Pseudomonas exotoxin (PE) requires proteolytic cleavage to generate a 37-kDa C-terminal fragment that translocates to the cytosol and ADP-ribosylates elongation factor 2. Cleavage within cells is mediated by furin, occurs between arginine 279 and glycine 280, and requires an arginine at both P1 and P4 residues. To study the proteolytic processing of PE-derived chimeric toxins, TGFα-PE38 (transforming growth factor fused to the domains II and III of PE) and a mutant form, TGFα-PE38gly279, were each produced in Escherichia coli. When assessed on various epidermal growth factor (EGF) receptor-positive cell lines, TGFα-PE38 was cleaved in a similar fashion to that of TGFα-PE38gly279. HB21scFv-PE40, which targets cells expressing the transferrin receptor, was cleaved in a similar fashion to that of TGFα-PE38 and nicked HB21scFv-PE40 exhibited increased toxicity for LoVo cells. In short-term experiments, the rate of reduction in protein synthesis by furin-nicked immunotoxins was increased compared with unnicked protein, indicating that cleavage by furin can be a rate-limiting step. We conclude that furin-mediated cleavage of PE-derived immunotoxins is important for their cytotoxic activity.

Pseudomonas exotoxin (PE) is a single chain protein toxin that is toxic for mammalian cells because of its ability to translocate to the cell cytosol and ADP-ribosylate elongation factor 2 (EF2) and inhibit protein synthesis (1). Once synthesized, the toxin folds to form a 3-domain protein composed of an N-terminal domain that mediates receptor binding, a middle domain that mediates translocation and has a prominent arginine-rich loop that allows proteolytic cleavage, and a C-terminal domain that has ADP-ribosylating activity and an endoplasmic reticulum (ER) retention sequence (2–4). In its native form, PE is a proenzyme, and in biochemical experiments, it requires a strong denaturant and reducing agent to reveal its enzyme activity (5). It is, therefore, of considerable interest to learn how toxin molecules unfold and are processed within cells without the use of harsh treatments.

The pathway that PE takes to the cytosol begins with its binding to the multi-ligand surface receptor known as the low density lipoprotein receptor-related protein (LRP), also known as the α2-macroglobulin receptor (6, 7). Binding leads to endocytosis via coated pits, which brings the toxin to the low pH environment of the endosomal compartment. There, the toxin is cleaved into an N-terminal fragment of 28 kDa and a C-terminal fragment of 37 kDa (8). Cleavage is between arginine 278 and glycine 280 (9) and occurs when the toxin is exposed to furin at pH 5.0–5.5 (10). Cells lacking furin exhibit a toxin-resistant phenotype. Toxin sensitivity can be restored by transfection with a cDNA encoding furin (11). Hydrolysis of the peptide bond between residues 279 and 280 leaves the toxin fragments joined by the disulfide bond linking cysteines 265 and 287. At a later step in the pathway, this bond is reduced by a mechanism that is yet unclear. Translocation of the reduced 37-kDa fragment to the cytosol requires the presence of a C-terminal endoplasmic reticulum retention sequence (4). In the cytosol, the 37-kDa fragment ADP-ribosylates EF-2 (12) inhibits protein synthesis and induces apoptosis (13).

To target the cell-killing activity of PE to specific populations of mammalian cells, the binding domain of the toxin is removed and replaced with cDNAs that encode antibody Fv fragments or receptor-binding ligands (14). New binding sequences are placed at the 5’-end of constructs and fused with domains II and III of PE. Depending on the presence or absence of subdomain Ib, these truncated forms of PE are called PE40 and PE38, respectively. In native PE, portions of domain I associate with domain III via several hydrogen bonds (2). The replacement of domain I by foreign sequences eliminates these interactions and changes toxin structure. This is reflected in the fact that immunotoxins exhibit full ADP-ribosylating activity, without the need of a denaturant.

The intracellular pathways of immunotoxin traffic have not been evaluated extensively. Differences in immunotoxin processing are likely to occur because receptors of various function are targeted. Also, truncated toxins are likely to behave differently than their full-length counterparts. When characterizing the cytotoxicity of TGFα-PE40 on various cancer cell lines, it was noted that this immunotoxin was 10–100-fold more active than native PE (15). The greater potency of the chimeric could...
have been due to differences in receptor number or binding affinity or to interactions with other cellular components. Here we characterize the proteolytic processing of PE-derived immunotoxins, report on comparisons with the cleavage of native PE, and speculate that the less stringent pH conditions needed to cleave chimeric toxins may contribute to their increased potency.

**MATERIALS AND METHODS**

**Cells**—The cell lines MCF7, HT29, KB, A431, LoVo, and the Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD) and were propagated in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and penicillin (50 units/ml) and streptomycin (50 μg/ml).

**Cloning of Mouse Furin**—To introduce the mouse furin sequence in the plasmid (Invitrogen Corp., San Diego, CA), HindIII and NcoI sites were added by polymerase chain reaction at the 5’- and 3’-ends of the furin gene from the pRK7musfur plasmid (16). The primers used were: 5’-GGTTTCAAGCTTGCAGATCGACTTAG-3’ and 5’-ATCGATGGCGCGCTAGATTACCATCGAGTT-3’. The resulting polymerase chain reaction fragment was digested with HindIII and NcoI and ligated into cleaved pRc/CMV vector. This resulting construct, pRcCMVfur1, encodes a truncated soluble form of mouse furin. (Rabbit muscle, amino acids 1–114)

**Transfection of CHO Cells and Production of Recombinant Furin**—CHO cells plated at a density of 3 x 10^5 cells/35-mm dish were transfected with 2.5 μg of the DNA construct pRcCMVfur1 by using Transfectam® (Promega). After 48 h, transfected cells were selected by treatment with Geneticin (G-418, Life Technologies, Inc.) at 1 mg/ml. The resulting polymerase chain reaction fragment was digested with HindIII and NcoI and ligated into cleaved pRc/CMV vector. This resulting construct, pRcCMVfur1, encodes a truncated soluble form of mouse furin. (Rabbit muscle, amino acids 1–114)

**Relative Toxicities of TGFα-PE38 and TGFα-PE38gly279**—PE and PE-derived chimeric toxins were produced in various cell lines and incubated overnight at 37 °C. At the end of the incubation period, the level of protein synthesis was determined by measuring the incorporation of [3H]leucine into cellular protein. Results are expressed as IC50 values.

**RESULTS**

Relative Toxicities of TGFα-PE38 and TGFα-PE38gly279—PE requires cleavage in domain II, between arginine 279 and glycine 280, to produce a C-terminal fragment capable of translocating to the cytosol and ADP-ribosylating elongation factor 2. By mutating either arginine 276 or arginine 279 (P4 and P1, respectively) to glycine, PE is rendered refractory to cleavage and non-toxic for cells (8). TGFα-PE38, a ligand toxin composed of TGFα fused to domains II and III of PE, is toxic for cells expressing the EGF receptor. To characterize the cell-mediated cleavage of this ligand toxin, arginine 279 was mutated to glycine to produce TGFα-PE38gly279.

With the exception of LoVo cells (see below), TGFα-PE38 was toxic for cells expressing the EGF receptor, with IC50 (the concentration of immunotoxin causing a 50% reduction in protein synthesis) values in the range of 0.1–2.4 ng/ml while TGFα-PE38gly279 was 146- to 1000-fold less active (Table I). This indicated that arginine 279 was important for toxicity, possibly to facilitate cell-mediated cleavage. Unexpectedly, TGFα-PE38gly279 was toxic for A431 cells, with an IC50 of 10 ng/ml. This toxicity was apparently caused by ligand-receptor interactions, a phenomenon that has been reported previously for A431 cells and other non-toxic forms of TGFα-PE (18). To confirm the ligand-receptor nature of this effect, we report that PEGgly279 exhibited very low toxicity for A431 cells with an IC50 of greater than 1000 ng/ml (Table I). Cells that do not express the EGF receptor, such as CHO, were resistant to both the wild-type and the mutant forms of the chimeric toxin (Table I).

**Susceptibility of TGFα-PE38 Toxins to Furin-mediated Cleavage**—Previously, we showed that PE was cleaved optimally by furin at pH 5.5, with no cleavage detected at pH values close to neutral (10). This result reflected an apparent pH-dependent change in toxin conformation and not the pH optimum of furin. Here we show that furin-mediated cleavage of TGFα-PE38 at pH 5.5 generated fragments of 35 and 11 kDa (Fig. 1). The 35-kDa fragment is equivalent to the 37-kDa fragment from native PE. The 11-kDa band was not resolved in this gel. No products were seen when TGFα-PE38gly279 was incubated with furin (Fig. 1). To determine if the cleavage of the chimeric toxins resembled that of the parent toxin, furin, and TGFα-PE38 were co-incubated over a wide pH range. Results indicated that cleavage had a broad acidic optimum (pH 5.0–6.5), but fragments were produced at all values tested from 4.5 to 8.5 (Fig. 1). The generation of the 35- and 11-kDa fragments was consistent with cleavage at arginine 279. N-terminal sequence analysis of the 35-kDa fragment confirmed this (data not shown).

**Toxicity of TGFα-PE38 Toxins on Furin-deficient Cells**—LoVo
is a human colon carcinoma cell line harboring two defective alleles of the furin gene (19, 20). Frequently, colon carcinomas and cell lines derived from them display high levels of EGF receptors, which potentially would make LoVo cells a good target for this ligand toxin. However, TGFα-PE38 had a relatively low activity against this line (IC50 = 37 ng/ml, Table I, and Fig. 2). This result was consistent with the hypothesis that furin-mediated cleavage was necessary for toxicity. To test this directly, TGFα-PE38 was cleaved by furin to produce a nicked toxin (held together via the disulfide bond joining cysteines 265 and 287) that was then added to LoVo cells for 24 h. Precleavage increased cytotoxic activity by 30-fold (Fig. 2). In short-term kinetic experiments, unnicked TGFα-PE38, used at concentrations ranging from 10 ng/ml to 1 μg/ml, exhibited little or no toxicity for LoVo cells while nicked toxin caused significant inhibition of protein synthesis (compare Fig. 3A with 3B). The greater activity of nicked TGFα-PE38 suggested that cleavage by furin was equivalent to processing by cells. An identical furin treatment of TGFα-PE38gly279 did not enhance toxicity, confirming that cleavage was necessary for activity (data not shown).

**Toxicity of Nicked TGFα-PE38 on Furin-expressing Cells**—Having shown that furin could cleave TGFα-PE38 and that furin-deficient cells were poorly sensitive to this toxin, we next analyzed the role of furin-mediated cleavage in cells known to express furin. To determine if furin-mediated cleavage was rate-limiting, we compared the toxicity of nicked and unnicked TGFα-PE38. When added to HT29 cells for 24 h, no apparent difference in toxicity could be detected (Fig. 4A). However, in shorter time periods, nicked ligand toxin was more active, suggesting that in *vitro* cleavage by furin mimics cellular processing and that furin cleavage can be rate-limiting (Fig. 4B). At a concentration of 100 ng/ml, nicked toxin reduced protein synthesis by 90% in 4 h. The same reduction in protein synthesis required an additional 3 h for the unnicked toxin.

**Susceptibility of PE Single Chain Immunotoxins to Furin-mediated Cleavage and Relevance to Toxicity**—To determine if other PE-based chimeric toxins were susceptible to cleavage by furin, a single chain antibody-toxin was assessed as a substrate. Specifically, the scFv fragment of the HB21 antibody directed to the human transferrin receptor fused to PE40 (21) was evaluated. At pH 5.5, furin-mediated cleavage generated fragments of 37 and 28 kDa (Fig. 5A). In *vitro*, HB21scFv-PE40 was cleaved by furin over the same pH range as TGFα-PE38 (data not shown), and the size of the fragments was consistent with a single cleavage event at or near the Arg279-
Furin-mediated Cleavage of Immunotoxins

Initial cleavage experiments with hTGFα-PE38 were performed at pH 5.5 because this was the optimal pH for cleavage of PE. In later experiments, we examined cleavage over a broad pH range. Unlike native PE, hTGFα-PE38 could be cleaved from pH 4.5 to 8.5. We speculate that this difference might allow greater production of the translocating fragment because furin is mostly expressed in the TGN (31–33), which has a pH range from slightly acidic to neutral. Previously, we found that the conformational change in PE, induced by acidic pH, is reversible, indicating that PE can be cleaved only in endosomes at pH 5.5 (10), while the broad pH range observed for the cleavage of the chimeric toxins suggests that they can be cleaved in any compartment expressing furin.

While our initial experiments were performed with the ligand toxin, hTGFα-PE38, we also evaluated the cleavage of an antibody-toxin fusion protein. Results with both kinds of immunotoxins were very similar, indicating that the composition of the binding domain did not unduly influence furin-mediated cleavage. Like hTGFα-PE38, HB21scFv-PE40 was poorly toxic for LoVo cells and similarly, exhibited an enhanced activity upon in vitro cleavage by furin and the addition of nicked immunotoxin.

The use of kinetic cytotoxicity assays confirmed that furin-mediated cleavage by cells can be a rate-limiting step in toxin action. As with PE, the addition of nicked protein provided a
resistance could be overcome by prior nicking of the immunotoxin with furin (34). Immunotoxins directed to other receptors on Daudi cells gave no indication of this partial resistance. This may indicate the existence of separate endocytic pathways that intersect to a greater or lesser extent with furin-containing organelles.

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