

## Proteolytic Activation of Bacterial Toxins by Eukaryotic Cells Is Performed by Furin and by Additional Cellular Proteases

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Before intoxication can occur, anthrax toxin protective antigen (PA), *Pseudomonas* exotoxin A (PE), and diphtheria toxin (DT) must be activated by proteolytic cleavage at specific amino acid sequences. Previously, it was shown that PA and DT can be activated by furin. In Chinese hamster ovary (CHO) cells, wild-type (RKKR) and cleavage site mutants of PA, each administered with a modified form of anthrax toxin lethal factor (the N terminus of lethal factor fused to PE domain III), had the following potencies: RKKR (wild type) (concentration causing 50% cell death [EC<sub>50</sub>] = 12 ng/ml) ≥ RAAR (EC<sub>50</sub> = 18 ng/ml) > FTKR (EC<sub>50</sub> = 24 ng/ml) > STRR (EC<sub>50</sub> = 49 ng/ml). In vitro cleavage of PA and cleavage site mutants of PA by furin demonstrated that native PA (RKKR) and PA with the cleavage sequence RAAR are substrates for furin. To characterize eukaryotic proteases that play a role in activating bacterial toxins, furin-deficient CHO cells were selected after chemical mutagenesis. Furin-deficient cells were resistant to PE, whose cleavage site, RQPR, constitutes a furin recognition site and to all PA cleavage site mutants, but were sensitive to DT (EC<sub>50</sub> = 2.9 ng/ml) and PA (EC<sub>50</sub> = 23 ng/ml), whose respective cleavage sites, RKKR and RVRP, contain additional basic residues. Furin-deficient cells that were transfected with the furin gene regained sensitivity to PE and PA cleavage site mutants. These studies provide evidence that furin can activate the three toxins and that one or more additional proteases contribute to the activation of DT and PA.

Many bacterial toxins are synthesized in a proform and require proteolytic activation before they can intoxicate cells (11). A number of toxins, including protective antigen (PA) from *Bacillus anthracis*, *Pseudomonas* exotoxin A (PE), and diphtheria toxin (DT), can be cleaved by eukaryotic proteases (11). While bound to an as yet unidentified eukaryotic receptor, PA becomes activated by proteolytic cleavage after the sequence RKKR-167 (16, 22). The receptor-bound fragment of PA then can bind one of two enzymatically active polypeptides, edema factor or lethal factor. The complex is endocytosed, the enzymatically active moiety translocates to the cytosol, and the target cell becomes intoxicated. PE (25) and DT (7, 23) are cleaved at the sequences RQPR-279 and RVRP-193, respectively, after which the domain containing ADP-ribosyltransferase activity is translocated. For each of these toxins, the requirement for -1 and either -4 or -6 basic residues as protease recognition sites has been established (14, 16, 37). The sequences at which cleavage occurs correspond to the amino acid sequence recognized by furin, a subtilisin-like, Ca<sup>2+</sup>-dependent processing endoprotease which recognizes the sequence, RXK/RR (2). Evidence that furin is required for the activation of PE derives from studies that used a furin-deficient mutant Chinese hamster ovary (CHO) cell line, RPE.40 (20, 21, 36). A role for furin in the processing of PA (15) and DT (15, 34) has been demonstrated.

Klimpel et al. (15) mutated the cleavage sequence in PA, RKKR, and found that an alternate sequence, RAAR, was cleaved by furin to produce an active toxin (16, 22). Watanabe et al. (35) demonstrated that in order to be cleaved by furin,

prorenin must contain, at minimum, -1 and -4 basic residues. Further characterization of the requirements for furin cleavage suggested that the -1 position must be occupied by arginine, that a basic amino acid at -2 is not essential, and that a basic residue at the -6 position may substitute for one at the -4 position (24, 35). The conclusion that furin requires a -4 basic residue has been challenged by recent reports that furin may cleave after dibasic sequences under some circumstances (4, 8). Given these requirements, it appears that classic furin recognition sequences are present in PE (arginine residues at the -1, -4, and -6 positions), DT (arginines at the -1, -2, and -4 positions), and PA (basic residues at the -1, -2, -3, and -4 positions). All three toxins are potential furin substrates, but DT and PA also contain dibasic or tetrabasic sequences, respectively, at their cleavage sites that could make them substrates for additional proteases.

Although PE is trafficked through the Golgi apparatus (6, 32, 39), PA (9, 12) and DT (31) have been shown to transfer their cytotoxic domains directly to the cytosol from acidified endosomes. Furin has been localized primarily to the *trans*-Golgi (5, 19), so PA and DT may not be exposed to significant amounts of furin. Therefore, we hypothesized that alternative proteases with specificities for multiple basic residues may be able to activate PA and DT. In order to address this hypothesis, we prepared PA proteins containing site-specific mutations in their cleavage sequences. To characterize the proteases that activate PA and DT, we isolated protease-deficient CHO cells. Data from experiments using protease-deficient cells and mutated toxins reported here suggest that eukaryotic cells contain at least two separate proteases that can cleave DT and PA.

### MATERIALS AND METHODS

**Materials.** PE and DT were purchased from List Biological Laboratories. *B. anthracis* PA was purified as described previously (18). Ethyl methanesulfonate (EMS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT),

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trypsin, cathepsin B, and aprotinin were obtained from Sigma (St. Louis, Mo.). Leupeptin was obtained from Bachem (Torrance, Calif.). Lipofectamine and geneticin (G418) were purchased from GIBCO-BRL Life Technologies, Inc. Precast 4 to 20% Tris-glycine gels were obtained from Novex (San Diego, Calif.). HyQ-CCM5 medium was purchased from HyClone (Logan, Utah).

**Preparation of PA mutated at residues 164 to 167.** Pairs of complementary deoxyoligonucleotides encoding the desired cleavage site sequences were synthesized on an Applied Biosystems PCR-Mate. These were ligated with the *Bam*HI-*Bst*BI fragment from pYS5 and the *Ppu*MI-*Bam*HI fragment from pYS6 and transformed into *Escherichia coli* DH5 $\alpha$  as described previously (16, 33). Plasmids containing the correct sequence were transformed into the *E. coli* *dam* *dem* strain GM2163. Unmethylated plasmid DNA was purified and electroporated into *B. anthracis* BH441 (*B. anthracis* UM44-1C9 containing Tn917 inserted into the major extracellular protease gene) by using a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, Calif.).

**Purification of native and mutated PA proteins.** Transformed *B. anthracis* BH441 expressing mutated PA proteins was grown in 6-liter batches in FA medium (33) containing 20  $\mu$ g of neomycin per ml in a BioFlo IV fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37°C. PA was purified by ammonium sulfate precipitation (0.47 g/ml of supernatant) at 4°C. The precipitated protein was centrifuged at 8,000  $\times$  g for 40 min, dissolved in buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5, and 5 mM EDTA, and dialyzed against 10 mM HEPES, pH 7.5. The dialysate was subjected to hydroxyapatite chromatography, eluted with 0.25 M potassium phosphate buffer, pH 7.0, and purified by MonoQ column chromatography (Pharmacia LKB Biotechnology, Piscataway, N.J.), using a gradient of 0 to 1 M NaCl in 10 mM BisTris propane-15 mM aminoethanol, pH 9.0. Purified PA mutants are designated according to the sequence at positions 164 to 167.

**Preparation of FP50.** FP50 is a fusion protein linking amino acids 1 to 254 from anthrax toxin lethal factor with domains Ib and III from PE (amino acid residues 362 to 608) and possessing the sequence LDER at the carboxyl terminus. Between the two toxin domains, the sequence TR is added. The amino terminus contains the added sequence MVP. The construct was prepared and purified using procedures that have been described previously (1).

**Cell culture.** CHO-K1 cells were obtained from the American Type Culture Collection. CHO and mutant cell lines derived from them were maintained in HyQ-CCM5 serum-free medium for CHO cells or  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) (GIBCO Laboratories, Grand Island, N.Y.) containing 10 mM HEPES, pH 7.3, and 50  $\mu$ g of gentamicin per ml. All cells were cultured in an atmosphere of 5% CO<sub>2</sub> at 37°C.

**Selection of toxin-resistant CHO cell mutants.** CHO-K1 cells were grown to 80% confluency in T-75 flasks. Cells were incubated at 37°C with 6  $\mu$ l of EMS per 20 ml of medium for 18 h. After being washed with fresh medium, the cells were plated in 100-mm-diameter dishes at 5  $\times$  10<sup>5</sup>/ml and incubated at 37°C for 5 days. The cells were challenged with 50 ng of FP50 per ml in combination with concentrations of PA RAAR or PA FTKR ranging from 100 to 1,000 ng/ml. After 36 h, the medium containing toxin was replaced with fresh medium. Surviving colonies were screened for sensitivity to PE, DT, PA, and cleavage site mutants of PA and were cloned by limiting dilution.

**Cytotoxicity assays.** PA proteins were assayed for functional activity on wild-type or mutant CHO cells with 50 ng of FP50 per ml. Cells at 10<sup>5</sup>/ml were transferred to 96-well plates (Becton Dickinson and Co., Lincoln Park, N.J.) 1 day prior to assay. FP50 with or without inhibitors was incubated with the cells for 4 h at 37°C, or FP50 was added to the cells just prior to the addition of PA. After the addition of PA, the assay mixture was incubated for 36 h at 37°C. Viability was determined using 0.5 mg of MTT per ml as described previously (27). The A<sub>540</sub> of the MTT product was measured using a microplate reader (Molecular Devices, Palo Alto, Calif.) and expressed as the percentage of absorbance in the absence of toxin. All data shown are representative of three or more assays.

**Partial purification of soluble furin.** Furin-deficient CHO cells were transfected with an expression plasmid, pMT3 PACESOL, which contains the gene encoding soluble human furin, and pED4NEO, a plasmid containing the gene encoding resistance to neomycin (29). Both were a kind gift from A. Rehemtulla (Howard Hughes Medical Institute, The University of Michigan Medical Center). Transfections were performed using Lipofectamine according to the manufacturer's instructions. Culture supernatant was harvested from growing cells, filtered through a 0.22- $\mu$ m-pore-size filter and adsorbed on Blue Sepharose CL-6B (Pharmacia). The eluted preparation was used as a source of crude, soluble furin. Digestion of 1  $\mu$ g of purified PA proteins was performed in a buffer containing 50 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM MgCl<sub>2</sub>, and 3 mM CaCl<sub>2</sub> for 4 or 16 h at 25°C. The digestion products were separated on 4 to 20% Tris-glycine gels and visualized by Coomassie blue staining or immunoblotting using polyclonal PA antiserum. In digestions performed in the presence of protease inhibitors, the inhibitor was incubated with the enzyme for 30 min at 25°C prior to the addition of PA.

**Transfection of furin-deficient cells with the furin gene.** FD11 cells were transfected with an expression plasmid, pMT3 PACE, which contains the gene encoding native human furin on an *Eco*RI-*Sal*I fragment, and pED4NEO, using Lipofectamine as described above. Transfected cells were selected and main-

TABLE 1. Sequences of cleavage sites in native and mutant toxins<sup>a</sup>

Toxin	Cleavage site
PE	RHRQPR
DT	RVRR
PA	SRKKR-167-STSS
Mutated PA	RAAR
	VFTKRSSL <sup>b</sup>
	STRR
	SSSR
	RRSS

<sup>a</sup> The cleavage site in PA encompasses residues 164 to 167 of the protein. All cleavage site mutants have replacements only in residues 164 to 167 with the exception of PA FTKR.

<sup>b</sup> PA FTKR was designed to be a substrate for PC1, and its sequence was derived from mouse prorenin. The residues replaced include 163 to 170.

tained in  $\alpha$ -MEM supplemented with 5% Fetal Clone II (HyClone) and 400  $\mu$ g of G418 per ml and were screened for sensitivity to PE and PA RAAR. Cytotoxicity assays were performed in serum-free medium as described above.

## RESULTS

**Effects of cleavage site sequence on toxin potency.** To investigate the sensitivities of wild-type CHO cells to native and mutant toxins, cytotoxicity assays were performed using toxins with varied cleavage sequences. The sequences at which proteolytic activation takes place in PE, DT, PA, and cleavage site mutants of PA are shown in Table 1. Wild-type CHO cells are extremely sensitive to anthrax toxin (PA plus 50 ng of FP50 per ml), PE, and DT (Fig. 1A). There is a range of sensitivities of CHO cells to native or cleavage site mutants of PA (Fig. 1B). Potencies (concentrations causing 50% cell death [EC<sub>50</sub>]) for each toxin are shown in Table 2. Native PA, which contains the cleavage site RKKR (EC<sub>50</sub> = 12 ng/ml) is the most potent, followed closely by the mutant of PA whose cleavage site has been changed to a minimal furin recognition sequence, RAAR (EC<sub>50</sub> = 18 ng/ml). These are followed, in order of decreasing potency, by PA mutants with dibasic sequences at the cleavage sites FTKR (EC<sub>50</sub> = 24 ng/ml) and STRR (EC<sub>50</sub> = 49 ng/ml). The cells were insensitive to a PA cleavage site mutant containing a single basic residue, SSSR (EC<sub>50</sub> > 1  $\mu$ g/ml), and to RRSS (EC<sub>50</sub> > 1  $\mu$ g/ml), which has Arg residues at the -3 and -4 positions. Thus, the order of potency of the anthrax toxin cleavage sequences is RKKR  $\geq$  RAAR > FTKR > STRR. Using the prorenin model system, Hatsuzawa et al. (13) showed that purified furin will not recognize dibasic sequences. However, Creemers et al. (8) demonstrated that furin could cleave dibasic sequences in von Willebrand factor (VWF). In contrast to results obtained using toxins, both of these groups used cells transfected with their chosen substrate and furin.

**Cleavage of PA and PA cleavage site mutants by partially purified, soluble furin.** In order to determine whether furin is responsible for activating PA mutants containing dibasic cleavage sequences, it was necessary to perform in vitro digestions of PA with furin. Purified PA and PA cleavage site mutants were incubated with soluble furin at ambient temperature, after which the digestion products were separated by gel electrophoresis. At 37°C, the soluble furin preparation lost activity more rapidly than at 25°C. The 63- and 20-kDa fragments of PA were identified on immunoblots (Fig. 2). Furin cleaved native PA (lane 2) in less than 1 h (data not shown) but required at least 16 h to cleave mutant PA with RAAR at its cleavage site (lane 4). Neither PA mutants containing dibasic cleavage sequences (lanes 6, 8, and 12) nor PA containing Arg at only the -1 position (lane 10) was cleaved by soluble furin. Cleavage of PA was inhibited by 10 mM ZnCl<sub>2</sub> (Fig. 3, lane 3),

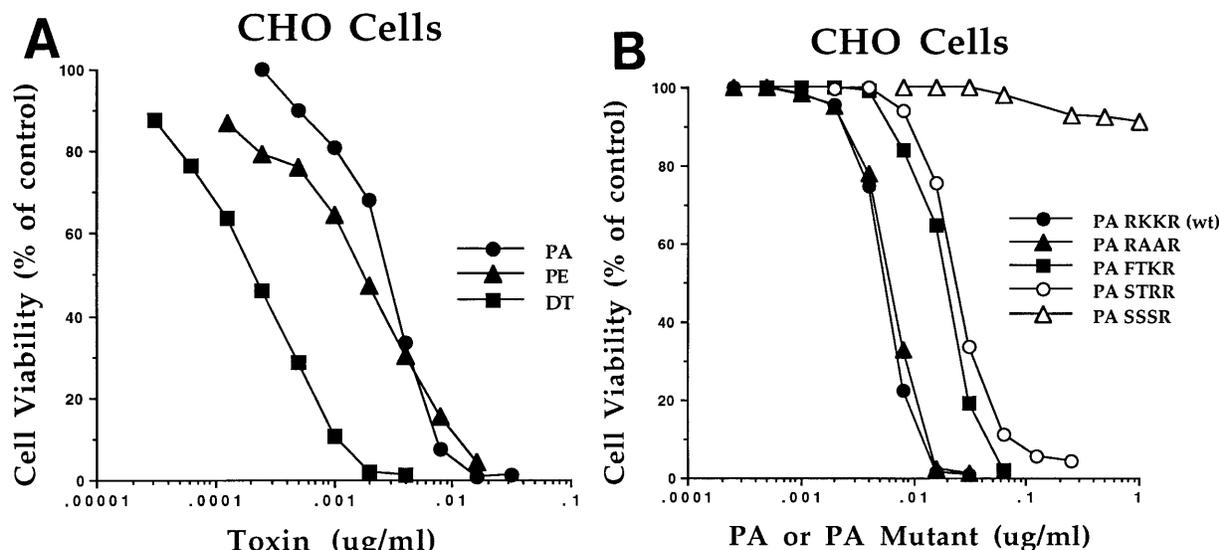


FIG. 1. Sensitivity of CHO cells to native and mutant toxins. CHO cells were incubated with toxins for 36 h as described in the text. PA or PA mutants were combined with 50 ng of FP50 per ml in all assays. WT, wild type.

100  $\mu$ M *p*-chloromercuribenzoic acid (Fig. 3, lane 4), and 10 mM dithiothreitol (Fig. 3, lane 6), but decreased only 44% after treatment with 10 mM leupeptin (Fig. 3, lane 5).

**Isolation of protease-deficient CHO cells.** To identify the eukaryotic proteases involved in bacterial toxin activation, it was desirable to obtain protease-deficient cell lines. Using EMS mutagenesis and selection with wild-type or mutant toxins as described in Materials and Methods, we obtained several types of mutant cells. A number of the lines were classified as elongation factor 2 mutants because they were cross-resistant to PE, DT, and PA in combination with FP50. Some mutants were classified as PA receptor mutants because they were resistant to PA that had been preincubated with trypsin. Several cell lines have been identified as PE receptor mutants because they cannot bind PE (8a). The remaining classes appear to be protease deficient. We chose to concentrate our efforts on cell lines designated FD1 (selected for resistance to PE) and FD11 cells (selected with a PA mutant containing FTKR at the cleavage sites). These cells were determined to be furin defi-

cient because in cell fusion experiments they failed to complement the defect in RPE.40, which is known to lack furin activity (20, 21a).

**Characterization of furin-deficient cells.** Cytotoxicity assays were used to characterize the furin-deficient cells. A complete characterization of the protease-deficient cells will be published separately (unpublished results). Because similar results

TABLE 2. Potencies of native and mutated toxins in CHO cells, FD11 cells, and FD11 cells transfected with furin

Toxin (cleavage site)	Sensitivities of CHO and CHO cell mutants to toxins ( $EC_{50}$ , ng/ml) <sup>a</sup>		
	CHO	FD11	FD11 + furin
PE (RQPR)	1.2 $\pm$ 0.05	>1,000	15 $\pm$ 0.71
DT (RVRR)	0.47 $\pm$ 0.28	2.9 $\pm$ 1.0	0.99 $\pm$ 0.01
PA (RKKR)(wt) <sup>b,c</sup>	12 $\pm$ 5.7	23 $\pm$ 9.0	16.0 $\pm$ 1.4
PA (RAAR) <sup>c</sup>	18 $\pm$ 7.7	>1,000	7.6 $\pm$ 3.2
PA (FTKR) <sup>c</sup>	24 $\pm$ 3.7	>1,000	16 $\pm$ 2.8
PA (STRR) <sup>c</sup>	49 $\pm$ 14	>1,000	14 $\pm$ 3.7
PA (SSSR) <sup>c</sup>	>1,000	>1,000	335 $\pm$ 21
PA (RRSS) <sup>c</sup>	>1,000	>1,000	125 $\pm$ 34

<sup>a</sup>  $EC_{50}$  is defined as the concentration of toxin that decreases MTT color by 50% from control color. The results represent the means of three or more experiments.

<sup>b</sup> Wild-type (wt) PA contains the sequence RKKR-167 at its cleavage site.

<sup>c</sup> PA and PA cleavage site mutants were combined with 50 ng of FP50 per ml in intoxication assays.

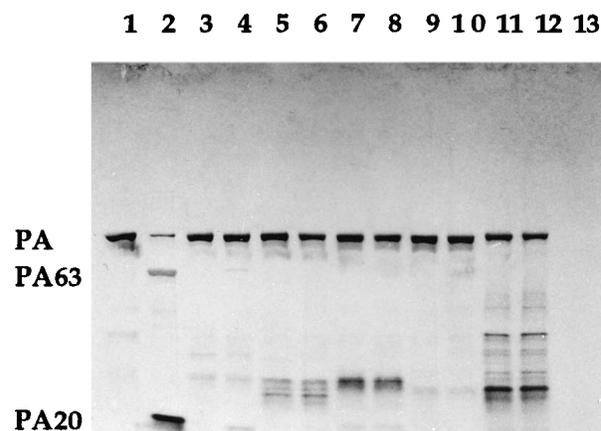


FIG. 2. Western blot of PA proteins digested with soluble furin. Native PA or cleavage site mutants of PA (0.5  $\mu$ g) were incubated with partially purified soluble furin as described in the text for 16 h at ambient temperature. The reaction was terminated by the addition of sodium dodecyl sulfate sample buffer and subsequent boiling for 3 min. The proteins were separated on 4 to 20% gradient gels and blotted with anti-PA polyclonal rabbit serum. Lane 1, PA (RKKR); lane 2, PA digested with furin; lane 3, PA RAAR; lane 4, PA RAAR digested with furin; lane 5, PA FTKR; lane 6, PA FTKR digested with furin; lane 7, PA STRR; lane 8, PA STRR digested with furin; lane 9, PA SSSR; lane 10, PA SSSR digested with furin; lane 11, PA RRSS; lane 12, PA RRSS digested with furin; lane 13, furin alone.

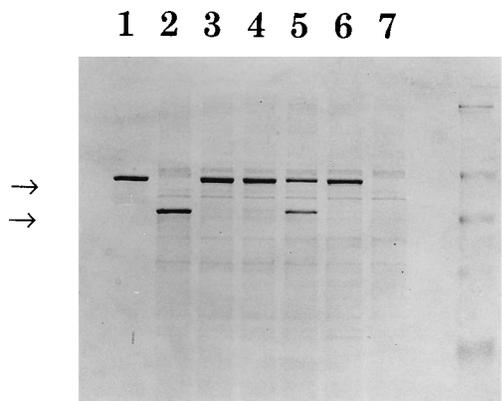


FIG. 3. Cleavage of PA by furin in the presence of protease inhibitors. Partially purified soluble furin (10  $\mu$ l) was incubated with inhibitors at the specified concentrations for 30 min at ambient temperature. PA (0.5  $\mu$ g) was added, and the mixture was incubated for 4 h. The reactions were terminated by the addition of sodium dodecyl sulfate sample buffer and subsequent heating. Digestion products were separated on 10% polyacrylamide gels and visualized by Coomassie blue staining. Bands corresponding to PA and PA63 are designated by arrows. Densitometry of the protein bands was performed on an LKB Ultrascan laser densitometer. Lane 1, PA; lane 2, PA digested with furin; lane 3, PA digested with furin plus 10 mM ZnCl<sub>2</sub>; lane 4, PA digested with furin plus 0.1 mM *p*-chloromercuribenzoic acid; lane 5, PA digested with furin plus 10 mM leupeptin; lane 6, PA digested with furin plus 10 mM dithiothreitol; lane 7, furin alone. Novex SeeBlue molecular mass markers are shown to the right of lane 7: 250, 98, 64, 50, 36, and 30 kDa.

were obtained for FD1 and FD11 cells, only data from experiments using FD11 cells are shown. FD11 cells are completely resistant to PE (Fig. 4A) and PA RAAR (Fig. 4B), as would be expected for furin-deficient mutants. The cells remain sensitive to PA and DT (Fig. 4A and Table 2). FD11 cells are resistant to PA mutants with dibasic cleavage sites (Fig. 4B). Cytotoxicity assays using RPE.40 gave identical toxin sensitivity profiles to those shown for FD11 cells (data not shown).

**Transfection of the furin gene into FD11 cells restores sensitivity to PE and PA cleavage site mutants.** Following transfection of the furin gene, FD11 cells became sensitive to PE and PA cleavage site mutants PA RAAR ( $EC_{50} = 7.6 \pm 3.2$

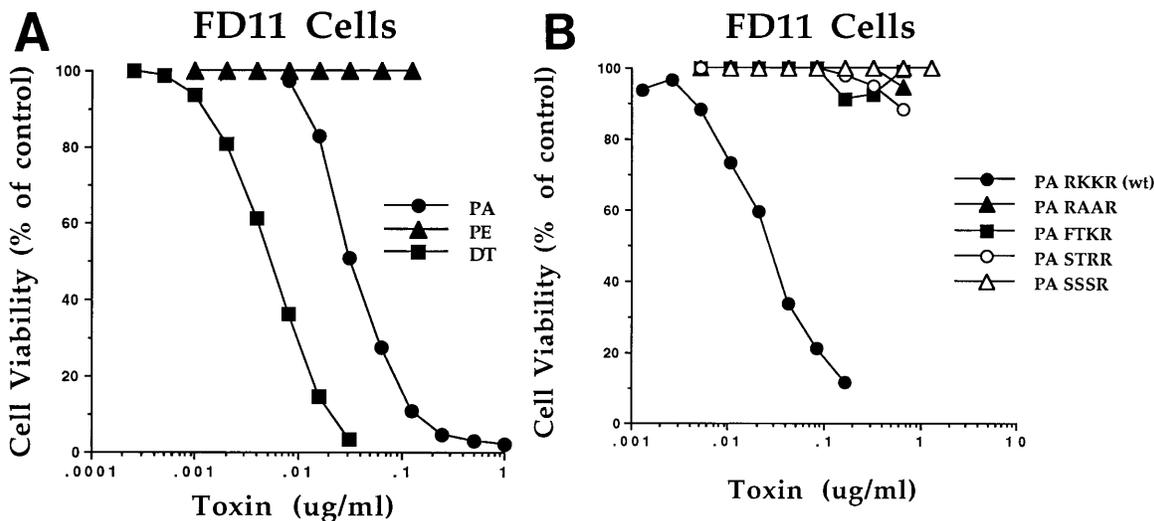


FIG. 4. Sensitivity of FD11 cells to native and mutant toxins. FD11 cells were incubated with toxins for 36 h as described in the text. PA or PA mutants were combined with 50 ng of FP50 per ml in all assays.

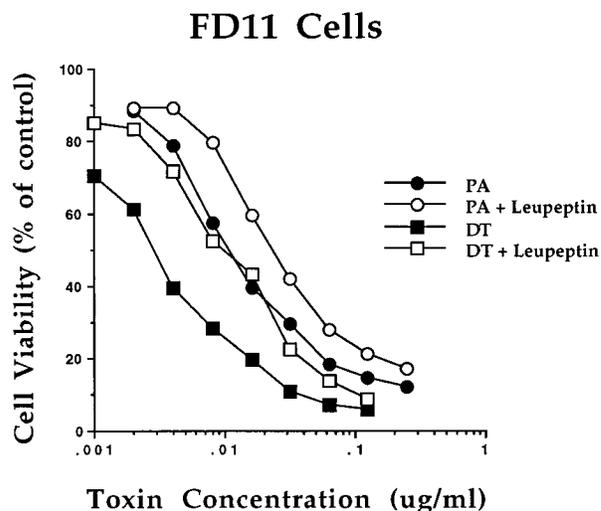


FIG. 5. Leupeptin partially protects FD11 cells from intoxication by PA plus FP50 and DT. FD11 cells were incubated with 10 mM leupeptin as described in the text. PA was combined with 50 ng of FP50 per ml.

ng/ml), PA FTKR ( $EC_{50} = 16 \pm 2.8$  ng/ml), and PA STRR ( $EC_{50} = 14 \pm 3.7$  ng/ml) (Table 2). Interestingly, the cells acquired some sensitivity to PA SSSR ( $EC_{50} = 335 \pm 21$  ng/ml) and PA RRSS ( $EC_{50} = 125 \pm 35$  ng/ml) as well. That the cells now expressed furin was further confirmed by their restored ability to cleave VWF (27a).

**Leupeptin partially protects FD11 cells from intoxication.** In an attempt to identify the proteases other than furin that activate bacterial toxins, FD11 cells were incubated with various protease inhibitors that might prevent toxin cleavage. Leupeptin (10 mM) consistently afforded partial protection from intoxication by PA plus FP50 and DT in FD11 cells (Fig. 5). Pepstatin and aprotinin had no protective effect at concentrations of up to 100  $\mu$ g/ml (data not shown).

**Cathepsin B sensitivity of PA and PA mutants.** Because leupeptin inhibits thiol proteases, we investigated the possibility that thiol proteases might activate PA. The major proteases

found in lysosomes are the cysteine proteases, cathepsins B, H, and L, and the aspartic protease cathepsin D (30). Cathepsin B and L activities of cell extracts from CHO and FD11 cells were compared. Using specific substrates for cathepsins B and L, we found the cells to contain equivalent levels of these enzymatic activities (data not shown).

## DISCUSSION

Many toxins require proteolytic cleavage in order to express full activity. For PE and DT, nicking releases a binding domain from an enzymatic domain, which translocates to the cytoplasm. For PA, nicking exposes a site to which an enzymatically active polypeptide binds. After endocytosis the enzymatic activity is delivered to the interior of the target cell. Each of these toxins becomes nicked at a defined site (Table 1). Site-directed mutagenesis of the amino acids within these sites demonstrated that proteolysis is required for toxicity (14, 16, 37).

Moehring et al. (20) demonstrated that RPE.40, a mutant CHO cell line completely resistant to PE, became sensitive after being transfected with furin cDNA, suggesting that furin is the only cellular protease able to activate PE. Data characterizing the protease that cleaves PE (10) as well as studies of cleavage site mutants of PE (14) are consistent with the claim that furin is the responsible enzyme. However, the cellular receptor for PE, the  $\alpha_2$ -macroglobulin receptor/low-density lipoprotein receptor-related protein (17), also requires processing by furin (38). PE resistance in furin-deficient cells is, therefore, probably also due to receptor dysfunction. The mutant CHO cell line used in this work, FD11, like RPE.40, is resistant to PE and becomes sensitive after transfection of furin cDNA (Table 2). The sensitivity of FD11 cells to PA and DT, however, was reduced only slightly from that of wild-type CHO cells (Table 2), providing strong evidence for the participation of other proteases in the activation of these toxins. These observations may reflect a difference in the accessibilities of the cleavage sites in DT and PA. Performing assays in serum-free medium did not alter the potencies of the various toxins, indicating that the proteases responsible for activating the toxins are supplied by the target cells and not by a component in the culture medium (data not shown). The small effect of furin deficiency on intoxication of cells by DT is in disagreement with data reported by Tsuneoka et al. (34), who found that LoVo cells, a furin-deficient human colon adenocarcinoma line, are resistant to DT. However, in their assays, cells were exposed to toxin for a relatively short period. In 30-h assays described herein, LoVo cells were sensitive to unnicked DT (data not shown).

FD11 cells were also resistant to PA mutants containing dibasic sequences at their cleavage sites. These findings have two possible explanations: (i) either these cells lack not only furin activity but also a second protease that recognizes dibasic sequences, or (ii) receptor-bound PA mutants containing dibasic cleavage sequences are normally activated by furin. The former explanation would be correct if furin processing was required by a cellular component involved in delivering to endosomes one or more proteases that cleave after dibasic sequences. Examples of this class of protease include the prohormone converting enzymes that activate peptide hormones and neuropeptides in neuroendocrine cells. Evidence supporting the latter explanation includes findings by Rehemtulla et al. (28) and Creemers et al. (8), who used cells transfected with furin and wild-type or mutated VWF and showed that furin could cleave after dibasic sequences. However, Hatsuzawa et al. (13) reported that furin cannot cleave mouse prorenin (which is cleaved after the sequence FTKR) unless an Arg

residue was added at the  $-4$  position. There may, therefore, be a conformational dependence of recognition of furin for its substrate, as has been suggested for the prohormone convertases (3). The furin preparation used in these studies cleaved only those substrates that contain Arg in both the  $-1$  and  $-4$  positions. That the preparation was not significantly contaminated with additional proteases was demonstrated by the observation that inhibitors of furin activity, but not leupeptin (Fig. 3, lane 5), protected PA from cleavage. In contrast to the relatively large amounts of cleaved substrate needed for detection of VWF processing, only a few molecules of cleaved toxin are required to kill a cell. Thus, cleavage of dibasic sequences by furin can be inefficient, yet still adequate for toxin activation.

After transfection with the furin gene, furin-deficient cells became somewhat sensitive to substrates containing atypical cleavage sequences (RRSS and SSSR), indicating that overexpression of the furin gene may lead to nonphysiologic cleavages. These findings may explain the results of Creemers et al. (8) and Breslin et al. (4), who reported that furin transfected into target cells cleaved mutated VWF and pro-opiomelanocortin, respectively, at sites that are not predicted to be recognition sequences for furin.

Cleavage of various toxin sequences by furin highlights the importance of secondary protein structure for enzyme recognition. PA, DT, and PE all contain the sequence RXKR at sites other than the site of proteolytic activation, yet these sites are not sensitive to furin. Characteristics such as overall flexibility, surface localization, and secondary structure are decisive factors which determine whether a primary sequence will be cleaved. Substrates for the prohormone convertases have been shown to have a  $\beta$ -bend N terminal to the cleavage site (3). PA, DT, PE, and a number of other furin substrates all contain a predicted protein turn immediately before and immediately after the primary sequence recognized by furin. In addition, each of the sites cleaved by furin is in a region of the proteins predicted to have a significant degree of flexibility (data not shown). The relatively extended site recognized by furin (RXK/RR) may predispose the enzyme to be sensitive to factors in addition to the primary protein sequence. The observation that furin is capable of cleaving a variety of primary sequences, including dibasic sequences, RXRXXR, and R-X-X-R, in some, but not all, proteins which contain an identical sequence of amino acid residues is not surprising.

All of the available evidence is consistent with furin playing an important role in activating PE, DT, and PA. DT and PA, however, have three and four basic residues, respectively, at their cleavage sites, making them substrates for additional proteases. These other proteases have also been suggested to play a role in processes other than intoxication, including cleavage of human immunodeficiency virus type 1 gp160 (26). Continued progress in locating, characterizing, and identifying these activating proteases is critically important to understanding toxin activation and, perhaps, the infectivity of human immunodeficiency virus and will depend upon finding more specific protease inhibitors and inactivating specific protease genes. Greater depth of understanding of how toxins interact with cells may result in new methods for blocking the action of the toxins in bacterial infections and in the design of new and effective fusion toxins for the treatment of human disease.

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