Structure/Function Analysis of Interleukin-2-Toxin (DAB\textsubscript{486}-IL-2)

We have used cassette and deletion mutagenesis to analyze the structural features of fragment B-related sequences in the fusion toxin DAB\textsubscript{486}-IL-2 (where IL-2 represents interleukin-2) that are necessary for the efficient delivery of fragment A to the cytosol of target cells. We demonstrate that whereas an intact disulfide bond between Cys\textsubscript{461} and Cys\textsubscript{471} may be required for the cytotoxic action of native diphtheria toxin, this bond is not required for the cytotoxic action of DAB\textsubscript{486}-IL-2. The in-frame deletion of the 97 amino acids from Thr\textsuperscript{387} to His\textsuperscript{466} of DAB\textsubscript{486}-IL-2 increases both the potency and the apparent dissociation constant ($K_d$) of the resulting fusion toxin for high affinity interleukin-2 receptor-bearing target cells. In contrast, the in-frame deletion of either the 191 amino acids between Asp\textsuperscript{291} and Gly\textsuperscript{483} or the 85 amino acids between Asn\textsuperscript{204} and Ile\textsuperscript{290} results in a 1000-fold loss in potency. These regions contain the putative membrane-spanning regions and the amphipathic membrane surface-associated regions of fragment B, respectively. These results indicate that the efficient delivery of the ADP-riboseyltransferase from DAB\textsubscript{486}-IL-2 to the cytosol requires the membrane-associating domains of fragment B. This function has been postulated to play a role in the diphtherial intoxication of eukaryotic cells. However, unlike native diphtheria toxin, fragment B sequences distal to Thr\textsuperscript{387} do not enhance the potency of DAB\textsubscript{486}-IL-2.

Moreover, the cytotoxic action of DAB\textsubscript{486}-IL-2, like that of native diphtheria toxin, requires receptor-mediated endocytosis, passage through an acidic compartment, and delivery of fragment A-associated ADP-riboseyltransferase to the cytosol of target cells (2).

To further our understanding of the entry of DAB\textsubscript{486}-IL-2 fragment A into the cytosol and to define the role that fragment B sequences play in this process, we have used cassette exchange and in-frame deletion mutagenesis to assemble a family of DAB-IL-2 variants. We demonstrate that whereas a Cys\textsuperscript{471} to Tyr\textsuperscript{471} mutation renders DT nontoxic, the analogous mutation in DAB\textsubscript{486}-IL-2 has little, if any, effect on the potency of this fusion toxin. Moreover, we show that fragment B sequences between Thr\textsuperscript{387} and His\textsuperscript{466} can be deleted from DAB\textsubscript{486}-IL-2 without a loss in biological activity, whereas the further deletion of Ser\textsuperscript{292} to Thr\textsuperscript{387}, a region which contains two putative membrane-spanning helices of fragment B, essentially abolishes cytotoxic activity. In a similar fashion, the deletion of fragment B sequences between Asp\textsuperscript{204} and Ile\textsuperscript{290} which include an amphipathic region(s) predicted to have membrane surface binding properties, also results in a marked loss of cytotoxic activity.

**MATERIALS AND METHODS**

**RESULTS AND DISCUSSION**

The nontoxic diphtheria toxin-related protein CRM1001 has been shown to result from a single G:A point mutation which changes Cys\textsuperscript{461} to Tyr\textsuperscript{471} (15). This mutation disrupts the disulfide bond between Cys\textsuperscript{461} and Cys\textsuperscript{471} and results in a loss of cytotoxic activity. Zucker (16) has shown by Schild analysis that CRM1001 blocks the action of native toxin on Chinese hamster ovary cells as effectively as CRM197. Recently, Dell'Arciprete et al. (17) have obtained similar results for Vero cells. Since CRM1001 retains ADP-riboseyltransferase activity and binds to receptors on Chinese hamster ovary cells (16) and Vero cells (17), its lack of cytotoxicity suggests that the fragment B disulfide bond may be required to retain a conformation necessary for efficient internalization and/or processing of receptor-bound native toxin.

To determine whether an intact disulfide bond between Cys\textsuperscript{461} and Cys\textsuperscript{472} was required for the biological activity of DAB\textsubscript{486}-IL-2, a 587-base pair ClaI-SphI restriction fragment from the tox-1001 allele was subcloned into plasmid pDW24

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to generate pDW26 (Fig. 1, Table 1). Following ligation and transformation, the DNA sequence of the tax-1001 portion of the new gene fusion was determined to verify that the Cys72

Escherichia coli

was grown in M9 minimal medium, and the mutant fusion toxin (designated DAB(1001)sr-IL-2) was purified from whole cell extracts by immunoaffinity chromatography and high pressure liquid chromatography.

The capacity of DABas-IL-2, CRM1001, and DAB(1001)sr-IL-2 to block [14C]leucine incorporation by high affinity IL-2 receptor-bearing HUT 102/6TG cells was determined by dose-response analysis. As anticipated from earlier studies (2), both native DT and DABas-IL-2 were highly toxic for these cells, whereas CRM1001 was found to be nontoxic. In marked contrast to CRM1001, however, the fusion toxin which carries the Cys72 to Tyr772 mutation (DAB(1001)sr-IL-2) was found to be as toxic for HUT 102/6TG cells as DABas-IL-2 (data not shown). These results demonstrate that whereas the fragment B disulfide bond between Cys461 and Cys471 may be required for the biological activity of native diphtheria toxin, this disulfide bond is not required for biological activity of the fusion toxin.

In addition to CRM1001, other nontoxic DT-related proteins have been used to study the structural requirements for the delivery of DT fragment A to the cytosol of target cells. For example, CRM45 (which lacks the diphtheria toxin receptor-binding domain but retains the membrane-associating domains of fragment B) (18-20) and CRM26 (which terminates upstream of these hydrophobic domains) have both been used by Bacha et al. (21) to form conjugate toxins. In this study, DT-related CRM96 and CRM45 were chemically conjugated to thryopterin-releasing hormone (TRH). Whereas both CRM26-TRH and CRM45-TRH were found to bind to the TRH receptor and to retain ADP-ribosyltransferase activity, only CRM45-TRH was found to be cytotoxic for TRH receptor bearing cells. These observations suggested that the membrane-associating domains of fragment B were necessary for the delivery of fragment A to the cytosol of target cells.

Since the disulfide bond between Cys461 and Cys572 was not essential for the cytotoxic action of DABas-IL-2, it was of interest to determine the DT fragment B sequences which were essential for the delivery of fragment A from the fusion toxin to the cytosol of target cells. Several in-frame deletion mutations were introduced into the fragment B encoding portion of the DABas-IL-2 toxin gene (Fig. 2). The first mutant (DABas-IL-2) was constructed by removing a 309-base pair HpaII-SphI restriction fragment from pDW24 and replacing it with the oligonucleotide linker 261/274 (Table 2) to generate plasmid pDW27 (Fig. 1). This linker restores fragment B sequences from Pro383 to Thr387 (amino acids 210-252 in DABas-IL-2) and allows for in-frame fusion to IL-2 sequences at this position. Thus, in DABas-IL-2, the 97 amino acids between Thr587 and Ile646 have been deleted, and the DT-related component of the fusion toxin is equivalent to CRM45.

In a similar fashion, a 191-amino acid in-frame deletion was constructed by removing a Ccll-SphI restriction fragment from pDW24 and replacing it with the oligonucleotide linker 292/293 (Table 2) to form plasmid pDW28, which encodes DABas-IL-2 (Fig. 1). In this case, the in-frame deletion encompasses the putative membrane-spanning helices that have been predicted to play a role in the delivery of fragment A to the eukaryotic cell cytosol (19, 20).

Following purification, DABas-IL-2 and DABas-IL-2 were found to have electrophoretic mobilities corresponding to apparent molecular masses of 57 and 47 kDa, respectively (Fig. 3). In dose-response analysis of HUT 102/6TG cells (Fig. 4), DABas-IL-2 and DABas-IL-2 exhibited IC50 values of ~4 x 10-10 and ~1 x 10-11 M, respectively. In marked contrast, the IC50 of DABas-IL-2 was ~1000-fold greater (~4 x 10-7 M). These results strongly suggest that fragment B sequences between Ser792 and Thr897 are essential for efficient delivery of fragment A, whereas sequences between Thr897 and His935 are not.

Since it was possible that the low potency of DABas-IL-2 for HUT 102/6TG cells was related to altered binding to the high affinity IL-2 receptor, a series of competitive displacement experiments using 111H-labeled recombinant IL-2 were conducted. As shown in Fig. 5, both DABas-IL-2 and DABas-IL-2 were found to have an apparent Kd ~3 times lower than that of DABas-IL-2 (Kd = 8 x 10-7 versus 2.5 x 10-4 M). However, it should be noted that whereas DABas-IL-2 binds more avidly to the high affinity IL-2 receptor than does DABas-IL-2, its cytotoxic activity is at least 1000-fold lower (Fig. 4). These results demonstrate that avid binding to the target receptor is not, in itself, sufficient for the biological activity of the DT-related IL-2 fusion toxins and that fragment B sequences between Ser792 and Thr897 are essential for a post-receptor binding event in the intoxication process. It is of particular interest to note that fragment B sequences from Ser792 to Thr897 contain the putative membrane-spanning helices that have been postulated to facilitate the membrane translocation of fragment A to the cytosol (19, 20). Moreover, this region of DT fragment B contains three of the four hydrophobic membrane-associating regions predicted by Elsdenber et al. (22).

Based upon hydrophobic photolabeling experiments, Papini et al. (23) have proposed that the first step in the diphtherial intoxication process involves the interaction of the toxin with the surface of the eukaryotic cell membrane. Lambotte et al. (20) have identified an amphipathic region of fragment B which is homologous to the surface lipid associating domain of apolipoprotein A-I. Therefore, it was postulated that this amphipathic region (amino acids 210-252 in DABas-IL-2) plays a role in the intoxication process. To test this hypothesis, the 85-amino acid encoding region from NsiI to Ccll was deleted from both pDW24 and pDW27 to form plasmids pDW30 and pDW31, respectively (Figs. 1 and 2 and Table 2). Plasmids pDW30 and pDW31 encode DAB(A205-289)sr-IL-2 and DAB(A205-289)sr-IL-2, respectively. Following ligation and transformation, the DAB-IL-2-related fusion proteins were expressed and purified as described above (Fig. 3). As shown in Fig. 4, the deletion of Leu449 to Val449 (which includes the amphipathic domain(s) of fragment B) results in an ~1000-fold loss of cytotoxic activity against high affinity IL-2 receptor-positive cells in vitro. In this instance, however, the deletion reduced the ability of the fusion toxin to compete for binding to the high affinity IL-2 receptor. For example, DABas-IL-2 was found to have a Kd of 2.5 x 10-4 M, whereas DAB(205-289)sr-IL-2 was found to have a Kd of 1 x 10-7 M. Comparison of the apparent dissociation constants of the DAB-IL-2 fusion toxins suggests that this region of fragment B may stabilize the interaction between the fusion toxin and the cell surface. Recent studies by Cabiaux et al. (24) of the secondary structure of DT interacting with acyl liposomes support such a model. Using polarized infrared spectroscopy, these investigators found that the amphipathic helical domain(s) of DT associated with the liposome surface at low pH such that the long axis of the helix was oriented perpendicular to the lipid acyl chains. Furthermore, the putative transmembrane domains of fragment B were found to associate parallel to the acyl chains of the liposomes. Although these experi-
ments lend credence to the binding and cytotoxicity data obtained for the four DAB IL-2 deletion mutants reported here, the deletions are large, and the results must be interpreted with caution. Site-directed mutagenesis experiments are currently underway to further define the structural features of both the amphipathic and membrane-spanning regions of DAB-IL-2 fragment B that are required for the delivery of fragment A to the cytosol of target cells.

Recently, Edwards et al. (25) have examined the receptor binding properties of a fusion toxin composed of transforming growth factor \( \alpha \) (TGF\( \alpha \)) and a truncated version of \textit{Pseudomonas} exotoxin A (PE40). Since the orientation of the functional domains of exotoxin A are opposite that of DT, receptor-binding domain substitution occurs at the N terminus. TGF\( \alpha \)-PE40 was found to bind to the epidermal growth factor receptor less effectively than native TGF\( \alpha \). Interestingly, the analysis of internal in-frame deletion mutants of TGF\( \alpha \)-PE40 demonstrated that the removal of the N-terminal 59 or 130 amino acids from the PE40 toxophore resulted in fusion proteins that were increasingly effective in their epidermal growth factor receptor binding properties. However, the truncated TGF\( \alpha \)-PE fusion toxins were less cytotoxic than full-length TGF\( \alpha \)-PE40.

In a similar study, Colombatti et al. (26) cross-linked antibody against the T3 antigen of human T-cells (UCHT1) to either native diphtheria toxin or DT-related mutant proteins. CRM45-like MapSA-UCHT1 conjugates were found to be 100-fold more potent than fragment A-UCHT1 conjugates. Surprisingly, full-length DT-UCHT1 conjugates were 100-fold more potent that the CRM45-like conjugates. Whereas it was previously shown that fragment B sequences contained within CRM45 were essential for the delivery of fragment A to the cytosol (21), the report of Colombatti et al. (26) strongly suggested that fragment B sequences downstream of Thr\( ^{357} \) enhanced the efficiency of fragment A entry into the cytosol.

In contrast to the results of Edwards et al. (25) and Colombatti et al. (26), the in-frame deletion of 97 amino acids from the fragment B domain of DAB\( ^{386} \)-IL-2 resulted in a fusion toxin that bound more effectively to the high affinity IL-2 receptor while retaining full cytotoxic potency for target cells. Although it is possible that IL-2 related sequences in DAB\( ^{386} \)-IL-2 partially compensate for the deleted fragment B sequences, the similarity in the dose-response curves of DAB\( ^{386} \)-IL-2 and DAB\( ^{395} \)-IL-2 suggests that any role sequences from Thr\( ^{357} \) to His\( ^{485} \) may play in the entry process is below the level of detection in the HUT 102/6TG assay system.

In conclusion, studies reported here further define the interaction between DAB-IL-2 fusion toxins and target lymphocytes. The removal of 97 amino acids between Thr\( ^{387} \) and His\( ^{485} \) alters the IL-2 conformation such that the IL-2 component of the fusion toxin binds to its receptor with higher affinity. Conversely, deletion of the amphipathic domain(s) contained within the Leu\( ^{288} \) to Val\( ^{298} \) region of fragment B results in a 4-fold loss of receptor binding affinity. This observation suggests that this region of fragment B may interact with the membrane surface and stabilize the interaction between the DAB-IL-2 fusion toxin and the cell. However, when the amphipathic domain(s) is deleted from DAB\( ^{395} \)-IL-2, the resulting fusion toxin binds to receptors as avidly as DAB\( ^{386} \)-IL-2, but is at least 1000-fold less cytotoxic. Thus, it appears that this membrane surface interaction may not only stabilize binding to the IL-2 receptor, but may be required to juxtapose the membrane-spanning domains of fragment B with the endocytic vesicle membrane for the efficient delivery of fragment A to the cytosol. Finally, deletion of the putative membrane-spanning helices from DAB\( ^{395} \)-IL-2 increases binding affinity, but abolishes cytotoxic activity.

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Structural and functional analysis of INT-2 homologues (INT-2): protein 3′ sequence required for the isolation of fragment A from the chromosomal target sites.

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AB c DEF H

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-L c L-67
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Figure 1. Partial restriction endonuclease digestion map of the structural gene encoding INT-2. Stippled boxes between the INT and INT-2 restriction endonuclease sites designate the INT-2 transgene fragment translated sequences which encode the neomycin resistance domain. The ambisense domain is bounded between the INT and INT-2 sites, and the polyadenylate spanning domains are bounded between the IST and IST sites. Restriction bands indicate the relative position of internal trinucleotide mutations.

Figure 2. BstNI-cleaved plasmid gel analysis of INT-2. Restriction enzymes: Alu I (A), BstNI (B), Dra I (C), Dde I (D), Hinc II (E), MucI (F), Mlu I (G), Pst I (H), and Pvu II (I). The DNA was loaded in 5 μg of DNA from each lane was loaded. The gels were dried and autoradiographed. The DNA size markers were: 4.7, 4.2, 3.6, 2.8, and 1.9 kb.

Figure 3. HindIII-cleaved plasmid gel analysis of INT-2. Restriction enzymes: Alu I (A), BstNI (B), Dra I (C), Dde I (D), Hinc II (E), MucI (F), Mlu I (G), Pst I (H), and Pvu II (I). The DNA was loaded in 5 μg of DNA from each lane was loaded. The gels were dried and autoradiographed. The DNA size markers were: 4.7, 4.2, 3.6, 2.8, and 1.9 kb.

Figure 4. Direct sequence analysis of INT-2. Restriction enzymes: BstNI (A), BstNI (B), Dde I (C), MucI (D), Mlu I (E), Pst I (F), and Pvu II (G). The DNA was loaded in 5 μg of DNA from each lane was loaded. The gels were dried and autoradiographed. The DNA size markers were: 4.7, 4.2, 3.6, 2.8, and 1.9 kb.

Figure 5. Summary of the predicted secondary structure of the INT-2 transgene fragment 3′ region.

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Figure 5. Summary of the predicted secondary structure of the INT-2 transgene fragment 3′ region.
Table 1: Bacterial strains used in this study.

| species | Tax product | reference |
|---------|-------------|-----------|
| E. coli (pHE6) | DBA459-1L-2 | this study |
| E. coli (pHE27) | DBA459-1L-2 | this study |
| E. coli (pHE28) | DAb459-1L-2 | this study |
| E. coli (pHE18) | DAb537-1L-2 | this study |
| E. coli (pHE30) | DAb200-2001-1L-2 | this study |
| E. coli (pHE31) | DAb200-2001-1L-2 | this study |

Confrontation: gibberelli (Gibor 1995) (K11000)

Table 2: Oligonucleotide linkers used in this study.

| Tax constant | Oligonucleotide | linker |
|--------------|----------------|-------|
| DBA459-1L-2  | 314 | 5' GCG GTC TGC GGC TAC GAT 3' |
|              | 314 | 5' CGC CTT TGC GGC TAC GAT 3' |
| DBA537-1L-2  | 292 | 5' CAT GCT GCC AGT 3' |
|              | 293 | 5' CAT GCC AGT 3' |
| DAb200-2001-1L-2 | 537 | 5' TAA ACC TAT 3' |
|              | 538 | 5' TAA ACC TAT 3' |
| DAb200-2001-1L-2 | 537 | 5' TAA ACC TAT 3' |

Table 3: Relative affinity of IL-2 and IL-3-2 related fusion proteins to display [125I]xyl-2 from high affinity IL-2 receptors on NIT 1D/NIT 1D cells.

| unlabeled ligand | apparent Kd | Rd DAB-1L-2/xyl-2 |
|-----------------|-------------|-------------------|
| rIL-2           | 1.7 x 10^-9 | -                 |
| DAB459-1L-2     | 2.5 x 10^-9 | 119               |
| DAB537-1L-2     | 8.0 x 10^-9 | 47                |
| DAB200-2001-1L-2| 8.6 x 10^-9 | 14                |
| DAB200-2001-1L-2| 1.0 x 10^-8 | 309               |
| DAB200-2001-1L-2| 2.9 x 10^-8 | 7.0               |
Structure/function analysis of interleukin-2-toxin (DAB486-IL-2). Fragment B sequences required for the delivery of fragment A to the cytosol of target cells.
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