The Cytoplasmic Domain of the Diphtheria Toxin Receptor (HB-EGF Precursor) Is Not Required for Receptor-mediated Endocytosis*

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Diphtheria toxin is believed to enter toxin-sensitive mammalian cells by receptor-mediated endocytosis employing the transmembrane cell surface precursor of heparin-binding epidermal growth factor-like growth factor as a receptor. To investigate the contribution of the receptor's cytoplasmic domain to the toxin internalization process, we constructed stable cell lines that express receptor molecules containing cytoplasmic domain mutations. Our results indicate that Tyr* and surrounding amino acid residues are important for high toxin sensitivity. Cells expressing mutant receptors are less sensitive to toxin and have fewer toxin-specific binding sites but internalize toxin at rates similar to those of cells expressing the intact receptor. This rate of internalization is much slower (1-2%/min) than that of classical endocytic receptors (10-50%/min). Our results are consistent with a model in which the cytoplasmic domain of the toxin receptor lacks a signal for rapid internalization. We suggest that toxin-receptor complexes, nevertheless, are internalized by receptor-mediated endocytosis by entrapment in clathrin-coated pits as part of bulk phase turnover of cell surface proteins. Although the rate is slow, successful intoxication occurs because a single internalized enzymatically-active toxin molecule is sufficient to inhibit protein synthesis in the cell.

Diphtheria toxin (DT) is a Corynebacterium diphtheriae exotoxin that inhibits protein synthesis of toxin-sensitive eukaryotic cells. DT is translated as a single polypeptide of 535 amino acid residues with a corresponding $M_r$ of 58,342. Limited proteolysis of the toxin yields a disulfide-linked polypeptide composed of two fragments: the enzymatic A fragment ($M_r$ 21,167) and the receptor-binding/translocation-mediating B fragment ($M_r$ 37,195) (1-4).

The cytotoxic action of DT occurs by the following steps: (i) binding of DT to specific cell-surface receptors, (ii) internalization of the toxin-receptor complexes, and (iii) translocation of the A fragment from acidic vesicles into the cytosol, where the toxin inhibits protein synthesis by ADP-ribosylation of elongation factor 2. Although all mammalian species possess elongation factor 2 that can be ADP-ribosylated by the A fragment of DT, not all mammalian cells are sensitive to DT (5). The available evidence suggests that the degree of DT sensitivity is proportional to the number of functional receptors present on the cell surface (6).

Existing evidence suggests that the DT receptor is internalized by receptor-mediated endocytosis in clathrin-coated pits. Dorland et al. (7) determined, by biochemical analysis, that internalization is a prerequisite for the expression of the toxin's biological activity. Keen et al. (8) demonstrated, using fluorescence microscopy analysis, that DT is concentrated and internalized in discrete vesicles, suggesting that internalization is a specific receptor-mediated endocytotic process. In addition, Morris et al. (9) determined that the toxin binds to DT-specific receptors and enters Vero cells by receptor-mediated endocytosis via clathrin-coated pits. Using biotin-labeled DT, they determined that the DT receptor was distributed over the entire cell surface and were able to detect the DT receptor in clathrin-coated vesicles. They suggested that for "productive intoxication," the toxin needs to be internalized by a receptor-mediated event through clathrin-coated pits.

Our laboratory has cloned the cDNA encoding a DT receptor from highly toxin-sensitive monkey Vero cells (10). The predicted amino acid sequence of the DT receptor is identical to that of the cell surface-expressed heparin-binding EGF-like growth factor (HB-EGF) precursor (11, 12). In addition to the signal sequence (residues 1-23), analysis of the predicted amino acid sequence of the DT receptor revealed three distinct domains: (i) an extracellular (residues 24-159), (ii) a transmembrane (residues 160-184), and (iii) a carboxyl-terminal cytoplasmic domain (residues 185-208) (10).

When a cloned receptor cDNA was transfected into wild-type DT-resistant mouse fibroblast L-M(TK-), cells, the resulting stable DT-sensitive (DT") mouse cell line, referred to as DT-II, possessed toxin sensitivity equal to that of Vero cells (i.e. IC$_{50} = 5$ ng/ml). However, the number of DT receptors/cell was found to differ between DT-II and Vero cells, the former displaying ~50,000 and the latter ~18,000 DT binding sites/cell (10).

Recently, a cDNA encoding the CD9 molecule was cloned from a Vero cell cDNA library and transfected into DT-II cells (13). The transfected cell line stably expressing CD9, referred to as DT-III, has an increased sensitivity to DT (IC$_{50} = 0.5$ ng/ml) and an increased number of DT binding sites/cell (>1,000,000). In contrast, when the CD9 cDNA was transfected into L-M(TK-), cells, the resulting transfected cell line, referred to as CD9-L-M(TK-), was not sensitive to DT and had no measurable DT binding sites (13).

The cytoplasmic domains of many receptors have been determined to be important for receptor-mediated endocytosis. In particular, multiple lines of evidence suggest that tyrosine or phenylalanine residues in the cytoplasmic domain are essential for rapid receptor internalization (14-18). There is evidence that these essential aromatic residues are part of tight $\beta$-turns in the low density lipoprotein receptor (19), in lysosomal acid phosphatase (20), in the insulin receptor (21), and in the trans-
ferrin receptor (22). Based on these and other observations, several consensus internalization motifs have been proposed (22-24) that have as common features a solvent-exposed loop formed by a β-turn that contains an important tyrosine. According to these motifs, 8-10 cytoplasmic amino acids are sufficient for an internalization signal. In addition, the tyrosine residue must be no closer than 7 residues from the transmembrane domain. Besides tyrosine residues, such turn-promoting residues as proline, glycine, serine, and aspartate are also present within the internalization sequences (22).

Examination of the DT receptor’s cytoplasmic domain reveals 2 tyrosine residues, at positions 186 and 192 (Fig. 1). Tyr186 is located too close to the transmembrane region to be an effective internalization signal (22, 23). The other tyrosine, Tyr192, is not only located 8 residues from the transmembrane domain but is also found within the sequence GGY, which has formed by a p-turn that contains an important tyrosine. Several mutations within the DT receptor cytoplasmic domain mutants. The name of the cytoplasmic domain mutants corresponds to the number of the amino acid residue(s) deleted, inserted, or substituted. Gaps in the lines represent deletions of the corresponding residue; the Δ symbol is the abbreviation for residue deletion. Amino acid insertions are designated with the symbol (∧). Amino acid substitutions are listed below the residue being replaced.

The DT receptor cDNA was subcloned from pDTS/Neo (a plasmid containing the cDNA originally cloned into the pUC119 vector) into the pUC119 vector by use of HindIII and XbaI restriction enzymes. The resulting vector is referred to as pUC119/DTS. The pUC119/DTS construct was used for all site-directed mutagenesis and subsequent DNA sequencing involving the DT receptor cDNA.

Three different types of modifications employing site-directed mutagenesis were performed on the DT receptor cDNA: deletions, substitutions, and insertions. The oligonucleotides were synthesized by the Molecular Cardiology Department at the University of Texas Southwestern Medical Center, Dallas.

Site-directed mutagenesis on the DT receptor cDNA was performed by use of the single oligonucleotide protocol followed by the KanKun selection method (29). Site-directed mutagenesis, including isolation of phagemid-containing uracil single-stranded DNA templates, was performed essentially as described by McClary et al. (30). Following in vitro synthesis, the potential mutants were transformed into E. coli TO1 cells and plated on low-melting-point agar containing 10 μg/ml ampicillin. The resulting colonies were screened by colony hybridization with a DNA probe specific for a fragment containing the internalization signal.

The vector was isolated, and the entire receptor sequence was determined by the method of Sambrook et al. (31). The complete nucleotide sequence of the mutant DT receptors was obtained in order to verify the entire receptor sequence.

The purpose of the current experiments was to determine the location of the internalization sequence of the DT receptor. Several mutations within the DT receptor cytoplasmic domain were generated by deletions, insertions, and substitutions within the normal sequence. The receptor constructs were transfected into mouse fibroblast cells, and the resulting mutant cell lines were analyzed for DT sensitivity, their capacity to bind radiiodinated DT, and the rate at which toxin is internalized. The data obtained indicate that mutations in and around Tyr192 decrease both DT sensitivity and the number of DT cell-surface receptors but do not affect the rate of internalization of the receptor. These results demonstrate that the cytoplasmic domain of the DT receptor is not required for internalization and suggest that the HB-EGF precursor is not acting as a classical receptor but rather as an occasionally endocytosed cell surface protein that DT utilizes to gain access to the cytosol.

**Experimantal Procedures**

**Materials**—All chemicals utilized were of the highest purity and obtained from previously reported sources (10, 13, 26, 27) with the exception of Pronase E, which was purchased from Sigma and insoluble heparinase (HIP), which was obtained from Calbiochem. Sequenase version 2.0 sequencing kit, bacteriophage T4 gene 32 protein, T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, phagemid vector pUC119, and restriction enzymes HindIII and XbaI were purchased from U.S. Biochemical Corp. DNA plasmid purification kits M13mp18 and QIAquick were purchased from Qiagen. [32P]Inulin (13-17 μCi/μg), α-32P-ATP (>1000 Ci/mmol), and [-3H]leucine (60 Ci/mmol) were obtained from Amerham Corp.

The pCDNA1Neo vector and E. coli strain C600 were purchased from Bio-Rad. DT was obtained from Connaught Laboratories (Ontario, Canada), purified, and radiiodinated as described elsewhere (28).

**Subcloning and Site-Directed Mutagenesis of the DT Receptor Gene**—The DT receptor cDNA was subcloned from pDTS/Neo to pcDNA1Neo, and the potential mutants were transformed into E. coli TO1 cells as described (10). Secondary cell receptors but do not affect the rate of internalization of the receptor. These results demonstrate that the cytoplasmic domain of the DT receptor is not required for internalization and suggest that the HB-EGF precursor is not acting as a classical receptor but rather as an occasionally endocytosed cell surface protein that DT utilizes to gain access to the cytosol.
seeded into a 100-mm culture dish at a cell density of 5 × 10⁴ cells/ml (total volume 10 ml/dish) and incubated overnight at 37 °C in 5% CO₂. On day 1, the cells were transfected with a mixture of 30 µg of sonicated salmon sperm carrier DNA and 10 µg of supercoiled pDNANeo/DTS cytoplasmic mutant DNA/plasmid. On day 2, the transfection mixture was removed, the cells were washed extensively with phosphate-buffered saline (PBS; 8.8 mM Na₂HPO₄, 1.2 mM KH₂PO₄, 140 mM NaCl, 10 mM KCl, 0.5 mM MgCl₂, and 1.0 mM CaCl₂ (pH 7.4)), and fresh nonselective medium (Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 50 units of penicillin/ml, 50 µg of streptomycin/ml, 2.5 µg of amphotericin B/ml, and 2 µg of transferrin) was added. On day 3, the nonselective medium was replaced with selective medium (nonselective medium containing 1 mg/ml of the neomycin analog G418). The selective medium was changed every 3–4 days. Individual colonies were visible on the transfection plates after an approximate 2-week incubation period. Potential mutant colonies were removed from the transfec- tion plates with the aid of trypsin-saturated discs. After removal, the colony-containing discs were transferred to a six-well plate containing selective medium, and the cells were expanded. At this time the cell lines were screened for DT sensitivity and binding.

Screening for DT Sensitive Transfectants—The cytoplasmic tailless mutant transfectants (Δ190–208 in both L-M(TK−) and L-M(TK−)/CD9 cell lines) were initially assumed to possess no DT binding sites but possibly DT resistance due to decreased receptor internalization. Use of the replica plate assay developed by Naglich et al. (10) demonstrated that the tailless transfectants actually possessed both toxin binding sites and toxin sensitivity (data not shown). Therefore, based on this information, subsequent transfections of the tailless and the other mutant cell lines were screened for DT sensitivity by the abbreviated cytotoxicity assay described below. The abbreviated cytotoxicity assay was found to be as effective as the replica plate method in screening for DTα cell lines. The abbreviated cytotoxicity protocol was also employed to subculture and purify each mutant cell line three times to ensure homogeneity of the cell lines.

Abbreviated Cytotoxicity Assay—This assay is similar to the cytotoxicity assay described below, except that only one concentration of DT (1 ng/ml) and a no DT control were employed. At 1 ng/ml of DT, L-M(TK−) and L-M(TK−)/CD9 display no inhibition of protein synthesis, while DTαI, DTαIII, and the DT receptor cytoplasmic mutants demonstrate a greater than 90% inhibition of protein synthesis. The 90% protein synthesis inhibition was used to differentiate between DTα cell lines and DTα cell lines. For each mutation, at least three individual DTα cell lines were isolated by this method and examined further in the complete cytotoxicity assay.

Cytotoxicity Assay—Confluent cell monolayers were washed twice with PBS. Culture medium containing the appropriate DT concentrations was added to the monolayers and incubated at 37 °C in 5% CO₂ for 1.5 h. At the conclusion of the incubation, the monolayers were washed twice with PBS, and ice-cold DT followed by the addition of the leucine-deficient minimum essential medium to the monolayers. After a 1-h incubation at 37 °C in 5% CO₂, [³H]leucine was added to the monolayers and incubated for an additional hour. The medium was removed, and the monolayers were washed twice with PBS, lysed, and counted for [³H]leucine incorporated into trichloroacetic acid-soluble precipitable material as described previously (33, 34).

DT Binding Assay—The ability of mutant receptor cell lines to bind radiolabeled DT was examined by procedures previously described (10, 13). Confluent monolayers were washed twice with ice-cold PBS, ice-cold binding medium (medium 195, 50 µg of bovine serum albumin/ml, 100 µg of gelatin/ml, and 20 µg Hepes pH 7.4) was added to the monolayers and preincubated for 30 min on ice at 4 °C. The preincubation medium was removed, and the monolayers were again washed with ice-cold PBS. Binding medium containing varying dilutions of [³H]labeled DT was added to the monolayers with or without a 200-fold excess of unlabeled DT. The monolayers were incubated for 4 h on ice at 4 °C. The monolayers were washed as above and solubilized into 0.2 N NaOH (200 µl/well). Cell-associated radioactivity was determined employing a Tri-Carb Analytical Gamma Trac 1200 γ counting system (10, 13). Specific binding was determined as the difference between the total radioactivity and the cell-associated radioactivity obtained when a 100-fold excess of unlabeled toxin was included. An acceptable level of nonspecific binding was 10–30%. Specific binding data were subjected to Scatchard analyses.

Internalization Protocol—The rate of internalization for the mutant receptor cell lines was determined employing a procedure previously described (35), with slight modifications. Trypsin-treated cell lines were seeded into 24-well plates and grown to confluency. On the day of the assay, the monolayers were washed twice with ice-cold PBS, ice-cold binding medium was added to the wells, and the monolayers were preincubated on ice for 30 min. The preincubation binding medium was removed, and the monolayers were again washed twice with ice-cold PBS. The monolayers were incubated for 4 h at 4 °C in fresh binding medium containing 250 ng/ml [³H]-labeled DT, either with or without a 200-fold excess of unlabeled DT. At the end of the binding step, the medium was removed, and the monolayers were washed twice with ice-cold PBS. A solution of Hank’s balanced salt solution with 4.2 mM NaHCO₃ and 40 mM Hepes pH 7.4 was added to the monolayers and incubated at 37 °C in 5% CO₂ for the internalization time points described below. This addition of NaHCO₃ and additional Hepes to Hank’s balanced salt solution was performed to counteract the decrease in pH that results from incubation of the mouse L-M(TK−)-derived cell lines at 37 °C. At various time points (0, 1, 2, and 3 h), the 24-well plates were centrifuged (600 × g) for 10 min at 4 °C, the media were withdrawn, and the amounts of trichloroacetic acid-soluble and trichloroacetic acid-in- soluble radioactivity in the media were determined. A solution of Hank’s balanced salt solution/NaHCO₃/Hepes containing 0.25 mg/ml Pronase E and 10 mg/ml IHP was then added to the monolayers and incubated at 4 °C for 1 h. The Pronase/IHP-released radioactivity (i.e. cell surface bound) was separated from the Pronase/IHP nonreleasable (i.e. cell- associated) radioactivity by a wash and the amounts of trichloroacetic acid-soluble and trichloroacetic acid-in- soluble radioactivity in the media were determined.

Results
Effect of Cytoplasmic Domain Mutations of the DT Receptor on DT Sensitivity—A summary of the mutations created in the cytoplasmic domain of the DT receptor is shown in Fig. 1. To determine the effect that the cytoplasmic mutations have on the biological activity of the DT receptor, the mutated receptor cDNAs were transfected into L-M(TK−)/CD9 cells, and the mutants obtained were tested for their sensitivity to DT. To minimize clonal variation, at least three independent cell lines representing each of the mutants were assayed. The mean IC₅₀ value of Δ190–208, a mutant lacking most of the cytoplasmic domain, was found to be 32 ng/ml of DT. In comparison to the mean IC₅₀ values of DTα-I, II, and III, this is an approximate 64-fold reduction in toxicity sensitivity (Table I). Three independent transfections of Δ190–208 revealed no discrepancies among their mean IC₅₀ values (data not shown).

In order to delineate residue(s) involved in the observed reduction of DT sensitivity, three scanning deletions of 6–7 amino acids each were constructed in the cytoplasmic domain of the DT receptor cDNA. Of the scanning deletion mutants, Δ190–195 displayed a reduction in toxin sensitivity of approximately the same degree (66-fold) as seen in Δ190–208. The other scanning deletion mutants, Δ196–201 and Δ202–208, were found to display only an ~5-fold reduction in sensitivity when compared with DTα-III cells (Table I).

The same DT receptor cytoplasmic deletions (Δ190–208, Δ190–195, Δ196–201, and Δ202–208) were transfected into L-M(TK−) cells, and their mean IC₅₀ values were determined. When the mean IC₅₀ values were compared with those of DTα-II cells, the same relative degree of reduction in toxin sensitivity was obtained as that observed with L-M(TK−)/CD9 cells (data not shown). These results suggest that the cytoplasmic domain of the receptor does not interact with CD9.

Since the mean IC₅₀ values for Δ190–208 and Δ190–195 are essentially the same, the region involved in maintaining high DT sensitivity is evidently within residues 190–195 of the DT receptor cytoplasmic domain. Within residues 190–195 is the sequence GGYG₉², which has been proposed to be the internalization sequence for the DT receptor (10). In order to verify the importance of this Ty92, a mutation referred to as ΔY192 was constructed in which this residue was deleted from the tyto-
Table I
Summary of cytotoxicity and binding data for the DT receptor cytoplasmic domain mutants

| Cell lines | IC50 (ng/ml) | 125I-labeled DT binding sites/cell (×10^10) | Kd (nM) |
|------------|-------------|------------------------------------------|---------|
| DT-III     | 0.5 ± 0.2   | 2.4 ± 13                                  | 30 ± 13 |
| L-M(TK-)/CD9/DTS | 0.8 ± 0.5 | 3.5 ± 18                                  | 15 ± 12 |
| Δ190-208   | 32 ± 18     | 1.4 ± 0.4                                 | 25 ± 16 |
| Δ190-195   | 33 ± 12     | 2.4 ± 2.0                                 | 28 ± 23 |
| Δ190-201   | 2.8 ± 1.0   | 4.1 ± 2.5                                 | 29 ± 12 |
| Δ200-208   | 2.6 ± 1.5   | 1.1 ± 0.8                                 | 28 ± 11 |
| ΔY192      | 22 ± 7.4    | 1.1 ± 0.4                                 | 12 ± 5.2 |
| Y190A      | 7.5 ± 4.4   | 4.1 ± 2.3                                 | 61 ± 32 |
| Y192A      | 4.3 ± 2.3   | 2.7 ± 1.7                                 | 23 ± 10 |
| 190PP191   | 30 ± 17     | 2.9 ± 2.5                                 | 25 ± 20 |
| R189P/G190P | 51 ± 20    | 1.2 ± 0.8                                 | 14 ± 2.2 |
| Y186A      | 0.5 ± 0.2   | 18 ± 10                                   | 18 ± 9  |
| DT-II      | 4.2 ± 1.8   | 0.8 ± 0.3                                 | 23 ± 14 |

* Values are the average obtained with at least three separate transfected cell lines stably expressing toxin sensitivity, and each cell line was analyzed in triplicate. 

† Mean 125I-labeled DT binding sites/cell, as determined by Scatchard analysis, are the result of at least three independent binding assays. 

plasmic domain. Cytotoxicity assays demonstrated that the mean IC50 values for Δ190-208, Δ190-195, and ΔY192 are similar; in fact, the IC50 ranges for these three mutants overlap, indicating no significant difference among these mutants (Table I). These data suggest that Tyr192 is important for maintaining the toxin hypersensitivity displayed by the DT-III cells.

To determine whether this tyrosine residue can be substituted by phenylalanine, as is the case with some other cytoplasmic domains (14, 15), the Y192F mutation was constructed and transfected into L-M(TK-)/CD9 cells. The mean IC50 value for this substitution is 7.5 ng/ml. Although this mutant is ~3-fold more sensitive to DT than ΔY192, complete sensitivity is not reconstituted since it is still ~15-fold less sensitive than DT-III cells (Table I). Furthermore, the improved sensitivity of Y192F, as compared with ΔY192, appears not to be specific since substitution of this tyrosine with alanine (Y192A mutant) results in a mean IC50 value of 4.3 ng/ml, indicating at least the same degree of reconstitution of sensitivity as in Y192F (Table I). Thus, neither phenylalanine nor alanine can completely replace Tyr192 of the DT receptor’s cytoplasmic domain, and the presence of a residue at this position results in a smaller decrease in toxin sensitivity than does a deletion of the residue (Table I).

The above-described cytoplasmic domain mutations reside in the proposed internalization sequence, GGY192. Since cell lines having a mutant receptor all showed a decreased toxin sensitivity, we next asked whether an increase in DT sensitivity could be achieved by modifying the cytoplasmic domain of the DT receptor to resemble the rapid internalization sequence, PPGY, present in lysosomal acid phosphatase (20). Two mutations were constructed: (i) 190PP191, where two prolines were inserted between Gly190 and Gly191, and (ii) R189P/G190P, where both Arg189 and Gly190 were replaced with proline (Fig. 1). The mean IC50 values obtained for 190PP191 and R189P/G190P are 30 and 51 ng/ml, respectively; these values represent a decreased DT sensitivity of 60- and 102-fold, respectively (Table I). Therefore, the presence of a PPGY sequence did not increase the sensitivity of the cells to DT.

Although Tyr186 (Fig. 1) was theorized to be too close to the predicted transmembrane domain to be an effective internalization signal (23), a mutant was constructed to determine whether such a mutation could affect toxin sensitivity. The mutation Y186A was constructed and transfected into L-M(TK-)/CD9 cells. The Y186A mutant possesses a mean IC50 value of 0.5 ng/ml, which is equal to that of DT-III cells (Table I). The lack of decreased sensitivity in Y186A lends support to the specificity of the above-described mutations in and around Tyr192 and demonstrates that not every mutation in the cytoplasmic domain of the DT receptor affects DT sensitivity.

The toxin sensitivities obtained with the cytoplasmic domain mutants have been compared with the sensitivity of DT-III cells (Table I). However, since DT-III cells were obtained by transfection of L-M(TK-)/CD9 cells first with pDTS (to yield DT-III cells (10)) and subsequently with pCD9 (13), it was necessary to demonstrate that transfection of L-M(TK-)/CD9 cells with pDTS also results in cells as toxin-sensitive as DT-III cells. Indeed, the mean IC50 value for the resultant cell line, L-M(TK-)/CD9/DTS, was found to be approximately that of DT-III cells (Table I).

Effect of Cytoplasmic Domain Mutations on the Number of DT Binding Sites—The decreased sensitivity to DT observed with the cytoplasmic domain receptor mutants is consistent with the mutations having an effect on receptor internalization. However, the decreased sensitivity could be due to a decrease in the number of toxin receptors at the cell surface, or a combination of decreases in receptor number and internalization rates. Therefore, we first measured the number of specific DT binding sites on the mutant cell lines. The 125I-labeled DT binding assay and subsequent Scatchard analysis of the binding data indicates that there is a decrease in the number of DT binding sites/cell for those DT receptor cytoplasmic mutants with a decreased toxin sensitivity; the number of binding sites ranges from 110,000 to 410,000 (Table I). In contrast, Y186A and L-M(TK-)/CD9/DTS, cell lines where no decrease in sensitivity is seen, possess a similar number of DT binding sites/cell as DT-III cells (Table I). Scatchard analysis was used to determine the Kd values for the DT-I, DT-II, and the DT receptor cytoplasmic mutant cell lines (Table I). The mean Kd values for DT-I, DT-II are 30 and 23 nM, respectively; these values are consistent with previously published reports on DT-I and DT-II (13). Even though the mean Kd values for DT-I, DT-II, and the DT receptor cytoplasmic mutants vary slightly from each other, the overlapping standard deviations suggest that the affinity of the receptor on these cell lines is not significantly different. It can be suggested, based on the similar mean Kd values for the DT-I and the DT receptor cytoplasmic mutant cell lines, that the cytoplasmic domain mutations do not cause alterations in the protein structure of the receptor’s extracellular domain that affect the binding of DT.

Effect of Mutations in the Cytoplasmic Domain on the Rate of Internalization—The decreased sensitivity to DT in the cytoplasmic domain receptor mutants, in addition to being due to a decreased number of toxin binding sites, could also be due to a decreased rate of DT receptor internalization. The internalization of 125I-labeled DT was employed as a measure of receptor internalization (35). DT-III cells and L-M(TK-)/CD9-derived toxin-sensitive cell lines are especially suitable for investigating the rate of internalization of 125I-labeled DT due to their relatively low nonspecific binding compared with that of many other toxin-sensitive cell lines (6, 13).

The rates of 125I-labeled DT internalization of DT-III cells and Δ190-208 cells, the most drastic deletion mutant, are shown in Fig. 2. Analysis of the internalization profiles sug-
Cytoplasmic Domain of the Diphtheria Toxin Receptor

The original hypothesis of this work was that the cytoplasmic domain, and in particular Tyr<sup>192</sup>, is important for internalization of the DT receptor. Mutations were constructed in the cDNA encoding the DT receptor and were transfected into the L-M(TK-)CD9 cell line. Cytotoxicity, binding, and internalization assays were performed on the isolated mutant cell lines. Our results suggest that DT<sup>III</sup> and Δ190–208 cell lines internalize DT at similar rates (Fig. 2). For comparison purposes, the 2-h 37°C incubation point was selected for calculation of internalization indices for these and the other cell lines. The internalization indices for DT<sup>III</sup>, DT<sup>II</sup>, and all the mutants, except Δ202–208, Y192A, Y192F, and R189P/G190P, were found not to differ significantly from each other (Fig. 3). The mutants Δ202–208, Y192A, Y192F, and R189P/G190P were found to have larger internalization indices. The internalization results strongly suggest that the decreased DT sensitivity in the mutant cell lines is not due to a decreased rate of toxin internalization.

**DISCUSSION**

According to their mean IC<sub>50</sub> values, the cytoplasmic domain mutants can be divided into two groups (Table I). Group I includes mutants with a modest reduction (5–10-fold) in DT sensitivity as compared with DT<sup>III</sup> cells. In contrast, Group II includes mutants with a much greater decrease in DT sensitivity (50–100-fold). Members of Group I, including Δ190–208, Δ190–195, ΔY192, 190PP191, and R189P/G190P, are representative of mutations in the cytoplasmic domain generated by deletion of Tyr<sup>192</sup> or alterations of the residues surrounding Tyr<sup>192</sup>. These results suggest that Tyr<sup>192</sup> is involved in maintaining the high-degree of toxin sensitivity demonstrated by the DT<sup>III</sup> cell line.

The DT receptor cytoplasmic domain mutations were found to possess a reduced number of 125<sup>I</sup>-labeled DT binding sites/cell (Table I). We suggest three possible explanations for the reduced number of toxin binding sites. The first possibility is that newly synthesized DT receptor cytoplasmic domain mutants are defective in their transport from the rough endoplasmic reticulum to the cell surface. A prerequisite for normal export of many newly synthesized proteins from the rough endoplasmic reticulum is proper assembly into their native conformation (37). In circumstances where proteins fail to achieve this requirement, they are retained and eventually undergo intracellular proteolytic degradation (38–43). However, the effect of the cytoplasmic mutations on the transport of membrane proteins to the cell surface varies with each individual protein. For example, removal of the cytoplasmic domain of the influenza hemagglutinin glycoprotein has no effect on transport (44); whereas, deletion of the cytoplasmic domain in the vesicular stomatitis virus G glycoprotein greatly slows its transport to the cell surface (45). The second possibility is that the mutant receptors are transported to the cell surface in a normal fashion and then are proteolytically processed to the mature form of HB-EGF at an accelerated rate. It has been shown, for example, that the cytoplasmic domain of the membrane-anchored proangiogenic factor β (proTGF-α) possesses a recognition sequence that influences the rate of cleavage from the membrane-anchored form to the mature soluble form. Alterations of this recognition sequence affect the rate of proteolytic cleavage at the extracellular domain of the proTGF-α (46). Third, the mutations in the cytoplasmic domain of the DT receptor could possibly affect the normal basolateral versus apical sorting for this receptor. Tyrosines in the cyto-

**Fig. 2. Comparison of the rate of internalization of DT for the DT<sup>III</sup> (top graph) versus Δ190–208 (bottom graph) cell lines.** A solution containing 250 ng/ml of 125<sup>I</sup>-labeled DT in the presence or absence of a 200-fold excess of unlabeled toxin was added to the monolayers. After a 4-h incubation at 4°C, the monolayers were washed and further incubated at 37°C. At the indicated time points, radioactivity was separated into four fractions as described under "Experimental Procedures," corrected for nonspecific binding, and graphed as the percentage of the total at each time point. Total radioactivity represents the percentage of the total radioactivity (internalized, degraded, and subsequently released DT) plus trichloroacetic acid-soluble radioactivity (internalized, degraded, and subsequently released DT) divided by cpm of 125<sup>I</sup>-labeled DT surface bound after a 2-h incubation at 37°C in 5% CO<sub>2</sub>. The higher the internalization index, the greater the internalization rate.

**Fig. 3. Internalization indices for the DT receptor cytoplasmic domain mutants.** The mean values of the internalization index (□) and the corresponding standard deviations (represented by error bars) were calculated from specific counts obtained from a minimum of three separate determinations, similar to those shown in Fig. 2, as follows: cpm of cell-associated (representing internalized and non-Pronase E/IHP released radiolabeled DT) plus trichloroacetic acid-soluble radioactivity (internalized, degraded, and subsequently released DT) divided by cpm of 125<sup>I</sup>-labeled DT surface bound after a 2-h incubation at 37°C in 5% CO<sub>2</sub>. The higher the internalization index, the greater the internalization rate.

According to their mean IC<sub>50</sub> values, the cytoplasmic domain mutants can be divided into two groups (Table I). Group I includes mutants with a modest reduction (5–10-fold) in DT sensitivity as compared with DT<sup>III</sup> cells. In contrast, Group II includes mutants with a much greater decrease in DT sensitivity (50–100-fold). Members of Group I, including Δ190–208, Δ190–195, ΔY192, 190PP191, and R189P/G190P, are representative of mutations in the cytoplasmic domain generated by deletion of Tyr<sup>192</sup> or alterations of the residues surrounding Tyr<sup>192</sup>. These results suggest that Tyr<sup>192</sup> is involved in maintaining the high-degree of toxin sensitivity demonstrated by the DT<sup>III</sup> cell line.

The DT receptor cytoplasmic domain mutations were found to possess a reduced number of 125<sup>I</sup>-labeled DT binding sites/cell (Table I). We suggest three possible explanations for the reduced number of toxin binding sites. The first possibility is that newly synthesized DT receptor cytoplasmic domain mutants are defective in their transport from the rough endoplasmic reticulum to the cell surface. A prerequisite for normal export of many newly synthesized proteins from the rough endoplasmic reticulum is proper assembly into their native conformation (37). In circumstances where proteins fail to achieve this requirement, they are retained and eventually undergo intracellular proteolytic degradation (38–43). However, the effect of the cytoplasmic mutations on the transport of membrane proteins to the cell surface varies with each individual protein. For example, removal of the cytoplasmic domain of the influenza hemagglutinin glycoprotein has no effect on transport (44); whereas, deletion of the cytoplasmic domain in the vesicular stomatitis virus G glycoprotein greatly slows its transport to the cell surface (45). The second possibility is that the mutant receptors are transported to the cell surface in a normal fashion and then are proteolytically processed to the mature form of HB-EGF at an accelerated rate. It has been shown, for example, that the cytoplasmic domain of the membrane-anchored proangiogenic factor β (proTGF-α) possesses a recognition sequence that influences the rate of cleavage from the membrane-anchored form to the mature soluble form. Alterations of this recognition sequence affect the rate of proteolytic cleavage at the extracellular domain of the proTGF-α (46). Third, the mutations in the cytoplasmic domain of the DT receptor could possibly affect the normal basolateral versus apical sorting for this receptor. Tyrosines in the cyto-

**Fig. 2. Comparison of the rate of internalization of DT for the DT<sup>III</sup> (top graph) versus Δ190–208 (bottom graph) cell lines.** A solution containing 250 ng/ml of 125<sup>I</sup>-labeled DT in the presence or absence of a 200-fold excess of unlabeled toxin was added to the monolayers. After a 4-h incubation at 4°C, the monolayers were washed and further incubated at 37°C. At the indicated time points, radioactivity was separated into four fractions as described under "Experimental Procedures," corrected for nonspecific binding, and graphed as the percentage of the total at each time point. Total radioactivity represents the percentage of the total radioactivity (internalized, degraded, and subsequently released DT) plus trichloroacetic acid-soluble, degraded DT in the medium (O); and trichloroacetic acid-precipitable DT in the medium (□).
plasmic domains of cell surface proteins have been theorized to be important for membrane sorting (47, 48). Interestingly, it has been reported that the DT cell surface binding proteins are located exclusively on the basolateral cell surface of the highly DT-sensitive and polarized Madin-Darby canine kidney cells (49). It would be tempting to postulate that Tyr^192 might be important for this basolateral sorting. However, since L-M(TK^-) cells are known to be nonpolarized and so well characterized DT-resistant polarized cell line is available, it is difficult at present to test this hypothesis. Regardless of the mechanism by which the number of receptors are reduced in the mutants, those that are located at the cell surface appear to be functional receptors in that the mutant cells are still quite toxin-sensitive, and their receptor affinities for DT are essentially the same as that of the toxin receptor on DT^-III cells (Table 1).

Comparison of the cytotoxicity and the binding data reveal no obvious correlation between toxin sensitivity and the number of ^125I-labeled DT binding sites/cell (Table 1). Certain mutants, such as ΔY192 (Group II) and Δ202-208 (Group I), were found to possess essentially the same number of ^125I-labeled DT binding sites/cell but varied greatly in their respective mean IC50 values. Conversely, other mutants, for example Δ196-201 and Δ202-208 (both members of Group I), possess different numbers of ^125I-labeled DT binding sites but have approximately the same mean IC50 values. Thus, the decrease in the toxin sensitivity for the mutant cell lines cannot be attributed solely to a corresponding reduction in the number of ^125I-labeled DT binding sites/cell.

This work was begun with the expectation that cytoplasmic domain mutants would internalize DT at a slower rate. However, the internalization indices for the DT receptor demonstrate that DT^-II, DT^-III, and most of the DT receptor cytoplasmic mutants internalize their DT receptors at the same rate (Figs. 2 and 3). Interestingly, four of the DT receptor cytoplasmic mutants, namely Δ202-208, Y192A, Y192F, and R189P/G190P, were found to accumulate DT at a slightly faster rate than the DT^-III cell line (Fig. 3). This slight increased rate of accumulation, however, did not produce increased DT sensitivity (Table 1).

The observed internalization rate for the DT receptor in both DT^-II and Δ190-208 cell lines is rather slow (~1%/min) (Fig. 2). A similar slow rate of internalization (1−2%/min) has been observed for both vesicular stomatitis virus G glycoprotein and a vesicular stomatitis virus G glycoprotein mutant lacking a tyrosine residue in the cytoplasmic domain (50). Slow rates have also been observed for mutants of several endocytic receptors from which internalization signals have been deleted (14, 16). In contrast, such high efficiency endocytic receptors as the transferrin receptor (51) and the low density lipoprotein receptor (52) internalize at a much faster rate (10−50%/min).

Other well characterized receptors such as the Fc receptor (53), fibronectin receptor (54), and CD4 protein of lymphocytes (55), which apparently lack efficient tyrosine-containing cytoplasmic internalization signals, are also slowly internalized. These receptors are constitutively internalized at rates typically near the rate of bulk membrane internalization (1−2%/plasma membrane taken up per coated pits per min) (25).

The internalization data presented here strongly suggest that the cytoplasmic domain of the DT receptor does not play an essential role in the internalization process. The slow rate of DT receptor internalization (Fig. 2) would suggest a rather inefficient mechanism for toxin internalization. The internalization mechanism, however, need not be very efficient since a single enzymatically-active A fragment of DT is sufficient to completely inhibit protein synthesis when introduced into the cytosol (56). The slow rate of DT internalization is in all probability a reflection of the fact that the toxin utilizes a cell surface protein, the HB-EGF precursor, as a receptor. This protein is a biochemically unique precursor that becomes processed at the cell surface into the mature growth factor; it may not have an internalization pathway other than an occasional entrapment into a clathrin-coated pit. If DT happens to bind to such an HB-EGF precursor/receptor molecule, the resulting complex can be internalized by receptor-mediated endocytosis. This internalized DT molecule could then insert into the membrane of an acidified endocytic vesicle and escape from the vesicle into the cytoplasm near the rate of bulk membrane internalization (1−2%/min).

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