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Expression of an Anti-CD3 Single-Chain Immunotoxin with a Truncated Diphtheria Toxin in a Mutant CHO Cell Line

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ADP-ribosylating immunotoxins are generally expressed in *Escherichia coli* and then refolded *in vitro.* Because the efficiency of the *in vitro* refolding process decreases with the number of protein domains and internal disulfide bonds, these immunotoxins have been generally limited to single-chain monovalent structures. We now show that using the hamster cell line CHO K1 RE1.22c (J. M. Moehring and T. J. Moehring, 1979, *Somat. Cell Genet.* 5, 453–468) that has been mutated to ADP-ribosylation insensitivity, a level of 4 μg/ml of a truncated anti-T cell immunotoxin, DT390-scFvUCHT1, can be secreted into the medium. This immunotoxin is glycosylated at the two potential N-linked glycosylation sites in the toxin moiety: positions 16–18 in the A chain and residues 235–237 in the B chain. The glycosylated immunotoxin is relatively nontoxic (IC_{50} 4.8×10^{-10} M). Removal of the N-linked oligosaccharides by *N*-glycosidase F treatment or mutations at the two N-linked glycosylation sites results in a highly active immunotoxin with an IC_{50} of 4×10^{-12} M toward $CD3^+$ Jurkat cells. This is a 12-fold increase in toxicity over the same immunotoxin harvested from *E. coli* periplasm without refolding. A single Asn^{235} Ala mutation that removed the B chain glycosylation was nearly as toxic as the double mutant. This suggests that B chain glycosylation is the major cause for the loss of toxicity. © 2000 Academic Press

Diphtheria toxin (DT)[2] is a 535-amino-acid protein composed of two subunits consisting of an A chain and a B chain connected by an arginine-rich and disulfide-bond-linked loop. The B chain contains two domains: a translocation (T) domain and a receptor-binding (R) domain (1). DT binds to heparin-binding EGF-like growth factor precursor on the cell surface (2) and undergoes receptor-mediated endocytosis. In the acidic environment of the endosomes, furin-cleaved DT (3) undergoes a conformational change, which promotes insertion of the T domain helices into the vesicle membrane, and the A chain is translocated and released into the cytosol (4). In the cytosol, the A chain catalyzes ADP-ribosylation of elongation factor 2 (EF-2), leading to inhibition of protein synthesis and cell death (5). This toxin-catalyzed activity occurs at a unique post-translational histidine derivative, diphthamide (5,6), found in a conserved amino acid (7) sequence in the EF-2 of all eukaryotes and archaebacteria (8).

Because of its well-defined structural and functional domains, and its efficiency at crossing eukaryotic cell membranes and initiating cell death, DT fragments have been used to construct immunotoxins (ITs) to target and kill specifically selected cells (9–12). ITs were initially generated by chemically linking a toxin to an antibody. But ITs made in this way are heterogeneous chimeric molecules, and it is difficult to produce ITs in large amounts because the antibody and toxin must be purified separately and then conjugated in a reaction that often has a low yield. More recently, ITs have been constructed by fusing coding sequences derived from toxins with those from antibodies and expressing in *Escherichia coli* (13–17). The genetic engineering procedure has advantages in economy and the potential to optimize the construct with respect to efficacy and freedom from side effects elicited by intact antibody.

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[2] Abbreviations used: DT, diphtheria toxin; DT390, N-terminal 390 residues of DT; scFvUCHT1, single-chain Fv fragments of UCHT1 antibody; CHO, Chinese hamster ovary; PEA, *Pseudomonas* exotoxin A; EF-2, elongation factor 2; ITs, immunotoxins; BSA, bovine serum albumin; DTT, dithiothreitol; TBS, Tris-buffered saline; PCR, polymerase chain reaction; B′ chain, DT T domain plus scFvUCHT1; IC_{50}, concentration required to inhibit protein synthesis to 50% of control.

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The expression of recombinant ITs in the prokaryote *E. coli* have resulted in high levels of accumulation in cytoplasm, but the expressed proteins are mainly insoluble and in the form of inclusion bodies. These require *in vitro* solubilization by denaturing agents and refolding procedures to obtain fusion proteins in an active form (17–19). When DT-based recombinant ITs were expressed as a secreted form, the yield of active protein was low and IT accumulation in the periplast was deleterious to the bacterial host (20–22). One of the significant advantages in the expression of heterologous genes in eukaryotes is that the expressed proteins are properly folded. However, in the expression of ADP-ribosylating agents, such as diphtheria toxin and *Pseudomonas* exotoxin A, the use of eukaryotes for high-level production has been restricted by their inherent sensitivity to these toxins. DT-resistant mutants have been selected from yeast (23). It has been shown that DT-resistant yeast (*Saccharomyces cerevisiae*) mutants that have a defect in one of the genes coding for the enzymes involved in the posttranslational synthesis of diphthamide could be used for expressing DT-based fusion immunotoxins (24). However, the activity of these fusion immunotoxins was not measured nor was the extent or possible effects of glycosylation reported. DT-resistant mutants have also been isolated from CHO cells. In this study, we used for the first time a DT-resistant Chinese hamster ovary cell line, CHO K1 RE1.22c (25), to express a truncated anti-T cell immunotoxin, DT390-scFvUCHT1, and we demonstrate that the mutant cells can be used to produce active fusion immunotoxins. CHO K-1 RE1.22c has a dominant point mutation in the first position of codon 717 of *EF-2* gene, resulting in an amino acid substitution of arginine for glycine. This mutation prevents addition of the side chain of diphthamide to histidine 715 of EF-2 and consequently makes *EF-2* non-ADP-ribosylatable by DT or PEA (26).

MATERIALS AND METHODS

Cell Lines and Vectors

Chinese hamster ovary cell line CHO-K1 was obtained from the American Type Culture Collection (ATCC CCL-61). The toxin-resistant cell line CHO-K1 RE1.22c was selected by Moehring and Moehring (25), and supplied to us by Dr. M. E. Digan (Novartis Pharmaceuticals, East Hanover, NJ) as was the mammalian expression vector SRαNeo that contains a neomycin-resistant gene and the SRα promoter as previously described (27).

Immunotoxins

The chemical conjugate of intact anti-human–CD3ϵ antibody UCHT1 and CRM9, a binding site mutant of diphtheria toxin immunotoxin, was made as previously described (12).

Recombinant immunotoxin, DT390-scFvUCHT1 was originally constructed by Thompson *et al.* (15). To facilitate secretion of the immunotoxin from CHO cells, a mouse κ-immunoglobulin signal peptide, METDTLLLWVLLLWVPGADAA (28), was used to replace the signal peptide of DT, creating sp-DT390-scFvUCHT1. This was done by a two-step PCR using *pfu* polymerase (Stratagene) and the following three primers: sp1, cgg gat cca GTC GAC atg gag aca gac aca ctc ctg tta tgg gta ctg ctg ctc tgg gtt cca; sp2, gta ctg ctg ctc tgg gtt cca ggt gcc gac gct gct *ggc gct gat gat gtt gtt gat;* and 3′DT-His, ata GAA TTC TTA gtg gtg gtg gtg gtg gtg *tga gaa gac tgt gag agt ggt gcc tt.* Primers sp1 and sp2 contained a *Sal*I site (uppercase), sequence for the signal peptide with overlapped sequences underlined, and sequence homologous to the 5′ end of DT (italics). Primer 3′DT-His had an *Eco*RI site (uppercase), a stop codon (bold uppercase), sequence for a His-tag, and sequence complementary to the 3′ end of DT (italics). The first PCR was performed on DT390-scFvUCHT1 with primers sp2 and 3′DT-His. This product was purified by agarose gel electrophoresis and then used as template for the second PCR with sp1 and 3′DT-His. The second PCR product was digested by *Sal*I and *Eco*RI and then inserted into vector SRαNeo downstream from the SRα promoter.

Transfection and Cell Culture

CHO cells were cultured in HyQ CCM5 medium (HyClone Laboratories, Logan, UT) and maintained in an incubator containing 5% CO_2. Transfection of CHO cells with immunotoxin constructs was carried out using LipoTAXI (Stratagene) transfection reagent and a protocol supplied by the manufacturer. Two days after transfection, cells were placed under selection with 1 mg/ml G418 (GIBCO/BRL) and allowed to grow for 4 days before being treated with trypsin. Cells were then split 1:10 and cultured in the wells of six-well plates (Corning Costar Corporation) to form colonies in selection medium. About 3 weeks after the start of G418 selection, cells of each well were pooled and transferred to T-25 or T-75 flasks (Corning Costar Corporation). When cells reached more than 80% of confluency, the culturing medium that had been in contact with cells for 48–72 h was collected and analyzed for the presence of immunotoxin protein.

SDS–PAGE and Western Blot Analysis

Immunotoxin containing samples were separated by SDS–PAGE using NuPAGE system (Invitrogen Corporation) under reducing (in the presence of 100 mM DTT) or nonreducing (without DTT) conditions. The separated proteins were transferred to nitrocellulose membrane by electroblotting. The membranes were blocked with 5% nonfat dry milk in TBS-Tween and incubated with goat polyclonal anti-diphtheria toxin

(15) as primary antibody. A rabbit anti-goat IgG antibody labeled with horseradish peroxidase (Santa Cruz Biotechnology) was used as a secondary antibody. The immunotoxin was visualized with SuperSignal chemiluminescent substrate (Pierce Chemical Company).

Site-Specific Mutation

A number of conservative single mutations that abolished either the toxin A chain or B chain glycosylation sites were introduced into DT390-scFvUCHT1 in pET-17b vector (Novagen). Each mutation was designed to introduce a new restriction site. The effects of these single mutations on the toxicity of DT390-scFvUCHT1 on Jurkat target cells were checked by performing *in vitro* transcription and translation using the T7-coupled transcription/translation system (Promega), quantitating the product by Western blots, and then applying the determined concentrations of the product to Jurkat cells in the inhibition of protein synthesis assay described blow. The single mutations screened were for toxin A chain residues Asn^{16} to Ala and Ser^{18} to Ala and for B chain residue Asn^{235} to Gln and to Ala. None of these mutations significantly affected the cytotoxicity compared to the wild-type DT390-scFvUCHT1 (data not shown). A single Asn^{235} to Ala and a double of Ser^{18} to Ala and Asn^{235} to Ala mutation were then introduced into sp-DT390-scFvUCHT1 in plasmid SRαNeo for expression in CHO cells. The oligonucleotide for Ser^{18} to Ala mutagenesis was GTG ATG GAA AAC TTT gCT agc TAC CAC GGG ACT AAA CC and created a new restriction site, *Nhe*I (nucleotide substitutions in lowercase and the new restriction site underlined). For the Asn^{235} to Ala mutagenesis, the oligonucleotide was G AGC GAA AGT CCg gcc AAA ACA GTA TCT GAG G and had a new restriction site, *Eae*I. These oligonucleotide-directed mutagenesis were performed using the MORPH site-specific plasmid DNA mutagenesis kit (5 Prime → 3 Prime, Inc.) and the protocol supplied by the manufacturer.

Purification of His-Tagged Immunotoxins by Ni^{2+} Columns

Purification of His-tagged DT390-scFv immunotoxins was performed with Ni–NTA resin from Qiagen and a protocol supplied by the manufacturer.

Tunicamycin Treatment

Immunotoxin-expressing CHO cells were cultured to about 70% confluency in HyQ CCM5 medium; this medium was then replaced with HyQ CCM5 medium supplemented with varying concentrations of tunicamycin for a 5-h period. After this initial incubation, the culturing media were then replaced by fresh tunicamycin containing media for 48 h before being collected for analysis.

Glycosidase Treatment

CHO-expressed immunotoxin was treated with peptide *N*-glycosidase F (PNGase F) under nondenaturing conditions. Digestion was carried out for 1 h at 37°C in a mixture containing 100 ng of Ni-column purified immunotoxin, 1 μl PNGase F (New England BioLabs) and 50 mM sodium phosphate, pH 7.5. The digested material was subjected to SDS–PAGE analysis and used for cytotoxicity assay.

Cytotoxicity Assay

The recombinant immunotoxin was quantified by Western blotting using a CRM9 preparation of known concentration as the standard determined by OD_{280nm} using $A_{0.1\%/cm} = 0.95$. In this case, a monoclonal antibody against A chain of diphtheria toxin (Accurate Chemical & Scientific Corporation) was used as the primary antibody and a goat anti-mouse antibody labeled with horseradish peroxidase (GIBCO/BRL) as the secondary antibody. Protein bands were visualized by color reaction with TMV membrane peroxidase substrate from KPL (Kirkegaard & Perry Laboratories). Repeat determinations of immunotoxin concentrations exhibited variations as high as 25%. To improve accuracy, quantification of glycosylated proteins was performed after PNGase treatment.

The cytotoxic activity of the immunotoxins was determined by their ability to inhibit protein synthesis in Jurkat cells after a 20-h exposure to immunotoxins as previously described. The data are presented as percentages of protein synthesis referenced to controls without immunotoxin. Each point is the mean of four to six replicates. Positive error bars are the calculated compounded percentage standard error.

RESULTS

Expression of DT390-scFvUCHT1 in CHO

On four separate occasions when wild-type CHO (CHO-K1) cells were transfected with sp-DT390-scFvUCHT1 containing plasmid DNA, only a few cells survived to form colonies 3 weeks after transfection. SDS–PAGE gel and Western blotting did not detect the presence of the immunotoxin protein in the culturing medium from these colonies. Cells from each well were then pooled after trypsin treatment and cultured to full confluency in separated T-25 flasks. Again upon analysis of culture medium, the target protein was still not detected. However, when the mutant CHO (CKO-K1, RE1.22c) cells were transfected with the construct, many cells survived to form colonies which covered about 50% of the area of each of the culturing wells 3 weeks after transfection. When the culturing medium was analyzed by SDS–PAGE gel and Western blotting, the target protein was detected in all four transfections. When the cells were pooled after trypsinization

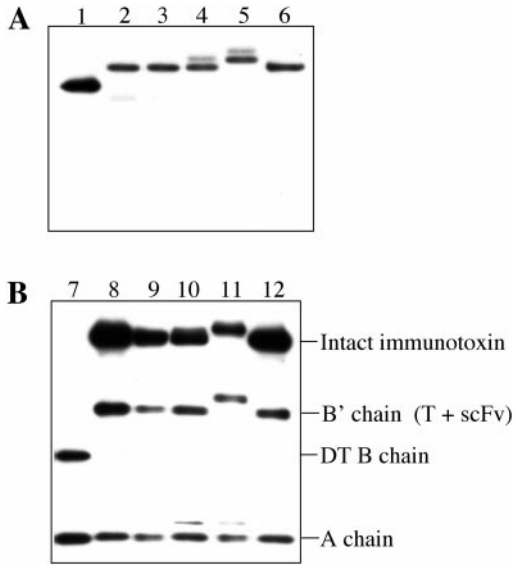

FIG. 1. Western analysis of CHO-expressed DT390-scFvUCHT1 immunotoxins. Protein samples were analyzed under nonreducing (A) and reducing conditions (B). Lanes 1 and 7, CRM9 nicked by trypsin; lane 2, *E. coli*-expressed DT390-scFvUCHT1; lanes 3 and 9, CHO-expressed DT390-scFvUCHT1 treated with PNGase F; lanes 4 and 10, CHO-expressed sm235 DT390-scFvUCHT1, lanes 5 and 11, CHO-expressed wild-type DT390-scFvUCHT1; lanes 6, 8, and 12, CHO-expressed dm DT390-scFvUCHT1.

and cultured to full confluence in separate T-75 flasks, the accumulation of the immunotoxin protein in the culturing medium of one T-75 flask reached 4 μg/ml during a period of 48 h with a medium volume to surface area ratio of 0.14 ml/cm^2. The mutant cells in the presence of the immunotoxin showed no obvious difference in cell morphology and growth as compared to those transfected with the vector alone.

N-Linked Glycosylation of CHO-Expressed Immunotoxin

The immunotoxin expressed by CHO cells migrated slower than that expressed in *E. coli* cytosol, indicating an increase in molecular size, and formed two distinct bands (Fig. 1, lanes 5 and 2). Compared with bacteria, CHO cells have posttranslational modifications. Glycosylation, particularly N-glycosylation, is the only process capable of causing a significant increase in molecular weight of the target protein. The acceptor group for N-glycosylation is an asparagine residue located within the sequence Asn-X-Ser or Asn-X-Thr. Protein sequence analysis revealed two potential N-glycosylation sites in the DT moiety of the immunotoxin: One such site is located at positions 16–18 (Asn^{16}Phe^{17}Ser^{18}) in the catalytic domain (A chain) and the other at positions 235–237 (Asn^{235}Lys^{236}Thr^{237}) in the transmembrane domain. To determine whether the immunotoxin expressed in CHO is N-glycosylated, the CHO-expressed immunotoxin was treated with PNGase F that cleaves the bond between the asparagine residue of the protein and the first residue of the carbohydrate chain. After the treatment, CHO-expressed immunotoxin migrated as one band to the same position as bacterial expressed product in SDS–PAGE (Fig. 1, lane 3), indicating that the slower migrating bands represent glycosylated molecules.

Removal of N-Linked Glycosylation

When a mutant immunotoxin gene (dmDT390-scFvUCHT1), which had mutations at the two potential N-glycosylation sites, Ser^{18} to Ala and Asn^{235} to Ala, was expressed in CHO K1 RE1.22c, only a single protein band was seen that migrated at the position of the bacterial expressed immunotoxin (Fig. 1, lane 6). In addition, the single mutation at position 235 (sm235 DT390-scFvUCHT1) in the B′ chain (T domain + scFvUCHT1) eliminated N-glycosylation of B′ chain, but had no effect on A chain glycosylation (Fig. 1, lane 4). These findings confirmed the presence of N-linked glycosylation at these two sites in the nonmutated immunotoxin expressed in CHO cells.

DT has a proteolytic furin cleavage site between the catalytic (C) and transmembrane (T) domains. Fragments of proteolytically cleaved DT are held together by the disulfide bond between C (A chain) and T domains (Fig. 1, lane 1). However, under reducing conditions, fragments (A chain and B chain) of "nicked" DT are separated and migrate as two bands in SDS–PAGE (Fig. 1, lane 7). Reducing SDS–PAGE revealed that some of the CHO-expressed immunotoxin were cleaved, generating A chain and B′ chain (Fig. 1B). It also showed that nearly all B′ chains were glycosylated (Fig. 1, lane 11), but only a fraction of A chains were glycosylated (Fig. 1, lanes 10 and 11). This glycosylation pattern is also demonstrated by presence of the two slower migrating bands in nonreducing SDS–PAGE (Fig. 1, lanes 4 and 5): the top fainter band is the products of N-glycosylation on both A and B′ chains, whereas the lower stronger one represents molecules glycosylated only on the B′ chain (Fig. 1, lane 5). Quantification of A chain glycosylation by densitometry showed that only about 14% of immunotoxin molecules secreted by CHO cells were N-glycosylated on A chains.

The N-linked glycosylation of CHO-expressed immunotoxins was also confirmed by the treatment with tunicamycin that inhibits core glycosylation of proteins in the ER (29). When the transfected cells were grown in the presence of 4 μg/ml or higher concentrations of tunicamycin, only one protein band, migrating like nonglycosylated protein was observed (Fig. 2). At higher concentrations of tunicamycin, cell growth and consequently accumulation of immunotoxin in the culturing media were affected. It was observed that with decreasing concentration of tunicamycin, the ratio of glycosylated proteins to nonglycosylated increased.

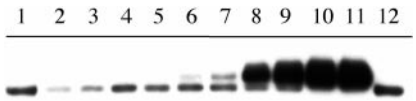

FIG. 2. Inhibition of tunicamycin on N-glycosylation of DT390-scFvUCHT1 immunotoxin expressed by mutant CHO cells. Cells were grown for 2 days in media containing various concentrations of tunicamycin. 10 μl of culturing medium of each tunicamycin concentration was taken for the nonreducing SDS–PAGE and Western-blotting analysis. Lanes 2 to 11, tunicamycin concentrations at 10, 8, 6, 4, 2, 1, 0.5, 0.25, 0.125, and 0 μg/ml; lanes 1 and 12, DT390-scFvUCHT1 expressed in *E. coli*.

Eventually, at concentrations 0.125 μg/ml or less, all immunotoxin molecules were glycosylated.

It appeared that N-glycosylation did not affect the level of protein expression and secretion. When the same transfection procedures were used, the expression of the nonglycosylation mutant dmDT390-scFvUCHT1 reached the same level as the wild-type counterpart. In addition, the expression of dmDT390-scFvUCHT1 in CHO-K1 RE1.22c did not affect cell growth and morphology.

Purification of DT390-scFvUCHT1 Immunotoxin

Partial purification of the CHO-expressed material was achieved by binding to a Ni^{2+} column. Based on Western blotting, over 90% of the applied immunotoxin in the medium was bound to and eluted from the column. When the same amounts of medium immunotoxin and purified material, as judged by Western blotting, were used in protein synthesis assay, both had the same toxicity. This indicated that all of the immunotoxin secreted from the expressing cells was active. In addition, the immunotoxin is very stable. When the culturing medium collected after a 48 h contact with cells was stored for more than 1 year at 4°C, the medium immunotoxin showed little (less than 5%) degradation, aggregation, and loss of activity.

Toxicity of CHO-Expressed Immunotoxin

Cytotoxicity assay showed that the CHO-expressed wild-type immunotoxin had a lower toxicity (IC_{50} 4.8×10^{-10} M). However, when the wild-type immunotoxin was treated with PNGase F to remove the N-linked oligosaccharide chains, the toxicity increased more than two logs (Table 1 and Fig. 3A). Furthermore, mutations at both of the two N-glycosylation sites had the same effect as PNGase treatment: dmDT390-scFvUCHT had the same toxicity as the PNGase treated nonmutated immunotoxin (Fig. 3B and Table 1). This result also showed that the amino acids substitutions that blocked the N-linked glycosylation did not affect the toxicity. Compared with dmDT390-scFvUCHT, the single mutant sm235DT390-scFvUCHT1 also had 14% of its molecules glycosylated at A chain and exhibited 0.3-fold lower toxicity than the double mutant (Fig. 3B and Table 1), and a repeat assay experiment (data not shown) showed 0.5-fold lower toxicity.

DISCUSSION

Our results showed that the mutant cell line, CHO-K1 RE1.22C, could tolerate high-level expression of an ADP-ribosylating immunotoxin. We could not establish stable immunotoxin-expressing cells from

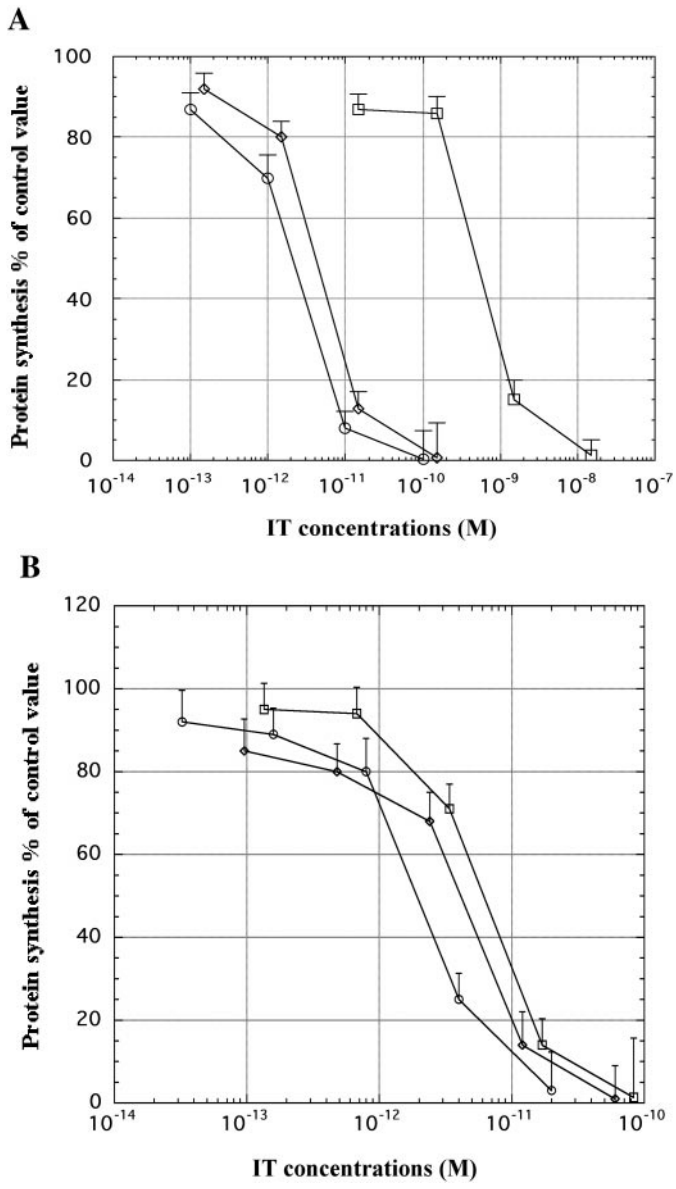

FIG. 3. The cytotoxic activity of CHO-expressed immunotoxins as determined by their ability to inhibit protein synthesis in Jurkat cells. UCHT1-CRM9 chemical conjugate was used as a standard in each assay (circles). (A) Toxicity of CHO-expressed DT390-scFvUCH1 immunotoxin with (diamonds) and without (squares) PNGase treatment. (B) The toxicity of smDT390-scFvUCHT1 (squares) and dmDT390-scFvUCHT1 (diamonds).

TABLE 1

Toxicity of DT390-scFvUCHT1 Immunotoxins from Different Sources

Immunotoxins	Sources	Toxicity (IC_{50})
DT390-scFv[a]	*E. coli* (periplast)	4.8×10^{-11}
DT390-scFv-His6	*E. coli TrxB*$^-$	4.0×10^{-12}
CRM9-UCHT1	Chemically conjugated	2.0×10^{-12}
DT390-scFv(His6)	CHO	4.8×10^{-10}
DT390-scFv(His6)	CHO, + PNGase	4.3×10^{-12}
dmDT390-scFv(His6)	CHO	4.1×10^{-12}
sm235DT390-scFv(His6)	CHO	5.9×10^{-12}

[a] This material was not purified (15).

transfected wild-type CHO cells, indicating that the *EF-2* gene mutation facilitated stable expression. This is in contrast to a previous report that indicated that low-level expression of a *Pseudomonas* exotoxin-based immunotoxin could be achieved in COS and MOLT-4 cells (30).

The protein produced by the mutant CHO cells behaved as homogeneous material during purification and displayed a constant specific activity toward target cells, indicative that both the toxin and the scFv domains were well folded. The potency of dmDT390-scFvUCHT1 immunotoxin is within twofold of the divalent chemical conjugate of intact anti-T cell UCHT1 antibody and CRM9 (Fig. 3B and Table 1). UCHT1-CRM9 can kill three logs of T cells *in vivo* (12) and this degree of *in vivo* T cell depletion can induce long-term tolerance to mismatched pancreatic islet and renal allografts in monkeys (31,32). Therefore, these immunotoxins have major clinical applicability. Reduced and nonreduced SDS gels of dmDT390-scFvUCHT1 containing medium harvested after 48 h showed very few breakdown products, indicating that CHO RE1.22c cells are a good candidate for scaled up expression of this and similar ADP-ribosylating immunotoxins. A particular advantage of this eukaryotic expression system is the presence of the ER protein folding machinery. We also see CHO cell expression of immunotoxins as providing an excellent standard on which to judge the efficacy of various protocols for refolding proteins from *E. coli* insoluble granules. In addition, we have evidence that a variety of recombinant multidomain divalent immunotoxins that are difficult to refold from *E. coli* can also be efficiently expressed in CHO RE1.22c cells (Thompson *et al.*, manuscript in preparation).

N-linked glycosylation of immunotoxin DT390-scFvUCHT1 resulted in a decrease of more than 100-fold in toxicity. Since only a fraction of A chain was glycosylated, most of this toxicity loss was caused by the B chain glycosylation. This indicates the N-linked oligosaccharide chain on the translocation domain, which functions to mediate the translocation of the N-terminal A chain across the endosomal membrane and into the cytosol, greatly reduced the efficiency of this process. Although most studies on T domain lipid insertion have focused on the hydrophobic TH8/TH9 helices, two helices at the amino-terminal region of the B chain, TH1 and TH5, have recently been shown to insert into the lipid bilayer under acidic conditions (33). The fact that the N-linked glycosylation at position 235 blocks translocation indicates that other helices such as TH2 and TH3 may also play an important role in the translocation process. The sm235DT390-scFvUCHT1, which had no glycosylation on the B′ chain and contained only about 14% of glycosylated A chain molecules, had a decrease in potency of between 0.3- to 0.5-fold when compared with that of the nonglycosylation mutant dmDT390-scFvUCHT1 and PNGase treated DT390-scFvUCHT1. This suggests that glycosylation on the A chain may also inhibit immunotoxin toxicity.

To understand why only a small fraction of the A chain molecules are glycosylated, we analyzed the three-dimensional structure of DT using the Cn3D program (34). Since the truncated DT without the recognition domain is likely to have an open structure similar to that of the dimeric form of DT, the structure of dimeric DT (35) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb/ (36)) with the PDB identification: 1DDT and presented in Fig. 4. The three residues recognized for A chain glycosylation ($Asn^{16}Phe^{17}Ser^{18}$) are partially blocked by atoms of the surrounding residues, particularly Phe^{17} which is buried inside the 3D structure of the DT (Figs. 4A and 4B). It is possible that this site cannot be recognized by the oligosaccharyl transferase when DT is completely folded. Glycosylation of this region may only occur when the sequence is just emerging in the ER lumen and has not been folded with the rest of the protein. In contrast the sequence for B chain glycosylation ($Asn^{235}Lys^{236}Thr^{237}$) is located in the loop between two helices (TH2 and TH3; (35)) and all three residues are on the surface of the DT molecule (Figs. 4A and 4C). This region would be susceptible to glycosylation throughout the ER and Golgi network.

It is not known at this stage whether or to what extent the CHO-expressed DT390-scFvUCHT1 immunotoxin is glycosylated by O-linked oligosaccharides. However, if O-linked glycosylation did occur, it had little effect on the toxicity since the toxicity of the CHO-expressed material is equal to purified material expressed in a *TrxB*$^-$ (thioredoxin reductase mutant) *E. coli* strain. This strain has a cytosolic oxidizing potential (37) and can provide small amounts (less than 1% of the expressed immunotoxin) of well-folded material (Liu *et al.*, to be published elsewhere). In addition, detailed analysis of N-linked and O-linked oligosaccharide structures from several CHO-produced glycoproteins has revealed oligosaccharide structures

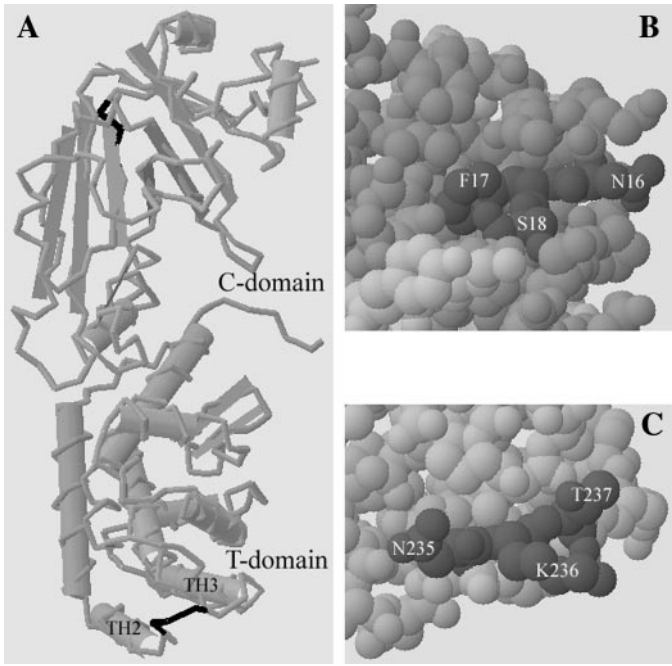

FIG. 4. 3D structures of DT C and T domains showing the N-linked glycosylation sites. The structures were rotated to a best angle to show the three-residue recognition sites for N-linked glycosylation (highlighted in black). (A) Protein backbones of DT C and T domains in tubular structure with three-dimensional objects showing β strands (planks) and α helices (cylinders). (B and C) show in "Space-fill" model the structures surrounding the glycosylation sites of C and T domains, respectively. Note the open surface structure of the T domain glycosylation site compared to A chain glycosylation site.

similar to those found on their native human glycoproteins counterparts (38). These findings have suggested the suitability of CHO-expressed recombinant proteins as pharmaceutical reagents.

Our studies have demonstrated that the mutant CHO K1 RE1.22c cells can be used as a host to express DT-based recombinant immunotoxins and provide information on how to produce highly active material with this system by removal of glycosylation sites. The most important advantage of this system over *E. coli* expression is that all of expressed protein is properly folded. The advantage of this eukaryotic system over *E. coli* is likely to increase as the number of domains and disulfide bonds in the recombinant protein is increased. The CHO expression system described here offers greater flexibility to design and express more complicated multidomain-recombinant immunotoxins.

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