Use of Synthetic Peptides and Site-Specific Antibodies To Localize a Diphtheria Toxin Sequence Associated with ADP-Ribosyltransferase Activity

JOAN C. OLSON

Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425

Received 11 May 1992/Accepted 15 September 1992

Diphtheria toxin (DT) and Pseudomonas aeruginosa exotoxin A have the same molecular mechanism of toxicity; both toxins ADP-ribosylate a modified histidine residue in elongation factor 2. To help identify amino acids involved in this reaction, sequences in DT that share homology with P. aeruginosa exotoxin A were synthesized and examined for a role in the ADP-ribosyltransferase reaction. By using this approach, residues 32 to 54 of DT were found to define an epitope associated with antibody-mediated inhibition of DT enzyme activity. This lends further support to the notion that residues in this region of DT are involved in the enzymatic reaction.

Most clinical symptoms of diphtheria are attributed to a toxin produced by pathogenic strains of Corynebacterium diphtheriae. This diphtheria toxin (DT) is one of many bacterial toxins whose molecular mechanism of action involves an ADP-ribosyltransferase activity (7). Other toxins having this activity, such as cholera toxin, pertussis toxin, Escherichia coli heat-labile toxin, Pseudomonas exoenzyme S, and Clostridium C2, C3, and iota toxins, exert different effects on cellular functions, and these differences reflect their substrate specificities.

Structure-function analyses of functionally related enzymes have, in many instances, led to the identification of functional sequence motifs. Although common sequence patterns can be identified in the enzymatically active region of ADP-ribosylating toxins having identical or closely related substrates, these same patterns are not apparent in ADP-ribosylating toxins having more distantly related substrates. An inability to identify a common sequence motif in the ADP-ribosylating toxins has precluded definitive identification of amino acids directly involved in this reaction.

Many approaches have been used to help confirm the identity of key functional residues in ADP-ribosylating toxins. Crystallographic studies of Pseudomonas exotoxin A (PE) (1), DT (6), and E. coli heat-labile toxin (16) have localized the active-site region of these toxins. Numerous amino acid alteration and site-directed mutagenesis studies have also led to the identification specific residues within ADP-ribosylating toxins that affect enzyme activity. Studies described in this report used synthetic peptides to help confirm the identities of residues in DT that are associated with the ADP-ribosyltransferase reaction. By using this approach, a region between residues 32 and 54 of DT was found to define an epitope that affects DT enzymatic activity.

Selection and immunological evaluation of DT synthetic peptides. Although DT and PE maintain the same molecular mechanism of action and a common substrate (7, 11), only limited sequence similarity between the two toxins has been identified (2, 4, 10, 19). The amino acid sequences within the enzymatically active region of DT and PE have been aligned in Fig. 1 to maximize recognition of sequence similarities. Since common sequences are likely candidates for common enzymatic functions, sequences in DT that are conserved in PE were synthesized and examined for involvement in the ADP-ribosyltransferase reaction. Sequences enclosed in boxes in Fig. 1 were synthesized by using 9-fluorenylmethoxycarbonyl amino acids, the Fmoc procedure, either by VetroGen Corp. (London, Ontario, Canada) or by the Protein Chemistry Facility at the Medical University of South Carolina. Peptides DT-18 (residues 15 to 31), DT-24 (residues 32 to 54), and DT-25 (residues 68 to 91) include sequence patterns shared by PE. DT-20 (residues 95 to 113) and PE-27 (PE residues 538 to 563) were synthesized as control peptides, with DT-20 representing a sequence poorly conserved in PE and PE-27 representing an exotoxin A sequence conserved in DT.

To assess whether the synthetic peptides defined DT epitopes, each peptide was assayed for recognition by polyclonal antisera against either intact DT or the A subunit of DT (DT-A). These antisera were prepared against purified DT (List Biologicals, Campbell, Calif.) or the A subunit of DT (DT digested with 1 μg of trypsin per ml for 15 min at 25°C); each protein was isolated by electrophoresis under reducing conditions (12) and transferred to nitrocellulose (17) for use as an immunogen (13). By using a modification of methods described by Voller and Bidwell (13, 18), a direct-binding enzyme-linked immunosorbent assay (ELISA) identified peptides DT-24 and to a lesser degree, DT-25 and DT-20 as including determinants recognized by DT antisera (Table 1). This supports the potential of the synthetic form of these sequences to accurately mimic epitopes expressed by intact DT. In comparison, peptide DT-18 was not recognized by either of the DT antisera, indicating that this sequence is hidden within the DT molecular structure or that this peptide does not accurately mimic the sequence it represents in intact DT.

To assess the cross-reactivity of sequence patterns shared between DT and PE, the reactivity of PE antiserum with DT peptides was also examined in a direct-binding ELISA. Although PE-specific antiserum (13) recognized PE-derived peptide PE-27, no cross-reactivity between sequence patterns shared by DT and PE was observed, either at the synthetic peptide level or at the level of intact toxins.

Functional evaluation of sequences in DT represented by...
synthetic peptides. DT synthetic peptides were initially evaluated for possible association with the ADP-ribosyltransferase reaction by using a peptide-antibody inhibition assay. This assay uses an antibody-combining site as a means of screening peptides for the potential to include epitopes associated with the enzymatic reaction. The premise of the assay is that if a synthetic peptide defines an epitope which is the same as that recognized by an antibody that inhibits enzyme activity, then preincubation of the antibody with that peptide should block the enzyme-inhibiting potential of the antiserum.

By using methods described by Carroll and Collier to assay DT ADP-ribosyltransferase activity (5), addition of 5 μl of DT-A antiserum to 50 ng of DT in a 100-μl volume was found to inhibit DT ADP-ribosyltransferase activity by 87.3%. When similarly assayed, the antiserum produced against intact DT in these studies was found to cause no inhibition of DT enzymatic activity. In the peptide-antibody inhibition assay, when 5 μg of peptide DT-18, DT-24, DT-25, DT-20, or PE-27 was preincubated with DT-A antiserum for 30 min prior to assay for inhibition of DT ADP-ribosyltransferase activity, one peptide, DT-24, almost completely blocked the enzyme-inhibiting potential of this antiserum (Fig. 2). These results support the close association of the sequence defined by DT-24 in the ADP-ribosyltransferase reaction. These results alone, however, are not sufficient to rule out involvement of the other DT peptide sequences in the enzymatic reaction. For a peptide to be recognized in this assay, the peptide, in addition to representing a sequence in close association the enzymatic cleft, must also accurately mimic the configuration of the respective sequence within intact toxin and be recognized by the antiserum used to define inhibiting epitopes.

The specificity for recognition of the DT-24 sequence in inhibition of enzymatic activity by the polyclonal DT-A

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**TABLE 1. Reactivity of DT and PE antiserum**

<table>
<thead>
<tr>
<th>Antiserum*</th>
<th>DT (T-DT)</th>
<th>PE (U-DT)</th>
<th>DT-18</th>
<th>DT-24</th>
<th>DT-25</th>
<th>DT-20</th>
<th>PE-27</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT</td>
<td>0.572</td>
<td>0.994</td>
<td>0.000</td>
<td>0.018</td>
<td>0.001</td>
<td>0.674</td>
<td>0.189</td>
</tr>
<tr>
<td>DT-A</td>
<td>0.480</td>
<td>0.939</td>
<td>0.009</td>
<td>0.018</td>
<td>0.004</td>
<td>0.655</td>
<td>0.086</td>
</tr>
<tr>
<td>PE</td>
<td>0.007</td>
<td>0.003</td>
<td>0.365</td>
<td>0.070</td>
<td>0.005</td>
<td>0.005</td>
<td>0.008</td>
</tr>
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</table>

* Immunogen used in antiserum preparation. Toxin antiserum were diluted 1:500 in the ELISA.

* Antibody reactivity represents binding to the indicated antigen; ELISA optical density at 450 nm minus background optical density is shown. ELISA wells were coated with 50 ng of toxin or 100 ng of peptide. Abbreviations: DT(T-DT), trypsin (1 μg/ml)-dithiothreitol (40 mM)-treated DT; PE(U-DT), urea (2 N)-dithiothreitol (40 mM)-treated PE; DT-18, DT-24, DT-25, and DT-20, DT peptides; PE-27, PE peptide as defined in Fig. 1.

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**FIG. 2.** Peptide blocking of antibody (Ab)-mediated inhibition of DT ADP-ribosyltransferase enzyme activity by using the peptide-antibody inhibition assay. The ADP-ribosyltransferase activity of 50 ng of DT was assayed in the presence of phosphate-buffered saline (−), a normal-serum control (NRS [normal rat serum]), or DT-A antiserum (DT-A). When indicated, DT-A antiserum was incubated with 5 μg of the specified peptide for 30 min prior to addition of DT and assay for enzymatic activity. Counts per minute (cpm) of [3H]ADP-ribose incorporated into the protein substrate are indicated, and each bar represents the mean of duplicate assays.
with DT-24 anti-DT-24
Anti-DT-A alone 0.748 248
900
at antiserum. These results support the direct association of residues 32 to 54 of DT in antibody-mediated inhibition of ADP-ribosyltransferase activity and correspondingly support the close or direct association of this sequence in DT with the ADP-ribosyltransferase reaction.

Effect of antibodies produced against DT-24 on cytotoxicity. Although most studies indicate that toxin-neutralizing antibodies inhibit toxicity by interfering with toxin-cell receptor interactions, the role of enzyme-inhibiting antibodies in toxinnonneutralization remains unclear. To determine whether inhibition of ADP-ribosyltransferase activity by DT-24 antibodies is associated with neutralization of DT toxicity, cytotoxicity assays were performed in the presence or absence of DT-24 antiserum. By using a modification of the DT cytotoxicity assay described by Carroll and Collier (5), 48-well tissue culture plates were seeded with 5 x 10⁴ Vero cells (CCL 81; American Type Culture Collection, Rockville, Md.) per well and incubated at 37°C for 3 h to allow the cells to reach approximately 80% confluency. At this time, 0.1 ng of DT per well was added in growth medium (supplemented with 5% fetal bovine serum and antibiotics in the presence or absence of a 1:20 to 1:100 dilution of DT-24 antiserum. DT cytotoxicity was assayed 18 h later. DT-24 antiserum caused a maximal 14.4% inhibition of DT toxicity over that of normal-serum-treated controls. Comparison of this with its 86.2% inhibition in DT enzymatic activity supports the notion that functional inhibition of enzyme activity by this antiserum is not associated with functional inhibition of DT cytotoxicity.

Because ADP-ribosylating toxins lack an obvious functional sequence motif, identification of key amino acids directly involved in the enzymatic reaction must rely on confirmation by multiple approaches. Site-specific antibodies induced via monoclonal antibody technology or synthetic peptide immunogens have previously been shown to be valuable tools in defining functional sequences in proteins. In the studies described here, synthetic peptides and synthetic peptide antibodies were used to confirm the close or direct association of amino acids between residues 32 and 54 of DT in the ADP-ribosyltransferase reaction. This is consistent with recent crystallographic studies of Choe et al. (6), who found that residues 32 to 54 are included within the CL2 loop of DT, which appears to cover the active-site region. Amino acid modification studies also support the idea of a close or direct association of residues in this sequence with the active site of DT, finding that alteration of Lys-39 (19) and Gly-52 (9) affects enzyme activity.

Although definitive identification of key residues directly involved in the DT enzymatic reaction remains controversial, amino acid modification studies currently favor the involvement of Glu-148 (3) in the DT catalysis reaction and His-21 (15) and Tyr-65 (14) in NAD⁺ binding. DT crystallographic studies (6) support these conclusions but also implicate Thr-42 in NAD⁺ binding. In comparison, crystallographic modeling studies of Brandhuber et al. (2) have predicted that Trp-50, Lys-39, Lys-51, and Try-54 of DT come in close association with NAD⁺ during the ADP-ribosylation reaction. Similar studies of Domenighini et al. (8) have indicated an opposite orientation of NAD⁺, with

<table>
<thead>
<tr>
<th>TABLE 2. DT antiserum binding and inhibition of enzyme activity in the presence of DT-24 peptide</th>
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</thead>
<tbody>
<tr>
<td>Antiserum</td>
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<tr>
<td>-----------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>NRS</td>
</tr>
<tr>
<td>Anti-DT-A alone</td>
</tr>
<tr>
<td>With DT-24[^d]</td>
</tr>
<tr>
<td>Anti-DT-24 alone</td>
</tr>
<tr>
<td>With DT-24</td>
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[^b] Binding was determined in a direct-binding ELISA (ELISA optical density at 450 nm minus background optical density is shown). Percent peptide blocking was calculated relative to antibody binding in the presence or absence of peptide.
[^c] DT ADP-ribosyltransferase activity was calculated as ADP-ribose incorporated into the substrate, cpm is counts per minute incorporated into ADP-ribose. Percent antibody inhibition is percent inhibition of DT activity in the presence of antibody, calculated relative to a normal-serum control. Percent peptide blocking is DT activity in the presence of antibody plus DT-24 peptide.
[^d] DT-24 inhibitor was added at concentrations calculated to optimize peptide inhibition, with 5 μg of peptide added to DT-24 activity assays and 2 μg of peptide added to DT-binding assays.

antiserum becomes evident in Table 2 by the high (83.2%) level of blocking of antibody-mediated inhibition of enzyme activity by peptide DT-24, associated with only a low (16.7%) level of blocking in DT-A antiserum binding to DT. Similar (11.8 and 15.8%) levels of blocking in binding of DT-A antiserum to DT were observed with peptides DT-25 and DT-20, which showed no effect in the peptide-antibody inhibition assay. These results point to the primary role played by residues within peptide DT-24 in mediating inhibition of enzyme activity by this DT-A antiserum.

Induction and analysis of DT-24 peptide antibodies. To determine directly whether antibodies against residues 32 to 54 of DT inhibit DT enzyme activity, antibodies were induced against this sequence as represented by the DT-24 peptide. For this, an N-terminal cysteine residue, added to the DT-24 peptide during synthesis, was used to cross-link peptide molecules. This form of the peptide, in the absence of a carrier protein, was then used to induce DT-24-specific antibodies. As shown in Table 3, antiserum induced by DT-24 reacted specifically with the homologous DT-24 peptide and DT, in both its native and trypsinized-reduced forms, but not with heterologous peptides or PE.

To assess the association of the DT-24 epitope with enzymatic activity, DT-24-specific antiserum was assayed for the potential to inhibit DT ADP-ribosyltransferase activity. As shown in Table 2, DT-24 antiserum proved as effective as DT-A antiserum in inhibiting ADP-ribosyltransferase activity. Preincubation of this antiserum with peptide DT-24 blocked its potential both to bind to DT and to inhibit DT enzymatic activity, confirming the specificity of this antiserum for residues 32 to 54 of DT. These results support the direct association of residues 32 to 54 of DT in antibody-mediated inhibition of ADP-ribosyltransferase activity and correspondingly support the close or direct association of this sequence in DT with the ADP-ribosyltransferase reaction.

<table>
<thead>
<tr>
<th>TABLE 3. Reactivity of DT24 peptide antiserum</th>
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<tr>
<td>Anti-</td>
</tr>
<tr>
<td>DT-24</td>
</tr>
</tbody>
</table>

[^a] DT-24 antiserum was prepared against a synthetic peptide representing DT residues 32 to 54 and was assayed at a 1:20 dilution.
[^b] Antibody reactivity represents binding to the indicated antigen; ELISA optical density minus background optical density is shown. Abbreviations and assay conditions are described in the Table 1 footnotes.
Glu-148, Glu-142, Asn-45, and Asp-47 of DT being involved in NAD\(^+\) binding.

The sequence which has been identified in these studies as being in close association with the ADP-riboseylation reaction includes the sequence pattern G--K--K--Q--D--WKGFY, which is conserved between residues 453 and 470 of PE (lysine residues are substituted for arginine in PE, and dashes represent nonidentical amino acids). The C-terminal portion of this sequence is highly conserved between the two toxins, and crystallographic and amino acid alteration studies are consistent with the notion that residues in this region are directly involved in the common enzymatic functions of DT and PE. As the approach described here using synthetic peptides has proven to be successful in screening sequences in DT for association with the active-site region, such an approach might similarly provide a means of facilitating the localization of active-site residues within other bacterial toxins.

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REFERENCES