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Furin-mediated Cleavage of *Pseudomonas* Exotoxin-derived Chimeric Toxins*

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***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 100–500-fold more toxic than TGFα-PE38gly279. In contrast to PE, where cleavage by furin is only evident at pH 5.5, furin cleaved TGFα-PE38 over a broad pH range, while TGFα-PE38gly279 was resistant to cleavage. TGFα-PE38 was poorly toxic for furin-deficient LoVo cells, unless it was first pretreated *in vitro* with furin. Furin treatment produced a nicked protein that was 30-fold more toxic than its unnicked counterpart. Using the single chain immunotoxin HB21scFv-PE40 as a substrate, furin-mediated processing of an antibody-based immunotoxin was also evaluated. 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)[1] 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 a 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 α_2-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 279 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

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[1] The abbreviations used are: PE, *Pseudomonas* exotoxin; EF2, elongation factor 2; EGF, epidermal growth factor; HB21scFv-PE40, single chain immunotoxin targeted to the human transferrin receptor; IC_{50}, the concentration that produces a 50% inhibition of protein synthesis; PE40, domains II, Ib, and III of PE; PE38, domains II and III of PE; scFv, single chain Fv fragment of a monoclonal antibody; TGFα, transforming growth factor α; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

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 pRc/CMV plasmid (Invitrogen Corp., San Diego, CA), *Hin*dIII and *Not*I sites were added by polymerase chain reaction at the 5′- and 3′-ends of the furin gene from the pRK7.musfurΔ1 plasmid (16). The primers used were: 5′-GGTTCTAAGCTTCTGCAGGTCGACTCTAG-3′ and 5′-ATCGATTGCGGCCGCTAGATTCATTCATCTCGAGT-3′. The resulting polymerase chain reaction fragment was digested with *Hin*dIII and *Not*I and ligated into cleaved pRc/CMV vector. This resulting construct, pRc/CMVfurΔ1, encodes a truncated soluble form of mouse furin (nucleotides 1–2142, amino acids 1–714).

Transfection of CHO Cells and Production of Recombinant Furin—CHO cells plated at a density of 3×10^5 cells/35-mm dish were transfected with 2.5 μg of the DNA construct pRc/CMVfurΔ1 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 cells were then cloned by limiting dilution. Colonies were subsequently screened for secretion of soluble furin. A 10-μl aliquot of supernatant from each colony was tested for its ability to cleave the mutant toxin PEala281 as described previously (10). The clone CHOfurΔ1 was maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. The culture medium was replaced by serum-free minimal essential medium that was harvested 48 h later. The protease was then purified from conditioned medium by high performance liquid chromatography using a nonporous DEAE resin. Peak fractions, detected by Western blotting using anti-furin polyclonal antibodies, were pooled and used for protease assays. The furin preparation had an activity ranging from $2–8 \times 10^{-7}$ units μl^{-1}. As defined previously, 1 unit of furin cleaved 1 μmol of the mutant PEala281 in 1 min at 23 °C (10).

Cleavage by Furin—Cleavage of toxins was conducted in a 20-μl reaction volume containing 200 mM sodium acetate, pH 4.5–6.0, 200 mM HEPES, pH 6.5–7.5, or 200 mM Tris-HCl, pH 7.5–8.5, 5 mM CaCl2, 2.5 μg of toxin and 8×10^{-6} units of furin. Samples were incubated at 23 °C for 20 h and reactions were terminated by adding SDS sample buffer. Digestion products were separated by SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie Brilliant Blue R-250.

Determination of Cleavage Site—Following TGFα-PE38 cleavage, the reaction products were separated by SDS-PAGE, and the proteins were transferred electrophoretically onto Immobilon-P membranes (Millipore) and stained with Ponceau S to identify the 35-kDa fragment. N-terminal sequence analysis was performed on the excised fragment. Automated Edman degradation analyses were carried out at the Protein Analysis Core Laboratory of the Cancer Center of Wake Forest University.

Toxin Expression—PE and PE-derived chimeric toxins were produced in *Escherichia coli* BL21/λDE3 as described previously (15). Upon induction with isopropyl-1-thio-β-D-galactoside, the expressed HB21ScFv-PE40 and TGFα-PE38 chimeric toxins accumulated in intracellular inclusion bodies. The inclusion bodies were denatured in 7 M guanidine hydrochloride and renatured in phosphate-buffered saline (PBS). The crude dialyzed protein preparation was then purified by sequential, Q-Sepharose (Pharmacia Biotech Inc.), Mono-Q (Pharmacia), and gel filtration chromatography as described previously (15, 17).

Determination of Toxic Activity—PE-related toxins were added to cells to determine their ability to inhibit protein synthesis. Purified proteins were diluted from concentrated stocks into PBS containing 0.2% human serum albumin and then added to cells for the indicated times. The cells were subsequently pulse-labeled with ^{3}H-leucine for 1 h and then washed with PBS. Proteins were precipitated with 12% trichloroacetic acid and then dissolved in 0.1 M NaOH. The radioactivity associated with the precipitated proteins was determined by scintillation spectroscopy. Controls consisted of cells that were not treated with toxin.

TABLE I
Cytotoxicity of TGFα-PE38 toxins

TGFα-PE38 and TGFα-PE38gly279 proteins were added to 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 [^{3}H]leucine into cellular protein. Results are expressed as IC_{50} values.

Cell line	Tissue of origin	IC_{50} (ng/ml)	
		TGFα-PE38	TGFα-PE38 gly279
MCF7	Breast	1.1	>1,000
HT29	Colon	2.4	350
LoVo	Colon	37	>1,000
KB	Epidermoid	0.1	310
A431[a]	Epidermoid	0.28	10
CHO	Ovary	>1,000	>1,000

[a] A431 IC_{50} (PE) = 5 ng/ml; IC_{50} (PEgly279) >1,000 ng/ml.

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[2] 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 IC_{50} (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 IC_{50} 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 PEgly279 exhibited very low toxicity for A431 cells with an IC_{50} of >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 TGFαPE38 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 TGFαPE38 Toxins on Furin-deficient Cells—LoVo

[2] The numbering system for amino acids in chimeric toxins reflects their location in the native toxin.

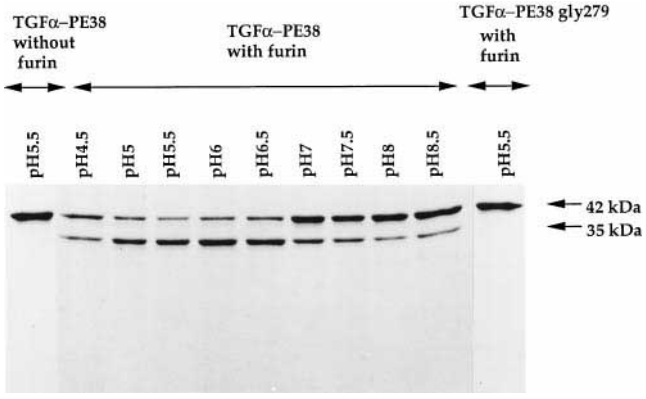

FIG. 1. **pH-dependent cleavage of TGFα-PE38 by furin.** TGFα-PE38 (2.5 μg) or TGFα-PE38gly279 (2.5 μg) was incubated with recombinant furin (8×10^{-6} units) at 23 °C for 20 h. The reaction products were analyzed by reducing SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. The 11-kDa fragment could not be seen on this gel.

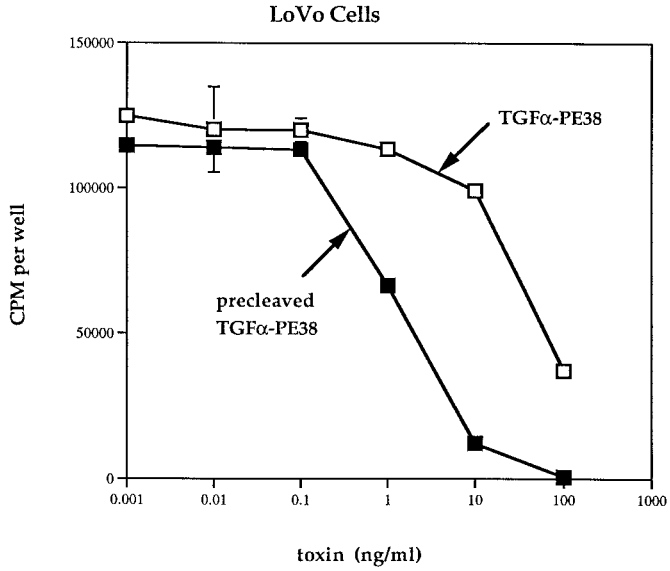

FIG. 2. **Toxicity of nicked and unnicked TGFα-PE38 on LoVo cells.** TGFα-PE38 (5 μg) was incubated for 20 h at 23 °C in 0.2 M sodium acetate, 5 mM $CaCl_2$, pH 5.5 without or with furin (1.6×10^{-5} units). The toxins were then added to LoVo cells at 37 °C for 24 h. The level of protein synthesis was determined by measuring the incorporation of [^{3}H]leucine into cellular protein. One representative experiment is shown. Data are the mean ± S.D. of quadruplicates. *CPM*, counts per min.

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 (IC_{50} = 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. 3*A* with 3*B*). 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 TGFαPE38 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. 4*A*). 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. 4*B*). 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. 5*A*). *In vitro*, HB21scFv-PE40 was cleaved by furin over the same broad 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 Arg^{279}-Gly^{280} peptide bond. To determine the relevance of furin cleavage, we compared the toxicity of nicked and unnicked immunotoxin on LoVo cells. Cleavage increased cytotoxic activity by 20-fold with the nicked immunotoxin having an IC_{50} of 1.2 ng/ml and the unnicked having 25 ng/ml. In short-term kinetic experiments, HB21scFv-PE40, at 10 ng/ml, reduced protein synthesis by 20% after 8 h on LoVo cells (Fig. 5*B*). When the immunotoxin was pretreated with furin, the protein synthesis rapidly decreased with 90% reduction after 8 h (Fig. 5*B*).

DISCUSSION

Furin is expressed widely and has been implicated in the processing of secreted proteins such as prohormones (22), growth factors (23), receptors (24), and viral glycoproteins (25). These proteins are synthesized as inactive precursors and must be proteolytically cleaved to become functionally mature. Similarly, *Pseudomonas* exotoxin (10, 11), diphtheria toxin (10, 16, 26, 27), and shiga toxin (28) require cleavage for maturation. For these toxins, it is necessary to produce enzymatically active fragments that translocate to the cytosol and inhibit protein synthesis. Furin appears to be one of the proteases that can mediate this cleavage (29). The protective antigen of anthrax toxin is also processed by furin (30).

A variety of ligand-toxin or immunotoxin fusion proteins have been made from these toxins. While the importance of specific arginine residues for the cell killing activity of DT-IL2 has been reported (26), the proteolytic processing of immunotoxins has not been extensively investigated. Here we report that furin is needed for the cleavage and activation of PE-derived recombinant immunotoxins, and this processing can be rate-limiting for killing cells. To cleave its substrates, furin requires basic residues (often arginines) at P1 and P4 positions. Since PE was cleaved after arginine 279, we investigated the role of this residue in mediating the toxicity of the chimeric toxin, TGFα-PE38. When TGFα-PE38gly279 was added to cells, it exhibited greatly reduced toxicity for all receptor-positive cell lines, indicating an important role for this residue. To investigate this, the wild-type and mutant chimeric toxins were incubated with recombinant furin, and the appearance of cleav-

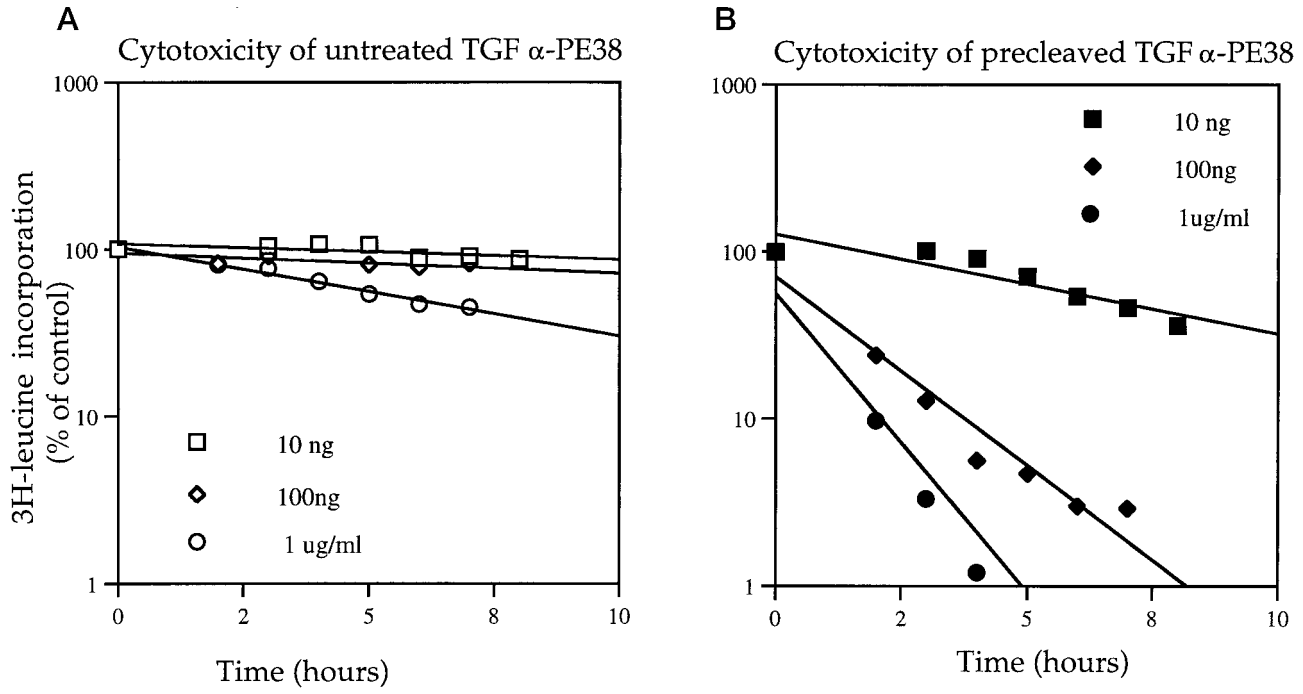

FIG. 3. **Kinetics of the toxicity by nicked and unnicked TGFα-PE38 on LoVo cells.** The level of protein synthesis was determined as described in Fig. 2. One representative experiment is shown. The values are the mean ± S.D. of quadruplicates and are expressed as percent of the control. *A*, TGFα-PE38 was incubated for 20 h at 23 °C in 0.2 M sodium acetate, 5 mM $CaCl_2$, pH 5.5 and then added to LoVo cells at three different concentrations (10 ng/ml, 100 ng/ml, and 1 μg/ml) for the length of time indicated. *B*, cleavage of TGFα-PE38 (5 μg) was performed at 23 °C for 20 h in 0.2 M sodium acetate, 5 mM $CaCl_2$, pH 5.5, using 1.6×10^{-5} units of recombinant furin. The precleaved TGFα-PE38 was then added to cells as indicated in *panel A*.

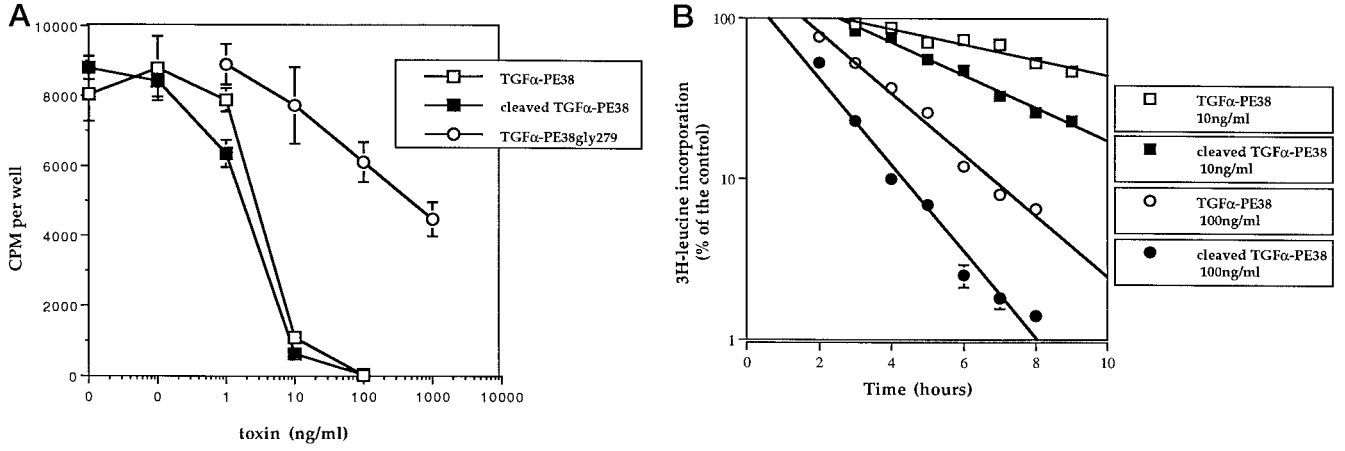

FIG. 4. **Toxicity of nicked and unnicked TGFα-PE38 on HT29 cells.** TGFα-PE38 was incubated for 20 h at 23 °C in 0.2 M sodium acetate, 5 mM $CaCl_2$, pH 5.5, with or without furin. The toxins were added to cells for various lengths of time, and the level of protein synthesis was determined by measuring the incorporation of ^{3}H-leucine into cellular protein. *A*, inhibition of protein synthesis after a 24-h incubation with TGFα-PE38, precleaved TGFα-PE38, or TGFα-PE38gly279. The values are the mean ± S.D. of quadruplicates expressed in counts/min/well (*CPM*). *B*, kinetics of inhibition of protein synthesis by nicked and unnicked TGFα-PE38. Chimeric toxins were added to HT29 cells at 10 ng/ml or 100 ng/ml for the length of time indicated. The results are the mean ± S.D. of quadruplicates and are expressed as percent of the control.

age products were monitored by SDS-PAGE. Furin cleaved TGFα-PE38 to produce fragments of 35 and 11 kDa while TGFα-PE38gly279 was not cleaved.

Initial cleavage experiments with TGFα-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, TGFα-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, TGFα-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 TGFα-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

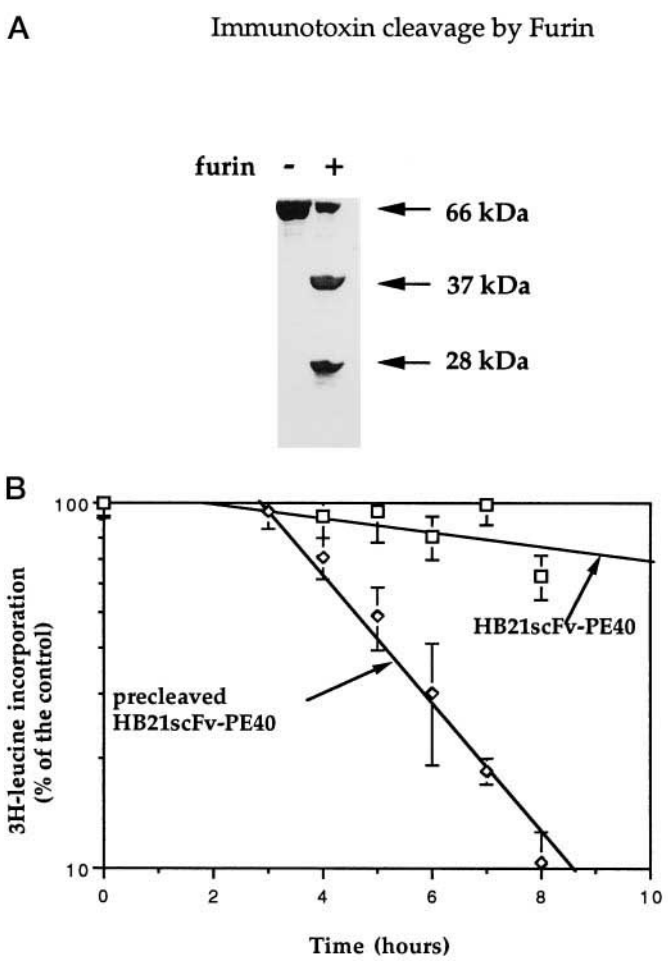

FIG. 5. **Toxicity of nicked and unnicked HB21scFv-PE40.** The cleavage of HB21scFv-PE40 was performed for 20 h at 23 °C in 0.2 M sodium acetate, 5 mM $CaCl_2$, pH 5.5. *A*, immunotoxin digestion products were analyzed by reducing SDS-PAGE. HB21scFv-PE40 (5 μg) was incubated without (−) or with 1.6×10^{-5} units of furin (+). *B*, inhibition of protein synthesis by nicked and unnicked HB21scFv-PE40 on LoVo cells. After the 20-h incubation, the toxins were added to LoVo cells at a concentration of 10 ng/ml for the length of time indicated. The level of protein synthesis was determined by measuring the incorporation of ^{3}H-leucine into cellular protein. Data are the mean ± S.D. of quadruplicates and are expressed as percent of control. □, HB21scFvPE40; ◇, cleaved HB21scFvPE40.

2–3 h headstart compared with unnicked toxin. Because of the length of this step, the effect of precleavage on furin-expressing cells was only noticeable for the first few hours after toxin addition. By 24 h, the cleavage step had become a small fraction of the total intoxication process.

The need for furin-mediated cleavage becomes an important consideration for immunotoxin design since cells that fail to express active furin or where furin is not part of the endocytic pathway are likely to be resistant to immunotoxin-mediated cell killing. Recently, we reported that Daudi cells were partially resistant to an anti-CD22 immunotoxin and that this 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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