The effects of helix breaking mutations in the diphtheria toxin transmembrane domain helix layers of the fusion toxin DAB\textsubscript{389}IL-2

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The fusion protein toxin DAB\textsubscript{389}IL-2 is composed of the catalytic and transmembrane domains of diphtheria toxin genetically linked to human interleukin 2 (IL-2). This fusion toxin is selectively toxic for eukaryotic cells which express the high-affinity form of the IL-2 receptor and the mechanism of intoxication parallels that of native diphtheria toxin. We used site-directed mutagenesis to introduce Pro residues into each of the three helical layers of the transmembrane domain. Although each of the mutations results in the complete loss of cytotoxic activity, individual mutants were found to vary with respect to channel formation in planar lipid bilayers, binding affinity and melting temperature. We propose that each of the three helix layers plays a critical role in the productive delivery of the catalytic domain to the cell cytosol.

Keywords: diphtheria toxin/fusion toxin/transmembrane domain

\section*{Introduction}

Diphtheria toxin (DT) is a 58 kDa, single-chain protein secreted by toxigenic strains of \textit{Corynebacterium diphtheriae}. Intoxication of sensitive cells by DT is dependent upon a number of processes. DT must first bind to its receptor and the charged receptors are then endocytosed in clathrin-coated pits. During endocytosis, the cellular protease furin has been shown to cleave DT at Arg193, which is located in a 14 amino acid protease-sensitive loop subtended by a disulfide bond between Cys186 and Cys201. The N-terminal 21 kDa fragment is composed of the transmembrane domain. The acidic environment of the C domain is known to induce conformational changes within the T domain that expose hydrophobic regions and a portion of the T domain inserts into the endosomal membrane, creating a channel of characteristic conductance (Mindell et al., 1994a,b; Silverman et al., 1994a,b). While the minimal helix requirements for establishment of an ion conductive pore are known (Silverman et al., 1994b), the requirements for the formation of a productive channel (i.e. a channel that leads to delivery of the C domain to the cell cytosol) are unknown. Previous work in this laboratory has shown that TH1–TH4 are not necessary for channel formation, but are required for efficient delivery of the C domain to the cell cytosol (vanderSpek et al., 1993). To understand further the structure–function relationships between the T domain and the productive delivery of the C domain, we have constructed a series of mutations in the DT-based fusion toxin DAB\textsubscript{389}IL-2. DAB\textsubscript{389}IL-2 consists of the C and T domains of DT genetically fused to interleukin 2 (IL-2). Since the R domain of native DT is not present, the fusion toxin is selectively targeted to only those cells that express the high affinity form of the IL-2 receptor (Williams et al., 1987, 1990). Helix-breaking Pro residues were introduced, one at a time, into individual \( \alpha \)-helixes of the T domain layers to explore the role of these structures on cytotoxicity (C domain delivery) and channel formation in planar lipid bilayers. The results indicate that in each case, introduction of Pro residues results in the loss of cytotoxicity. While channel formation in planar lipid bilayers among individual mutants varied, some mutants formed channels with normal conductances, other mutants formed abnormal channels and others did not form channels at all. Receptor binding affinities were determined and fluorescence spectroscopic studies and ADP-ribosyltransferase assays performed to determine the effects of the Pro mutations on protein structure. Mutations in the second helix layer appeared to result in the greatest structural perturbations. The results, along with data from previous studies, suggest that the first helix layer is involved with stabilization of the T domain on the membrane surface and the product pore is formed by the second and third helical layers. The first helix layer provides a specific topologic orientation for efficient insertion of the C-terminal end of the C domain into the pore.

\section*{Materials and methods}

\textbf{Plasmids, bacterial strains and fusion toxin products}

A schematic representation of the T domain of DAB\textsubscript{389}IL-2 is shown in Figure 1. The transmembrane helices, the helix layers and the amino acid residues that were changed are indicated. The pET11d derivatives (Novagen) encoding DAB\textsubscript{389}IL-2 with mutations in the T domain were created by PCR mutagenesis of the wild-type DAB\textsubscript{389}IL-2 gene, followed by cassette exchange using unique \textit{Nsi\textsubscript{I}} and \textit{Sph\textsubscript{I}} sites located in the T domain that encompass the mutated sites (Ausbubel et al., 1991). All constructs were sequenced using a Sequenase reagent kit (US Biochemical). The resulting mutant fusion toxins are listed in Table I.

\textbf{Escherichia coli} strain JM101 was used for plasmid DNA

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preparation and strain HMS174 (Novagen) was used for expression of DAB_{389}IL-2 and mutant proteins.

**Oligonucleotides**

Oligonucleotides were synthesized using an Applied Biosystems Model 391 PCR Mate DNA synthesizer. The oligonucleotides were removed from the columns and deprotected as described by the manufacturer. The oligonucleotides were then dried under vacuum, resuspended in TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0) and their concentrations were determined by measuring the absorbance at 260 nm.

**Polymerase chain reaction (PCR)**

PCR was performed using a Perkin-Elmer reagent kit. The cycle conditions were 1 min at 95°C for strand separation, 1 min at 37°C for primer hybridization and 1 min at 72°C for synthesis of complementary strands. A total of 25 cycles were performed.

**Expression and purification of DAB_{389}IL-2 and transmembrane domain mutants**

Expression of DAB_{389}IL-2 and the T domain mutants DAB(T2-14P)_{389}IL-2, DAB(L248P)_{389}IL-2, DAB(L264P)_{389}IL-2, DAB(A283P)_{389}IL-2, DAB(A304P)_{389}IL-2, DAB(V314P)_{389}IL-2, DAB(L339P)_{389}IL-2 and DAB(L368P)_{389}IL-2 was under control of the T7 polymerase promoter in derivatives of pET11d. The plasmids encoding DAB389 IL-2 and the related mutants were transformed into E.coli HMS174. For expression, 1 l of Luria broth, containing 0.2% maltose and 100 µg/ml ampicillin, were inoculated with 5.0 ml of an overnight culture. The cultures were incubated at 37°C, with shaking, until an OD600 of ~0.3 was reached, then glucose was added to 0.2%. The cultures were incubated until an OD 600 of ~1.0 was reached, MgSO4 was added to a final concentration of 10 mM and 2×10^9 plaque-forming units/ml of the λ coliphage derivative CE6 were added. Coliphage CE6 encodes the T7 RNA polymerase which induces recombinant protein expression. The bacterial cultures were incubated an additional 2 h at 37°C with shaking and the bacteria were pelleted by centrifugation. In each instance, recombinant proteins were expressed in insoluble inclusion body form and were purified, denatured and refolded as described previously (vanderSpek et al., 1993). Once purified and refolded, the fusion protein toxins were concentrated to 50–100 µg/ml using a Filtron concentrator with an M_t = 10 000 cut-off filter (Filtron, Northboro, MA).

Following concentration, the fusion toxins were further purified by DEAE-Sepharose ion-exchange chromatography (Pharmacia Biotech) to separate monomeric from aggregate forms. The proteins were applied to the column and washed exhaustively with 10 mM phosphate buffer, pH 7.2, and eluted with a linear 0–0.8 M KCl gradient in the same buffer. All protein concentrations were determined using Pierce protein assay reagent and proteins were analyzed for purity by 12% SDS–PAGE, followed by staining with Coomassie Brilliant Blue.

**Cytotoxicity assays**

HUT 102/6TG cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone), 2 mM glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. For cytotoxicity assays, 5×10^4 cells were seeded into each of 96 V-bottomed wells in a microtiter plate (Linbro). The fusion toxins to be tested were diluted in the above medium and added such that the final volume in each well was 200 µl and the fusion toxins ranged in concentration from 10^{-7} to 10^{-12} M. The cells were incubated for 18 h at 37°C in a 5% CO2 environment. The microtiter plates were then centrifuged at 170 g for 5 min, to pellet the cells, and the medium was carefully aspirated from above the cell pellets. The cells were resuspended in 200 µl of leucine-free minimal essential medium (Life Technologies) supplemented with 1.0 µCi/ml [¹⁴C]leucine (280 mCi/mmol) (DuPont NEN), 2 mM glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were incubated for 90 min and then the plates centrifuged at 170 g for 5 min. The medium was removed and the cell pellets were resuspended in 60 µl of 0.4 M KOH. After incubation for 10 min at room temperature, 140 µl of 10% TCA were added to each well. After incubation for 10 min at room temperature, precipitated proteins were collected on glass fiber filters using a PhD cell harvester (Cambridge Technology, Watertown, MA). Radioactivity was assessed by standard methods. All assays were performed in quadruplicate and medium alone served as a control.

**Table 1. Comparison of IC_{50} values, binding affinities, conductances and enzyme activity for DAB_{389}IL-2 and related mutant proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>T domain helix mutated</th>
<th>IC_{50} (M)</th>
<th>K_d (pM)</th>
<th>Conductance (pS)</th>
<th>Activity (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAB_{389}IL-2</td>
<td>Wild-type</td>
<td>1.0×10^{-12}</td>
<td>2900</td>
<td>40</td>
<td>171</td>
</tr>
<tr>
<td>DAB(T214P)_{389}IL-2</td>
<td>1</td>
<td>&gt;10^{-7}</td>
<td>2500</td>
<td>43, 57</td>
<td>410</td>
</tr>
<tr>
<td>DAB(L248P)_{389}IL-2</td>
<td>3</td>
<td>&gt;10^{-7}</td>
<td>5000</td>
<td>42</td>
<td>291</td>
</tr>
<tr>
<td>DAB(L264P)_{389}IL-2</td>
<td>4</td>
<td>&gt;10^{-7}</td>
<td>4000</td>
<td>45</td>
<td>222</td>
</tr>
<tr>
<td>DAB(A283P)_{389}IL-2</td>
<td>5</td>
<td>&gt;10^{-7}</td>
<td>5000</td>
<td>50</td>
<td>359</td>
</tr>
<tr>
<td>DAB(A304P)_{389}IL-2</td>
<td>6</td>
<td>&gt;10^{-7}</td>
<td>55</td>
<td>50</td>
<td>1710</td>
</tr>
<tr>
<td>DAB(V314P)_{389}IL-2</td>
<td>7</td>
<td>&gt;10^{-7}</td>
<td>35</td>
<td>—</td>
<td>205</td>
</tr>
<tr>
<td>DAB(L339P)_{389}IL-2</td>
<td>8</td>
<td>&gt;10^{-7}</td>
<td>6000</td>
<td>—</td>
<td>479</td>
</tr>
<tr>
<td>DAB(L368P)_{389}IL-2</td>
<td>9</td>
<td>&gt;10^{-7}</td>
<td>25</td>
<td>40</td>
<td>342</td>
</tr>
<tr>
<td>Free fragment A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>819</td>
<td></td>
</tr>
</tbody>
</table>
**Schild analysis**

Schild analyses were performed to determine the apparent binding affinities of the transmembrane domain mutants (Schild, 1957; Itleson and Gill, 1973). The wild-type form of the fusion toxin DAB389 IL-2 was incubated with HUT 102/6TG cells as described above, in the presence of 1×10^{-7}, 5×10^{-8}, 1×10^{-8} or 5×10^{-9} M of mutant protein. The mutants DAB(T214P)389 IL-2, DAB(L248P)389 IL-2, DAB(L264P)389 IL-2, DAB(A283P)389 IL-2, DAB(A304P)389 IL-2, DAB(V314P)389 IL-2, DAB(L339P)389 IL-2 and DAB(336P389 IL-2) were competitive inhibitors of DAB389 IL-2. The concentration of DAB389 IL-2 at the IC_{50} in the presence and absence of inhibitor was determined and the apparent K_{i} calculated using the equation T/T_{0} = 1 = I/K_{i}, where T is the IC_{50} of DAB389 IL-2 without inhibitor, T_{0} is the IC_{50} concentration of DAB389 IL-2 in the presence of inhibitor and I is the concentration of the inhibitor used.

**Channel-forming activity**

Channel-forming activities of the mutants of DAB389 IL-2 were determined using a planar lipid bilayer membrane system (vanderSpek et al., 1993; Silverman et al., 1994b). The membranes were formed across 50–100 µm apertures that had been made in polystyrene cups. A 1% hexane solution of lecinthin type IIS (Sigma) with the neutral lipids removed (Kagawa and Racker, 1971) was used to coat both sides of the aperture and allowed to dry. The outside of the aperture was then coated with a 1.5% squalene solution prepared in light petroleum. The cup was placed in the back chamber of a block prepared by Warner Instruments (Hamden, CT). A buffer solution (1 M KCl, 2 mM CaCl2, 1 mM EDTA, 50 mM HEPES, pH 7.2) was added to the cup to above the level of the aperture (0.5 ml). The front chamber of the block was filled with 1.0 ml of the same buffer solution, except with 30 mM MES, pH 5.3, instead of the HEPES. A 50 µl aliquot of the lecinthin hexane solution was layered on top of the buffer in the front chamber and the hexane was allowed to evaporate. The buffer in the front chamber was then lowered and raised above the level of the aperture and the planar lipid bilayer was formed. DAB389 IL-2 and its mutants were added to the front chamber at concentrations ranging from 20 to 730 ng/ml. A voltage of +60 mV was applied across the membrane using voltage clamp conditions. The back chamber of the block, containing the cup, was held at virtual ground and the voltages refer to the front chamber to which the proteins were added. Current was monitored using standard methods (Jakes et al., 1989). Channel conductances were determined using the equation g = IV, where g is the conductance, I is the current flowing through the membrane and V is the voltage applied across the membrane.

**Fluorescence spectroscopy**

Fluorescence was measured using an Aminco Bowman Series 2 luminescence spectrometer (Spectronic Instruments, Rochester, NY). The fluorescence intensity was measured at 290 nm excitation and an emission scan from 300 to 390 nm, at a rate of 2 nm/s, was performed. DT, DAB389 IL-2 and related mutants were diluted to between 50 and 100 µg/ml in 20 mM phosphate buffer, pH 7.2. A refrigerated circulating water-bath was used to maintain the temperature of the cuvette holder and a 10 min incubation was performed after each temperature change, to allow the sample temperature to equilibrate. Samples were initially scanned at 5°C, then the temperature was raised in 5°C increments to 60°C and the intrinsic fluorescence was scanned at each increment. The results are reported as a comparison of emission at 330 and 350 nm. The effects of increasing temperature upon fluorescence intensity (F) are reported as temperature versus F_{330}/F_{350}.

**Catalytic domain activity**

Crude EF-2 was prepared from wheat germ extract as described by Chung and Collier (1977). The amount of radioactivity incorporated into trichloroacetic acid (TCA)-precipitable material from [adenine-32-P]nicotinamide adenine dinucleotide (NAD) in the presence of the crude EF-2 was used as a measure of ADP-ribosyltransferase activity. The reaction was performed in 70 µl of 10 mM Tris–HCl, pH 8.0, 1 mM EDTA to which were added 20 µl of the crude EF-2 preparation, 10 µl [adenylate-32-P]NAD (50 µCi/ml, specific activity 800 Ci/mmol adjusted to 3 µM with unlabeled NAD) and 10 µl (10 ng/µl) of DAB389 IL-2 or mutant fusion toxin. Free fragment A (catalytic domain) was used as a control. The reactions were incubated at 25°C for various times: 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 min. The reactions were stopped by addition of 500 µl of 10% TCA and the precipitate was collected on glass microfiber filters (Whatman GF/A). The TCA-soluble material was removed by rinsing with 10% TCA and then 100% ethanol. The filters were air dried and counted using standard methods. The results were recorded as counts per minute (cpm) as a function of time. The results reported here are the number of EF-2 ribosylated by 1 mol of DAB389 IL-2 or the related mutants, per minute (Table I).

**Results**

Figure 1 depicts the amino acid sequence of the T domain, the residues in the T domain α-helices that were individually changed to proline and the T domain helical layers. DAB389 IL-2 (wild-type), DAB(T214P)389 IL-2 and DAB(L248P)389 IL-2 (mutations in the first helical layer), DAB(L264P)389 IL-2 (mutation in helix TH4 between the first and second helical layers), DAB(A283P)389 IL-2, DAB(A304P)389 IL-2 and DAB(V314P)389 IL-2 (mutations in helical layer 3) were expressed and purified as described in Materials and methods. The purified proteins were separated by denaturing SDS–PAGE on 12% SDS gels and stained using Coomassie Brilliant Blue (Figure 2). The proteins all migrated at ~57.7 kDa, which is in agreement with their calculated molecular weights. Western blot analysis was also performed and all the proteins were immunoreactive with polyclonal anti-DT antibody (data not shown).

When assayed on HUT 102/6TG cells, DAB389 IL-2 was...
found to have an IC$_{50}$ of 1.0×10$^{-12}$ M. In marked contrast, none of the T domain mutants were cytotoxic and the IC$_{50}$s were >10$^{-7}$ M (Figure 3A and Table I). Schild analysis was performed to determine if the loss of cytotoxicity resulted from a loss of IL-2 receptor binding affinity by the mutants (Figure 4A and B and Table I). Based on this analysis, the mutants were grouped into one of two types. The first group, with mutations in T domain helixes 1, 3, 4, 5 and 8, possessed apparent binding affinities ($K_d$) ranging from 2000 to 6000 pM. These values are similar to the $K_d$ of 2900 pM found for DAB$_{389}$ IL-2 (Kiyokawa et al., 1991). The second group, however, including mutations in T domain helices 6, 7 and 9, had apparent $K_d$s ranging from 25 to 55 pM. Despite this ~50–110-fold increase in apparent binding affinity for the IL-2 receptor, these mutants were completely non-toxic for HUT 102/6TG cells.

In the intoxication process, under acidic conditions, the T domain of diphtheria toxin is known to insert into the endocytic vesicle membrane and form a channel. In vitro, the transmembrane domain of diphtheria toxin is also known to form characteristic channels in planar lipid bilayers. We therefore analyzed the channel-forming ability of DAB$_{389}$ IL-2 and the related Pro substitution mutants. As expected from earlier studies, upon addition of 20 ng/ml of DAB$_{389}$ IL-2 to the front chamber, characteristic channels of 40–43 pS spontaneously formed (Mindell et al., 1992; vanderSpek et al. 1993). The mutations in helix layer 1 resulted in proteins that still formed channels. In the case of the T214P mutation two channels measured were found to have conductances of 43 and 57 pS. DAB(L248P)$_{389}$ IL-2 formed a few briefly open channels with a conductance of 42 pS. The mutant fusion toxin DAB(L264P)$_{389}$ IL-2 in which the Pro mutation is between helix layers 1 and 2 was found to form slightly larger channels than wild-type. Mutations in helix layer 2 resulted in no, or very abnormal, channel formation. The helix layer 3 mutations were of interest. The TH8 mutation resulted in a protein that no longer formed channels. This was expected as it is known that TH8 and TH9 alone will form channels with the characteristic 42 pS conductance (Silverman et al., 1994b).

Since the introduction of Pro point mutations is known to be helix breaking and may affect the refolding and stability of a given mutant fusion toxin, we measured and compared the effect of these mutations in DAB$_{389}$ IL-2 on intrinsic fluorescence. In these experiments we determined the fluorescence intensity ratio at 300 and 350 nm as a function of temperature. Since this analysis measures the environment around absorbing Trp amino acids, they serve as an indirect
The Trp residues in DT are located at positions 51 and 153 in the C domain, 206 and 281 in the T domain and 398 in the native DT receptor binding domain. As can be seen in Figure 5, the 330/350 nm fluorescence intensity ratio for DAB389 IL-2 was less than that of native DT and most of the unfolding occurred between 30 and 50°C. The Trp substitutions in helical layers 1 and 3 did not markedly alter the fluorescence intensity ratio and their thermal denaturation curves were found to be similar to that of DAB389 IL-2 (data not shown). The Pro substitution mutants that were found to differ from DAB389 IL-2 were those in the second helical layer. The TH5 mutation DAB(A283P)389 IL-2 exhibited less fluorescence intensity at lower temperatures and the red shift in fluorescence with increasing temperature was between 30 and 40°C and was not marked. The TH6 mutation DAB(A304P)389 IL-2 exhibited a denaturation curve similar to that of wild-type. The TH7 mutant DAB(V314P)389 IL-2 appeared to have the most temperature-sensitive structure and initially exhibited the same fluorescence intensity as wild-type DAB389 IL-2, but then showed a sharp decrease in the fluorescence intensity ratio between 30 and 35°C. Additionally, the fluorescence intensity ratios at the higher temperatures were lower than those of wild-type DAB389 IL-2 (Figure 5).

The ADP-ribosyltransferase activity of the C domain was also determined as a means of assessing the effects of the mutations on C domain folding and availability. One mole of the free fragment A (C domain) catalyzed the ADP-ribosylation of 819 mol of EF-2 under the conditions of the assay and 1 mol of wild-type DAB389 IL-2 catalyzed the ADP-ribosylation of 171 mol of EF-2. This indicates that C domain alone was more available for ribosyltransferase activity than when it was constrained by the structure within the fusion toxin. Compared with wild-type fusion toxin, the Pro mutations had similar activities ranging from 1.2 to 2.8-fold greater, with the exception of the helix layer 2, TH6 mutation which possessed 10-fold greater activity (Table I). This result indicates the C domain in the TH6 mutant was in a more ‘relaxed’ conformation than even C domain alone.

Discussion

The function of the T domain of DT is to facilitate the delivery of the C domain to the cell cytosol where it ADP-ribosylates elongation factor 2. The mechanisms of this delivery include the formation of an ion-conducting channel with a characteristic conductance of 50–42 pS (Mindell et al., 1992; vanderSpek et al., 1993). In an earlier study we showed that while channel formation is required for the productive delivery of the C domain to the cytosol, it is by itself not sufficient (vanderSpek et al., 1993). In order to probe further the structure–function relationships within the transmembrane domain that are required for delivery of the C domain to the cytosol, we used PCR site-directed mutagenesis to introduce Pro residues, one at a time, into various regions of the T domain helix layers of the DT-based fusion toxin DAB389 IL-2. We then studied the effects of these mutations on cytotoxicity and channel formation in planar lipid bilayers.

Pro residues were introduced as they are known to disrupt helical structures and to produce 20–30° kinks in α-helices (Chou and Fasman, 1974; Barlow and Thornton, 1988; Richardson and Richardson, 1988; Sankararamakrishnan and Vishveshwara, 1990). To determine the effects of the Pro mutations on conformation, three different methods were employed. Binding affinities were determined to assess conformational effects on the IL-2 receptor binding domain, ADP-ribosyltransferase assays were performed to determine if the mutations affected the C domain activity and fluorescence spectroscopic studies were performed to determine the effects on conformation surrounding Trp residues. As the temperature was raised, DT, DAB389 IL-2 and the related fusion toxins were denatured (melted) and Trp residues became more exposed to the aqueous solution, resulting in lower fluorescence intensities. The Trp residues in DT are located at positions 51 and 153 in the C domain, 206 and 281 in the T domain and 398 in the native DT receptor binding domain. The Trp residues in DAB389 IL-2 and the related mutants are located at positions 51 and 153 in the C domain, 206 and 281 in the T domain and 398 in the native DT receptor binding domain. The Trp residues in DAB389 IL-2 and the related mutants are located at positions 51 and 153 in the C domain, 206 and 281 in the T domain and 398 in the native DT receptor binding domain. The results for native DT showed marked denaturation between 45 and 55°C. This result is in excellent agreement with the transition temperature midpoint of 48°C obtained by Zhao and London (1986) (Figure 5). In contrast, the melting curve for the fusion protein toxin DAB389 IL-2 indicates that this molecule is less tightly folded than DT and melted occurring between 30 and 50°C. The fluorescence spectroscopic melting studies were used to assess the overall conformation of all three domains.

The results presented here lend further insight into the functions of the three helix layers of the T domain.

**Helix layer 1 mutations**

The first helix layer is composed of helices TH1–TH3. In the first mutation, DAB(T214P)389 IL-2, a Thr residue present in TH1 was exchanged with a Pro residue (Figure 1). DAB-(T214P)389 IL-2 was not cytotoxic, but formed noisy channels,
with conductances higher than those formed by the wild-type DAB389 IL-2. The fluorescence spectroscopic studies of DAB(T214P)389 IL-2 indicate that this mutant possessed a melting curve similar to that of wild-type DAB389 IL-2. There is a Trp present at position 207 near the start of the first helix layer and it is interesting that the introduction of the Pro residue at position 214 did not measurably disrupt the environment of this residue.

DAB(T214P)389 IL-2 catalyzed the ADP-ribosylation of EF-2 with activity similar to that of DAB389 IL-2, indicating that C domain activity was not affected by the mutation. The free fragment A (C domain of diphtheria toxin) possessed a catalytic activity approximately 5-fold greater than that of DAB389 IL-2, probably due to steric constraints placed upon the C domain by the remainder of the protein (Table I). The binding activity of DAB(T214P)389 IL-2 was also similar to that of the wild-type and all these results suggest that the folding of this protein, with respect to environment of Trp residues, C domain activity and R domain receptor binding affinity, did not differ appreciably from wild-type (Figure 4 and Table I).

DAB(L248P)389 IL-2, the TH3 mutant, was not cytotoxic, formed channels with 42 pS conductance and possessed a binding affinity, ADP-ribosyltransferase activity and melting curves similar to those of wild-type. DAB(L264P)389 IL-2, containing a mutation in TH4 which connects the first and second helix layers, was also not cytotoxic, formed channels with conductance of 45 pS and possessed a binding affinity, ADP-ribosyltransferase activity and melting curve similar to those of wild-type (Table I).

In all cases, the helix layer 1 mutations were not cytotoxic, they formed channels (although in the case of the TH1 and TH4 mutations with slightly different conductances) and possessed binding affinities, ADP-ribosyltransferase activities and melting curves similar to those of the wild-type fusion toxin. These results are in agreement with those of our previous work in which amino acid residues 204–263 were deleted from DAB389 IL-2. This deletion effectively removed the first helix layer and a portion of TH4 from the protein (vanderSpek et al., 1993). The mutant protein DAB(A282–263)389 IL-2 formed channels with wild-type conductances, but was not cytotoxic although the binding affinities were similar to those of wild-type DAB389 IL-2. Additionally, when the missing amphipathic helices were reconstructed using different amino acid residues possessing similar properties, some cytotoxicity was restored. These results are all consistent with the first helical layer being a non-specific, membrane surface anchor that aligns the second and third helix layers for insertion into the membrane. In the absence of the first helix layer, or upon insertion of a helix-breaking Pro residue, the channels formed are non-productive and do not lead to delivery of the C domain to the cell cytosol. Since it is known that the delivery of a single molecule of the C domain to the cytosol is lethal for the cell (Yaimaizumi et al., 1978), it is possible that this region of the T domain plays an additional role in the intoxication process. The architecture of the first helical layer of the T domain on the membrane surface may also orient the C-terminal end of the C domain for efficient insertion into the channel. As described by Mark Twain (1884), this problem is not unlike that faced by Huckleberry Finn: does one bring the thread to the eye of the needle, or the eye of the needle to the thread?

Helix layer 2 mutations

The mutants with Pro substitutions in the second helix layer, DAB(A283P)389 IL-2 (TH5 mutation), DAB(A304P)389 IL-2 (TH6 mutation) and DAB(V314P)389 IL-2 (TH7 mutation), were not cytotoxic and channel formation was abnormal. Only DAB(A304P)389 IL-2 (TH6) formed channels and they possessed larger conductances (50 pS) than those observed for wild-type DAB389 IL-2.

DAB(A283P)389 IL-2, the TH5 mutation, appears to exist as a loosely folded molecule with slight denaturation occurring between 30 and 40°C (Figure 5). There is Trp residue at position 282 and it is not surprising that an Ala–Pro mutation at residue 283 should result in exposure of the Trp residue. DAB(A304P)389 IL-2 possessed a 44 and ADP-ribosyltransferase activity that were not substantially different from those of wild-type. DAB(A304P)389 IL-2 (TH6 mutation) possessed a melting curve similar to that of DAB389 IL-2. However, it was more active in the ADP-ribosylation assay (10-fold) and the binding affinity was better (50-fold), indicating that folding differences existed that affected the C domain and the receptor binding domain. DAB(V314P)389 IL-2 (TH7 mutation) exhibited a striking denaturation between 30 and 35°C, possessed ADP-ribosyltransferase activity similar to that of wild-type and had an 80-fold better binding affinity. Overall, these results suggest that the insertion of Pro residues into the second helix layer affects the conformation of the fusion protein. These changes in protein conformation, in turn, are likely to affect the shape of the channel formed, resulting in a change in conductance.

Previous studies have shown that only helix layer 3, composed of TH8 and TH9, need be present for the formation of channels with characteristic conductances (Silverman et al., 1994). Additionally, we have shown that deletion of a portion of TH9 results in the formation of smaller channels and a decrease in cytotoxicity (vanderSpek et al., 1994). While only TH8 and TH9 are required for channel formation in planar lipid bilayers, these helixed by themselves cannot form a productive channel (i.e. a channel capable of facilitating the delivery of the C domain). The current findings indicate that the second helix layer is also required for effective C domain delivery to the cell cytosol and that the introduction of the Pro residues resulted in no or abnormal channel formation. Although the first helix layer may act as a membrane surface anchor, it appears that the second helix layer is more intimately associated with channel formation. Choe et al. (1992) proposed that TH5 and TH6 in the second helix layer, in addition to TH8 and TH9, insert into the endosome membrane upon acidification of the endosome. Work by Cabiaux et al. (1994) indicated that TH6 and TH7 probably form one transmembrane helix. Overall the indications are that the second helix layer is likely to participate in the formation of a productive channel.

Helix layer 3 mutations

The Pro substitution mutations in helix layer three yielded some surprising results. DAB(L339P)389 IL-2 (TH8 mutation) is the putative channel-forming region and, as expected, this mutant did not form channels and was not cytotoxic. The binding affinity, denaturation curve and ADP-ribosyltransferase activity did not differ greatly from those of wild-type DAB389 IL-2 (Table I). DAB(L368P)389 IL-2, the TH9 mutation, was not cytotoxic although it possessed ADP-ribosyltransferase activity and a denaturation curve similar to those of wild-type, a binding affinity 70-fold better than that of wild-type and formed channels with normal conductance. This result was unexpected as we anticipated that the introduction of a Pro residue into the middle of TH9 would have resulted in a
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mutant that did not form channels. In a previous study, we deleted a portion of TH9 beginning with a Phe residue at position 369. DAB(Δ369–387)389 IL-2 formed smaller channels with a 30 pS conductance. The binding affinity was similar to that of wild-type DAB389 IL-2 and the cytotoxicity was 2000-fold less than that of wild-type. We reasoned that some of the 30 pS channels were able to facilitate delivery of the C domain to the cytosol. In this study, replacement of a Leu residue at position 368 with a Pro resulted in channel formation with wild-type conductance. Even though channels were formed, however, they were not productive channels, and DAB(L368P)389 IL-2 was not cytotoxic. Although DAB-(L368P)389 IL-2 formed a channel with wild-type conductance, it is possible that the shape of the channel was changed so that C domain delivery was affected. The fact that the binding affinity was changed indicates that the protein conformation was in some way changed.

Although the precise molecular mechanism for productive delivery of the DT C domain to the cytosol is not fully understood, it is apparent that each of the helical layers of the T domain participates in the process. The helix layer 2 and 3 helices, TH5, TH6, TH7, TH8 and TH9, are each required, in an undisrupted form, for the formation of channels that lead to productive delivery of the C domain to the cytosol. Observations made in the present study support the hypothesis that TH5, TH6 and TH7 form one transmembrane hairpin and TH8 and TH9 form another transmembrane hairpin. The two transmembrane hairpins form the foundation of a channel that is capable of facilitating the delivery of the C domain to the cell cytosol. In addition, the results presented here support and extend the hypothesis that the first helical layer serves to stabilize the T domain on the membrane surface and to orient the C terminal end of the C domain for efficient insertion into the channel for delivery to the cytosol.

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