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Intracellular Localization and Degradation of Diphtheria Toxin

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The internalization of surface-bound diphtheria toxin (DT) in BS-C-1 cells correlated with its appearance in intracellular endosomal vesicles; essentially no toxin appeared within secondary lysosomal vesicles. In contrast, internalized epidermal growth factor (EGF) was localized within both endosomal and lysosomal vesicles. Upon preincubation of cells with leupeptin, a lysosomal protease inhibitor, a threefold increase in the accumulation of EGF into lysosomes was observed. Under identical conditions, essentially all of the diphtheria toxin remained within endosomes (less than 2% of the intracellular diphtheria toxin accumulated in the lysosomal fraction), indicating that the inability to detect diphtheria toxin in lysosomes was not due to its rapid turnover within this vesicle. Following internalization of EGF or DT, up to 40% of the ligand appeared in the medium as TCA-soluble radioactivity. EGF degradation was partially leupeptin-sensitive and markedly NH_4Cl-sensitive, indicating lysosomal degradation. In contrast, DT A-fragment degradation was resistant to these inhibitors, while B-fragment showed only partial sensitivity. These data suggest that the bulk of endocytosed diphtheria toxin is localized within endosomes and degraded by a pathway essentially independent of lysosomes.

Key words: epidermal growth factor, receptor-mediated endocytosis, non-lysosomal degradation, lysosomotropic amines

Many macromolecules, including diphtheria toxin, appear to enter the cell by receptor-mediated endocytosis. While the binding of diphtheria toxin to cell surface receptors and the effects of various chemical agents on the intoxication of cells have been described in some detail [1,2], the intracellular trafficking and the mechanism for delivery of the toxin to its cytosolic target protein, elongation factor-2, remain unclear.

Experiments with lysosomotropic amines suggest that diphtheria toxin gains access to the cytosol upon acidification of an intracellular vesicle [2]. Sandvig and Olsnes have proposed that diphtheria toxin may enter cells upon acidification of a prelysosomal vesicle [3]. Support of this hypothesis has come from two recent observations: i) a mutant CHO

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cell line resistant to DT, pseudomonas toxin, and several viruses was shown to be defective in endosome acidification [4]; and ii) Draper and coworkers showed that diphtheria toxin kills sensitive cells under conditions which inhibit endosome-lysosome fusion [5]. In this study, we show that the delivery and degradation of diphtheria toxin proceeds via a pathway different from that of epidermal growth factor. The bulk of intracellular diphtheria toxin was localized within intermediate-density vesicles and subsequently degraded in a NH_4Cl-resistant manner. These data suggest that a nonlysosomal pathway is responsible for the intracellular trafficking and degradation of diphtheria toxin in BS-C-1 cells.

MATERIALS AND METHODS

Diphtheria Toxin Preparation

Diphtheria toxin was obtained from Connaught Laboratories (Toronto, Canada) and purified as described elsewhere [6]. "Nicked" nucleotide-free monomeric diphtheria toxin (DT) was used throughout this study.

Preparation of ^{125}I-DT

^{125}I-DT or ^{125}I-DT A-fragment (DTA) was prepared with lactoperioxidase as previously described [7] or with Iodobeads (Pierce Chemical Company, Rockford, IL). Iodobead-catalyzed iodination was performed by incubation of 38 μg of DT with two Iodobeads and 1 mCi Na-^{125}I (Amersham Corp., Arlington Heights, IL) in 25 mM Tris-saline, pH 7.5 (TBS) for 15 min at 22°C. The ^{125}I-DT was separated from free ^{125}I by chromatography on a 1 × 10-cm column of P10 (Bio-Rad Laboratories, Richmond, CA).

Preparation of Differentially Radiolabeled DT

A subunit exchange reaction [8] was used to prepare DT that was preferentially radiolabeled in either fragment A (DTA) or fragment B (DTB).

DT radiolabeled in DTA (DT [^{125}I-A]). 38 μg of ^{125}I-DTA (specific activity 4–5 × 10^6 CPM per pmol) and 38 μg DT were incubated separately in 20 mM dithiothreitol for 10 min at 37°C. The proteins were mixed, dialyzed overnight at 5°C, and chromatographed over Sephacryl S-200. SDS-PAGE of the dialyzed mixture showed that greater than 98% of the radiolabeled fragment A migrated as DT.

DT radiolabeled in DT B-fragment (DT [^{125}I-B]). 38 μg ^{125}I-DT (specific activity 4–5 × 10^6 CPM per pmol) and 380 μg DTA were incubated separately in 20 mM dithiothreitol for 10 min at 37°C. The proteins were mixed, dialyzed overnight at 5°C, and chromatographed over Amicon Green A (Amicon Corporation, Danvers, MA) [6]. SDS-PAGE of the DT fractions showed the presence of about 90% of the radiolabel within DTB and about 10% within DTA.

Preparation of ^{125}I-EGF

Five micrograms of EGF (receptor grade, Collaborative Research, Lexington, MA) was incubated in TBS, pH 7.5, with 1.0 mCI Na-^{125}I and two Iodobeads for 15 min and desalted on a P10 column.

Cells and Culture Conditions

BS-C-1 cells (African green monkey kidney cells) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagles MEM (DMEM) supplemented with 15% fetal calf serum (FCS; Grand Island Biologicals Company, Grand Island, NY). Cells used for experiments were subcultured by a 1 to 2 split by trypsin treatment; all experiments were performed 48 hr after seeding of cells.

Removal of Surface-Bound Diphtheria Toxin

DT was released from the cell surface by proteolysis with 0.5% trypsin and 0.01% sodium EDTA in phosphate-buffered saline (PBS; GIBCO Grand Island, NY). The digestion was performed at 5°C and stopped by adding 1 ml of FCS. Cells were then washed four times in PBS.

Removal of Surface-Bound EGF

EGF was released from the cell surface by acid treatment with 2 M glycine-HCl in saline as previously described [9].

Incubation of Cells With Leupeptin

Cells treated with 60 μM leupeptin for 18 hr prior to incubation with either DT or EGF. At the appropriate time, cells were chilled to 5°C, treated to remove surface bound ligand, washed three times in medium, and refed with DMEM at 37°C with 60 μM leupeptin. At the indicated times, 1 ml of medium was removed, cleared of nonadherent cells by centrifugation at 500g, and treated with 10% TCA. After 30 min, the samples were centrifuged and supernatant fluid and precipitate were assayed for radioactivity using a Packard Gamma counter.

Subcellular Fractionation

Following removal of receptor-bound DT or EGF, cells were disrupted by Dounce homogenization and fractionated on a 22% Percoll gradient as previously described [10].

RESULTS

^{125}-I-DT binding to BS-C-1 cells at 5°C was both specific and saturable with respect to toxin concentration (Fig. 1) and time (Fig. 2). Scatchard analysis showed the presence of 32,000 diphtheria toxin receptors per cell with an apparent equilibrium dissociation constant of 0.7 nM.

BS-C-1 cells were incubated with ^{125}I-DT for 8 hr at 5°C, washed, warmed to 37°C, and assayed for presence of protease-resistant toxin at the times indicated (Fig. 3). Initially, less than 5% for the cell-associated toxin was resistant to protease release, indicating that essentially all of the toxin was cell surface-associated. Upon warming to 37°C, surface-bound toxin was chased into a protease-resistant form and within 55 min about one-half of the total cell-associated toxin was in a protease-resistant form.

The subcellular location of the protease-resistant DT was determined by Percoll density fractionation (Fig. 4). The appearance of toxin in a protease-resistant form correlated with its presence in an intermediate-density fraction (1.035 gm/ml) characteristic of endosomal vesicles. Toxin was not detected in heavy-density fractions which contained β-hexosaminidase activity, a marker enzyme of secondary lysosomes.

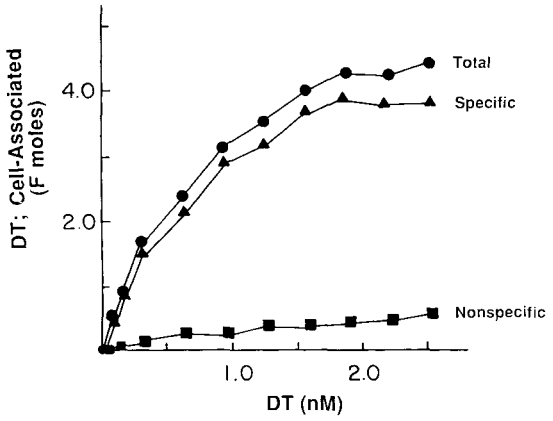

Fig. 1. Association of diphtheria toxin with BS-C-1 cells as a function of toxin concentration. BS-C-1 cells were washed and incubated at 5°C in media containing the indicated concentration of ^{125}I-DT (3.0 × 106 CPM per pmol) alone (total cell-associated radiolabel, ●) or with a 100-fold excess of nonradiolabeled DT (nonspecific cell-associated radiolabel, ■). After 16 hr at 5°C, the cultures were washed, solubilized in 0.1 N NaOH, and assayed for radiolabel by liquid scintillation. Specific association (▲) was determined as the difference between the total and the nonspecific radiolabel. Scatchard analysis showed the presence of 32,000 diphtheria toxin receptors per cell and an apparent equilibrium dissociation constant of 0.7 nM.

To determine whether the failure to detect diphtheria toxin in secondary lysosomal vesicles was due to rapid turnover in the lysosome, we examined the effect of leupeptin, a thiol-protease inhibitor [11], on the distribution of DT and epidermal growth factor (EGF) within intracellular vesicles. EGF has previously been shown to be internalized by a receptor-mediated pathway and delivered to lysosomes [10–13]. Following a 90-min incubation at 37°C, ^{125}I-EGF was distributed in both intermediate- and heavy-density

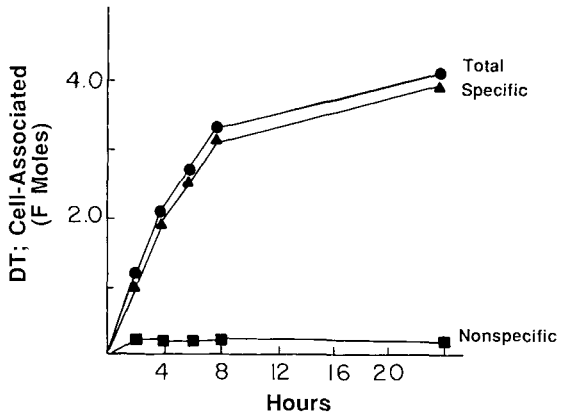

Fig. 2. Association of diphtheria toxin with BS-C-1 cells as a function of time. BS-C-1 cells were washed and incubated at 5°C in media containing 1.6 nM ^{125}I-DT alone (total cell-associated radiolabel, ●) or with a 100-fold excess of nonradiolabeled DT (nonspecific cell-associated radiolabel, ■). At the indicated times, cells were washed, and cell-associated radiolabel determined as described in Figure 1. Specific association (▲) was determined as the difference between the total and the nonspecific radiolabel.

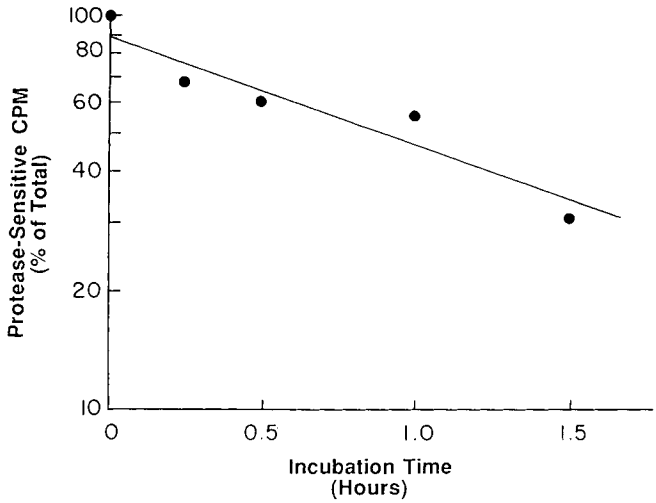

Fig. 3. Internalization of ^{125}I-DT into a protease-resistant compartment. BS-C-1 cells were incubated with 1.6 nM ^{125}I-DT at 5°C for 8 hr, washed, and incubated in growth media at 37°C. At the indicated times, the cells were treated for 15 min with PBS containing 0.5% trypsin and 0.01% EDTA at 5°C to release surface-bound diphtheria toxin. Following centrifugation, protease-resistant toxin (pellet) was measured (●).

fractions (Fig. 5a). Pre-incubation of cells with leupeptin resulted in a threefold increase in EGF in the lysosomal fraction. Following a 90-min incubation of cells with ^{125}I-DT at 37°C, DT was present only in intermediate-density, endosomal vesicles; none was detected in the heavy-density fractions (Fig. 5b). Pre-incubation of cells with leupeptin had a negligible effect on the intracellular distribution of diphtheria toxin; essentially all of the toxin remained within the intermediate density fraction, with less than 2% of the total cell-associated radioactivity shifted into heavy-density fractions. Leupeptin treatment resulted in less than a 10% increase in the total cell-associated radioactivity of either ^{125}I-DT or ^{125}I-EGF.

We also observed differences in the rate of DT and EGF degradation in the presence of NH_4Cl or leupeptin. Cells were incubated with either ^{125}I-DT or ^{125}I-EGF for 15 min at 37°C, washed, and treated to remove surface-bound ligand, and then incubated at 37°C alone or in the presence of NH_4Cl or leupeptin. Degradation was measured as the release of TCA-soluble radiolabel into the culture medium. In the absence of inhibitor, 25% and 45% of the total internalized ^{125}I-DT (specifically labelled in the A- or B-fragment, respectively) and 40% of the total internalized ^{125}I-EGF appeared in the culture medium as TCA-soluble radiolabel during a 2-hr chase (Fig. 6). At the end of the 2-hr chase, less than 5% of the total cell-associated label remained in the cell fraction. EGF degradation was markedly sensitive to inhibition by NH_4Cl (Fig. 6A). However, NH_4Cl had no effect on the degradation of the A-fragment of DT (Fig. 6B) and caused an only slight inhibition in the rate of degradation of the B-fragment (Fig. 6C). Leupeptin had no effect on the degradation of the DT A-fragment, and caused similar inhibition in the rate of degradation of DT B-fragment and EGF. Figures 4–6 are representative of three or more experiments each.

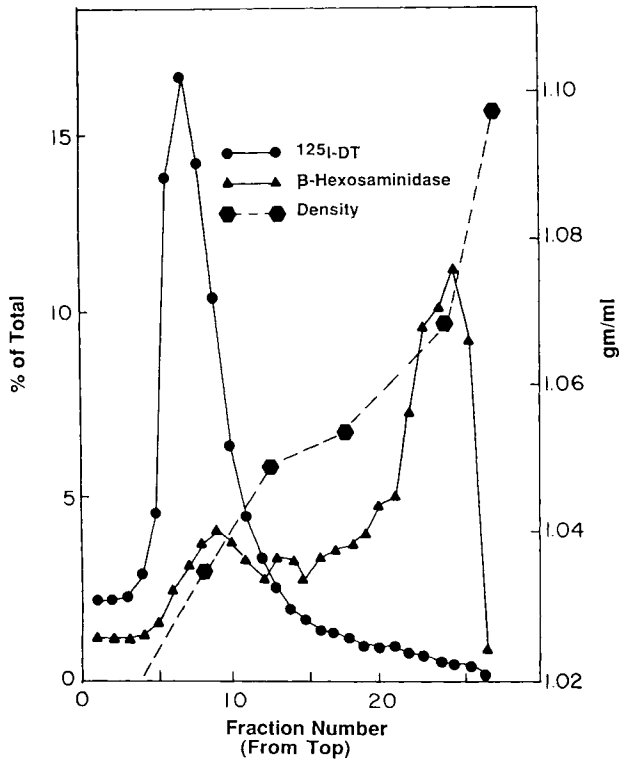

Fig. 4. Internalization of ^{125}I-DT into an intermediate-density vesicle. BS-C-1 cells were incubated at 37°C with 3.2 nM ^{125}I-DT for 25 min, washed, and treated to remove surface-bound DT. Cells were then disrupted by Dounce homogenization and fractionated on a 22% Percol gradient [10]. Internalized ^{125}I-DT (●), β-hexosaminidase activity (▲), and gradient density (⬢) are shown as indicated.

DISCUSSION

Our data show that, following internalization, diphtheria toxin is localized intracellularly within vesicles of intermediate density similar to endosomes. We did not detect diphtheria toxin within heavy-density vesicles, and, under conditions which caused the accumulation of EGF into the lysosomes, less than 2% of the toxin was shifted into the lysosomal vesicle fraction. Thus, internalized diphtheria toxin is localized within vesicles of the density of endosomes which are apparently responsible for its intracellular trafficking.

Other studies, measuring the effect of lysosomotropic amines on toxin action, have suggested that the toxin gains access to the cytosol of mammalian cells through an acidic compartment [2]. This compartment was believed to be the lysosome before the discovery of the acidic endosome. Subsequently, Sandvig and Olsnes [3] suggested that the A-fragment of DT may pass to the cytosol from a prelysosomal vesicle or endosome. Two recent studies support the role of the endosome in the cytosolic delivery of the A-fragment. First, a mutant CHO cell line resistant to DT, pseudomonas toxin, and several viruses was found to be defective in endosome acidification [4]. Secondly, Draper and

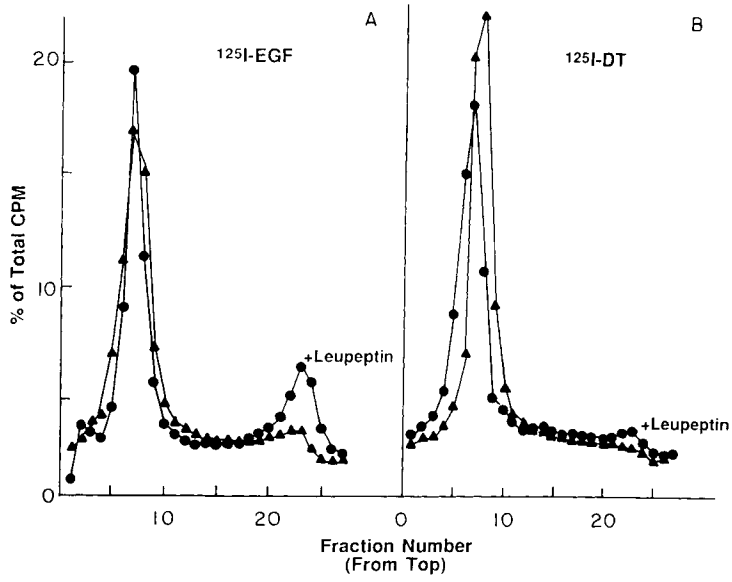

Fig. 5. Effect of leupeptin on the subcellular localization of ^{125}I-DT and ^{125}I-EGF. BS-C-1 cells were preincubated for 18 hr alone (▲) or within leupeptin (●). Next, cells were incubated at 37°C with 0.2 nM ^{125}I-EGF (**A**) or 3.2 nM ^{125}I-DT (**B**). Cells preincubated with leupeptin also had leupeptin present during this incubation. After 90 min, cells were disrupted and fractionated as described in Figure 4.

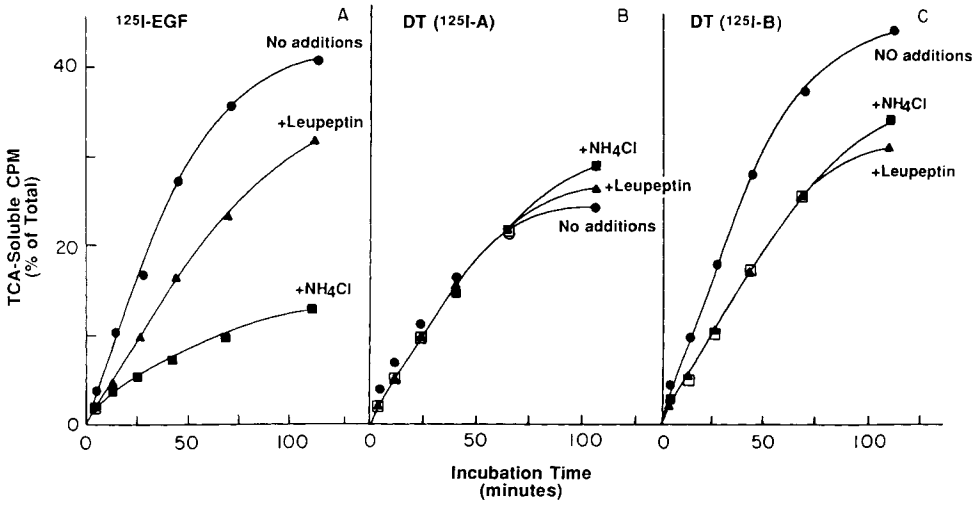

Fig. 6. Effect of NH_4Cl or leupeptin on the degradation of ^{125}I-DT or ^{125}I-EGF. BS-C-1 cells were incubated at 37°C with 0.2 nM ^{125}I-EGF (**A**) or 3.2 nM ^{125}I-DT, A-fragment radiolabeled (**B**) and B-fragment radiolabeled (**C**). After 15 min, the cultures were washed and treated to remove surface-bound ligand and refed alone (●), with 15 mM NH_4Cl (■), or with 60 μM leupeptin (▲). At the indicated times, medium was removed and precipitated with cold 10% TCA. TCA-soluble radiolabel was measured by gamma counting.

coworkers [5] showed that diphtheria toxin kills cells under conditions which inhibited endosome-lysosome fusion. Thus, the endosome and not the lysosome is postulated as the acidic compartment where the A-fragment is transferred across the cell membrane into the cytosol.

As previously reported by others [2], we observed an increase in the accumulation of ^{125}I-DT and a decrease in the appearance of TCA-soluble radioactivity in the medium of cells *loaded* with DT in the *presence* of NH_4Cl (data not shown). Under these conditions, we suggest that NH_4Cl inhibits endosome acidification and transfer of DT to the cytosol, the compartment where degradation takes place. The data presented here were the result of DT loaded and internalized in the *absence* of NH_4Cl. Subsequently, under these conditions, A-fragment degradation was not inhibited by NH_4Cl or leupeptin, and B-fragment degradation was only slightly inhibited. We suggest that the bulk of the internalized toxin had been delivered to the cytosol and thus degradation was NH_4Cl resistant. In contrast, degradation of EGF was markedly inhibited by NH_4Cl and showed some inhibition by leupeptin. These results suggest that following internalization in the absence of NH_4Cl, the bulk of DT and EGF are trafficked intracellularly by different pathways.

Other studies have shown that EGF is internalized and degraded via the endosome-lysosome vesicle pathway [10–13]. The slight inhibition in the rate of B-fragment degradation (DT-^{125}I-B) in the presence of NH_4Cl indicates that some of the B-fragment, unlike the A-fragment of DT, is degraded via an NH_4Cl-sensitive pathway. This may account for the small amount of diphtheria toxin present in heavy-density vesicles following incubation with leupeptin, as indicated in Fig. 4. At this time it is not possible to determine whether this (alternate) pathway is physiologically relevant or the result of the iodination procedure producing structurally aberrant B-fragment. Also, it suggests that the A-fragment and some of the B-fragment of DT are degraded by different intracellular pathways.

Degradation of DT may occur in the endosome, where proteolytic degradation has recently been described [14], or in the cytosol by the ubiquitin-dependent pathway [15]. Whole toxin might reach the cytosol if large doses of toxin lead to disruption of the endosome and release of its contents into the cytosol, as has been demonstrated following uptake of adenovirus [16]. This hypothesis has been proposed by Hudson and Neville [17], who suggest on the basis of kinetic evidence that toxin is released into the cytosol as a bolus upon vesicle disruption. On the other hand, Yamaizumi and coworkers [18] have reported that DTA, when introduced into the cytoplasm as the monomer form, is relatively stable in the cell cytosol. Further work is needed to distinguish between the endosome and the cytosol as sites for degradation for DT.

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