectin-binding domain and the DT-binding domain. It was important to determine if these smaller glycoprotein receptors shared other characteristics with the 150,000 dalton glycoprotein receptor; specifically, we tested if the binding of DT to the smaller glycoproteins was inhibited by the P site ligand inositol hexaphosphate. The results of this experiment are also shown in Fig. 1. The presence of inositol hexaphosphate (10 mM) greatly inhibited the interaction of DT with the 150,000 dalton glycoprotein receptor in the nonpapain-treated control sample (Fig. 1 upper panel, ~ 85% inhibition) and with the residual uncleaved 150,000 dalton glycoprotein receptor in the papain-treated sample (Fig. 1 lower panel, ~73% inhibition). In contrast, the interaction of DT with the 88,000 dalton and 74,000 dalton glycoproteins was only minimally inhibited by inositol hexaphosphate (Fig. 1 lower panel, ~12% inhibition). Qualitatively similar results were obtained when ATP, another P site ligand, was tested (data not shown).

The finding of a DT-binding domain that is insensitive to the presence of inositol hexaphosphate bound to the toxin suggested to us the existence of a site on the toxin other than the P site that can, under certain conditions, interact with the DT receptor. We have designated this other site on the toxin the X site (see model Fig. 2), and we suggest that this site might be located within the 17,000 dalton carboxy-terminal receptor-binding region of the B fragment that is absent in CRM 45 protein (11). The DT-binding domain which interacts with the X site and is located within the papain-generated 74,000-88,000 dalton glycoprotein fragments, has been designated the X' site (Fig. 2). The previously postulated polyanionic site on the receptor (5,7,8,14), which binds to the polycationic P site and to other polycationic molecules, has been designated the P' site (Fig. 2). This model (Fig. 2) envisions the P-P' interaction to be the primary toxin-receptor interaction since the presence of inositol hexaphosphate almost completely inhibits toxin-receptor interaction (Fig. 1 upper panel), and

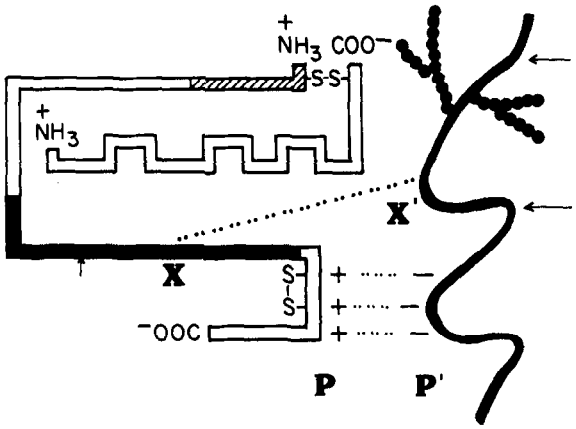

Fig. 2. Model for diphtheria toxin-receptor interaction. The toxin molecule with its disulfide-linked A fragment (amino-terminal) and B fragment (carboxy-terminal) is shown on the left, and the 150,000 dalton DT-binding glycoprotein is shown on the right. The hatched area at the amino-terminal end of the B fragment represents a region which is similar to phospholipid-binding domains of apolipoproteins (17); the dark area in the middle of the B fragment represents the hydrophobic domain, part of which has the properties of a transmembrane protein (17); the vertical arrow is the approximate point where CRM 45 protein (45,000 daltons) terminates. The X site we postulate to be within the 17,000 dalton carboxy-terminal receptor-binding region of the B fragment and to be responsible for binding of the toxin to the X' site on the receptor. The P site is the highly cationic site located within the 8,000 carboxy-terminal region of the B fragment (8). The X' site is located within the 74,000-88,000 dalton DT-binding glycoprotein fragments derived by papain cleavage (approximately at the points indicated by the horizontal arrows) of the 150,000 dalton receptor. The P' site is the previously postulated highly anionic site on the receptor that binds to the P site on the toxin and to other polycationic molecules (5,7,8).

envisions the X-X' interaction to be a secondary (weaker) interaction between the toxin and the receptor. This X-X' interaction, however, becomes prominent and readily apparent when the solubilized receptor is treated with papain, and the glycoproteins containing the X' site, free from the P' site, are generated. The X-X' interaction may also become a primary interaction in the case of some mutant toxin molecules (CRMs) which due to their mutation(s) might have a more exposed X site than wild type toxin molecules and thus be able to bind to the X' site of the cell surface DT receptor. For instance, the nontoxic CRM 197 protein, which has an altered enzymatic A fragment and a B fragment that can bind to cell surface receptors (15,16) but that does not bind ATP (9), may be in a conformation such that the P site is not exposed (explaining the lack of ATP binding) but the X site is exposed, permitting its interaction with the toxin recep-

tor (with the X' site). Alternatively, the X-X' interaction may not be part of the initial toxin-receptor interaction per se but may be part of the subsequent entry step of the toxin. This latter possibility would still be consistent with the observed polyphosphate-mediated protection of cells from the lethal effects of DT since the polyphosphates are known to act by binding to the highly cationic P site and preventing the initial toxin-receptor interaction.

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