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Clostridium septicum Alpha-Toxin Is Proteolytically Activated by Furin

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Clostridium septicum **alpha-toxin is secreted as an inactive 46,450-Da protoxin. The protoxin is activated by proteolytic cleavage near the C terminus, which eventually causes the release of a 45-amino-acid fragment. Proteoytic activation and loss of the propeptide allow alpha-toxin to oligomerize and form pores on the plasma membrane, which results in colloidal-osmotic lysis. Activation may be accomplished in vitro by cleavage with trypsin at Arg_{367} (J. Ballard, Y. Sokolov, W. L. Yuan, B. L. Kagan, and R. K. Tweten, Mol. Microbiol. 10:627–634, 1993), which is located within the sequence KKRRGKR_{367}S. A conspicuous feature of this site is a recognition site (RGKR) for the eukaryotic protease furin. Pro-alpha-toxin (AT^{pro}) that was digested with trypsin or recombinant soluble furin yielded the 41,327-Da active form (AT^{act}). A mutated alpha-toxin in which the furin consensus site was altered to KKRSGSRS at the cleavage site (AT^{SGSR}) was cleaved and activated by trypsin but not by furin. In cytotoxicity assays, wild-type Chinese hamster ovary (CHO) and furin-deficient CHO (FD11) cells were killed by AT^{pro} but not by AT^{SGSR}. Both cell types were killed by AT^{SGSR} that was preactivated with trypsin. Propidium iodide uptake assays revealed that FD11 cells were approximately 22% less sensitive to AT^{pro} than were CHO cells. AT^{pro}-induced cell lysis of FD11 cells, assessed by propidium iodide uptake, was partially prevented by leupeptin (5 mM) and completely prevented by antipain (2.5 mM). The inhibition by antipain suggested the presence of cysteine or serine proteases that could also activate AT^{pro}. These findings demonstrate that furin is involved in the activation of *C. septicum* alpha-toxin on the cell surface but that alternate eukaryotic proteases can also activate the toxin. Regardless of the activating protease, the furin consensus site appears to be essential for the activation of alpha-toxin on the cell surface.**

Clostridium septicum is a gram-positive, motile, spore-forming anaerobe that is implicated as the cause of traumatic and nontraumatic gas gangrene, necrotizing enterocolitis, and pericarditis (15, 17, 20, 23). Although the organism produces several extracellular factors, it secretes a single lethal extracellular toxin which has been designated alpha-toxin (6). Alpha-toxin is secreted as an inactive protoxin (AT^{pro}) of 46.5 kDa and requires proteolytic activation which yields a 41.3-kDa cytolytically active form (AT^{act}) (4). Ballard et al. demonstrated that activation could be accomplished in vitro with trypsin, which nicks the toxin at R_{367}, near the C terminus (4). Activation leads to oligomerization of the toxin (4) into a prepore complex (21) which then inserts into the membrane to form the pore. The pore has been estimated to be approximately 1.6 nm in diameter (4).

C. septicum alpha-toxin has both mechanistic and sequence similarities to the pore-forming toxin aerolysin produced by *Aeromonas hydrophila* (3, 4). These toxins exhibit about 27% identity and 72% similarity over a region of nearly 400 residues of their primary structures. As with alpha-toxin, aerolysin also requires proteolytic activation (at K_{427}) to form an active, cytolytic complex. Proteolysis of AT^{pro} releases a 5.1-kDa C-terminal peptide; similarly, proteolysis of aerolysin releases a 4.5-kDa C-terminal peptide. Both alpha-toxin and aerolysin contain the amino acid sequence RXXR at the cleavage site. This sequence is a potential recognition sequence for the eukaryotic protease furin and may be an important site for the in vivo activation of these toxins.

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Furin is a ubiquitously expressed, subtilisin-like serine protease (5). Furin, also called PACE, is a member of a growing family of processing enzymes that includes the prohormone convertases (5). Furin functions within the constitutive secretory pathway. The prohormone convertases act to process prohormones and neuropeptides prior to their release from secretory granules; they are often located in discrete populations of cells. Furin plays a role in processing proreceptors and other proproteins. Although the majority of furin is found in the Golgi apparatus, it is also found on the plasma membrane, where it remains anchored by a transmembrane domain (7, 18). Membrane-bound furin has been implicated in the activation of anthrax toxin, protective antigen, and diphtheria toxin on the surfaces of target cells (9, 16, 25).

No study has examined the activation of alpha-toxin on the surfaces of susceptible cells. In this study we have examined the role for cell surface furin in the activation of *C. septicum* alpha-toxin. Using recombinant-derived native pro-alpha-toxin (AT^{pro}), alpha-toxin mutated at the furin recognition sequence (AT^{SGSR}), and cells that are deficient in furin expression, we show that furin can activate the native toxin. However, furin-deficient cells remained partially sensitive to the toxin, demonstrating the presence of alternate proteases that can activate wild-type AT^{pro}. Regardless of the activating protease, it appears that the furin consensus site is essential for activation.

MATERIALS AND METHODS

Materials. Alpha minimum essential medium and HyQ-CCM5 medium (serum-free) were purchased from HyClone (Logan, Utah). Propidium iodide, 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin, and antipain were obtained from Sigma Chemical Co. (St. Louis, Mo.). Leupeptin was obtained from Boehringer (Mannheim, Germany). Fetal bovine serum was purchased from GIBCO-BRL Life Technologies, Inc. (Grand Island, N.Y.).

Construction of alpha-toxin cleavage site mutant. Plasmid pFKO1 was derived from pET22b (Novagen) by the placement of a PCR-generated construct of alpha-toxin which contained the altered furin site into the *Nco*I and *Xho*I restriction sites. pFKO1 contains the gene for alpha-toxin, which has been engineered such that the alpha-toxin signal peptide was replaced with the product of the signal peptide coding region of the *pelB* gene (MKYLLPTAAAGLLL LAAQPAMAMVTNL; the underlined sequence is the amino terminus of alpha-toxin) and a hexahistidine tag was added to its carboxy terminus (NNILEHH HHHH; the underlined sequence is the C terminus of native alpha-toxin). The native activation site (DKKRRGKRSVD; underlined residues denote the consensus furin site) was replaced with the sequence DKKRSGSRSVD, which effectively eliminated the furin cleavage site. The furin knockout construct was generated by the overlap mutagenesis technique of Ho et al. (11), using the appropriate primers and the substitution of 2.5 U of *Pfu* polymerase for 5 U of *Taq* polymerase. This change in the thermostable polymerase was necessitated by the high level of misincorporation we experienced using *Taq* polymerase. After cleavage of the PCR product with *Nco*I and *Xho*I, the fragment was cloned into pET22b to generate pFKO1.

Purification of recombinant alpha-toxin. *Escherichia coli* BLR(DES) cells, which contained the gene for the recombinant alpha-toxin on plasmid pBRS10 (encoding alpha-toxin with a wild-type activation site) or the plasmid containing the gene for the furin site derivative of alpha-toxin (pFKO1) were grown, and the respective toxins were purified as described previously by Sellman et al. (21).

Purification of soluble furin. The CHO cell line 90-80 A-1 5.0 was provided by A. Rehemtulla. This cell line is transfected with a plasmid containing genes encoding C-terminally truncated soluble furin and adenosine deaminase. Growth in 11 AU medium containing deoxycoformycin (14) caused amplification of both genes. Cells were then grown in serum-free medium, the supernatant was collected and filtered, and aprotinin was added to 2 μg/ml. Frozen aliquots were stable for more than 1 year.

In vitro digestion of toxins with trypsin or furin. Purified wild-type or mutant AT^{pro} (1 μg) was incubated with 1 ng of trypsin in a buffer containing 25 mM HEPES (pH 7.4), 1 mM $CaCl_2$, and 0.5 mM EDTA for 30 min at 37°C or with partially purified furin in a buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, and 3 mM $CaCl_2$ for 1 h at 25°C. Products from the digestion were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (4 to 20% polyacrylamide) (Novex, San Diego, Calif.) and stained with Coomassie blue.

Cytotoxicity assays with in CHO and FD11 cells. CHO and FD11 cells (9) were maintained in alpha minimal essential medium supplemented with 5% fetal bovine serum and 50 μg of gentamicin per ml (CHO medium) in an atmosphere of 5% CO_2 at 37°C, as described previously (9). Products from trypsin or furin digests were immediately diluted with CHO medium and added to CHO or FD11 cells previously plated at 2×10^5 cells/ml in 96-well microtiter plates. The plates were incubated for 1 h at 37°C. Cytotoxic concentrations of toxins were assessed by the addition of 0.5 mg of MTT per ml as described previously (9). The data shown are representative of three or more assays. The FD11 cells are derivatives of CHO cells that lack only the ability to produce furin.

Hemolysis assay. The hemolytic activity of alpha-toxin was determined as described previously by Ballard et al. (2). Briefly, AT^{pro} (6 μg) was added to 0.5% washed erythrocytes in a total volume of 1 ml of TBS (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) and incubated for 15 min at 37°C. The cells were then washed to remove unbound protoxin (three times with 1.5 ml of TBS each time). The erythrocytes (with bound protoxin) were resuspended in a total volume of 1 ml, which included TBS, 2 mM $CaCl_2$, and trypsin (100 ng) or furin (500 μl). The samples were placed at 37°C, and 100-μl samples were removed at 0, 15, 30, 60, 90, and 150 min. After each sample was removed, the intact erythrocytes were pelleted and 100 μl of the supernatant was diluted into 900 μl of TBS. This sample was analyzed for hemoglobin content at 540 nm. Hemoglobin release is directly correlated with cell lysis.

Assay for propidium iodide uptake. CHO or FD11 cells were detached from the surfaces of T-75 flasks by the addition of Hanks balanced salt solution containing 3 mM EDTA. CHO medium was added and the cells were pelleted and then resuspended at a concentration of 10^6 cells/ml in HyQ-CCM5 medium. In experiments using protease inhibitors, the inhibitors were added and the cells were incubated for 0.5 h at 37°C. Propidium iodide, dissolved at 10 ng/ml in HyQ-CCM5, was added to the cells, followed immediately by the addition of 0.5 μg of AT^{pro} per ml. The cells were monitored for fluorescence with a FACScan (Becton Dickinson).

RESULTS

Cleavage of wild-type (AT^{pro}) alpha-toxin or the furin knockout mutant of alpha-toxin (AT^{SGSR}) with trypsin or furin. Products from trypsin or furin digests of AT^{pro} and AT^{SGSR} containing the sequences KKRRGKRS (wild-type cleavage site) and KKRSGSRS (furin site knockout), respectively, were separated on an SDS-polyacrylamide gel (Fig. 1). AT^{pro} and AT^{SGSR} exhibited masses of approximately 46.5 kDa each (Fig. 1, lanes 1 and 6, respectively). Both toxins were

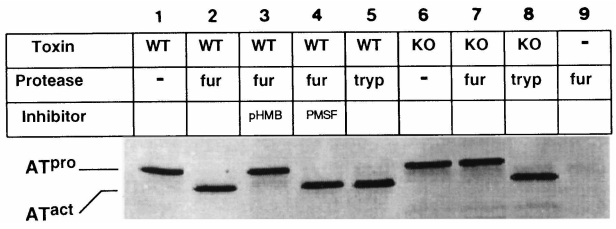

FIG. 1. SDS-polyacrylamide gel (4 to 20% polyacrylamide) of wild-type *C. septicum* AT^{pro} and a cleavage site mutant of AT^{pro} cleaved with trypsin (tryp) or furin (fur). Wild-type (WT) and furin knockout (KO) mutant (AT^{SGSR}) alpha-toxins at 200 μg/ml were digested with 0.5 μg of trypsin per ml or a 1:40 dilution of furin for 60 min at 23°C in a solution containing 75 mM NaCl, 5 mM potassium phosphate, 12 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM $CaCl_2$, 0.5 mM $CaCl_2$, 2 mM 2-mercaptoethanol, and 50 μg of ovalbumin per ml (pH 7.5). Inhibitors added to the indicated reaction mixtures were 4 mM *p*-hydroxymercuribenzoate (pHMB) (this concentration was needed to be in excess over the 2-mercaptoethanol) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF).

substrates for trypsin (Fig. 1, lanes 5 and 8), yielding digestion products of identical size. Only AT^{pro} was cleaved to the 41,327-Da species by furin (Fig. 1, lanes 2 and 7). Cleavage of AT^{pro} by furin was prevented by the inclusion of *p*-hydroxymercuribenzoate (Fig. 1, lane 3), which has been shown to inhibit furin activity (9, 24), but not by phenymethylsulfonyl fluoride (Fig. 1, lane 4), an inhibitor of trypsin-like enzymes.

Cytotoxic activity of AT^{pro} or AT^{SGSR} cleaved with trypsin or furin. Products from trypsin or furin digestion of AT^{pro} or AT^{SGSR} were tested for potency on wild-type CHO cells (Fig. 2A) or the furin-deficient CHO cell line FD11 (Fig. 2B). The concentrations of toxin that killed 50% of the cells (EC_{50}) are shown in Table 1. In both wild-type CHO and FD11 cells, AT^{pro} was cytotoxic in the nanograms-per-milliliter range. The cytolytic activity of AT^{SGSR} was 1000-fold less toxic than wild-type toxin in the absence of trypsin. Cleavage of the native toxin with trypsin or furin did not alter the EC_{50} dramatically from the value seen with undigested alpha-toxin. The change in the EC_{50} for AT^{SGSR} was negligible when the toxin was incubated with furin, but it was decreased 100-fold by cleavage with trypsin.

Activation of erythrocyte-bound alpha-toxin by trypsin and furin. AT^{pro} was incubated with washed erythrocytes followed by addition of trypsin or furin (Fig. 3). Neither AT^{pro} nor trypsin caused hemolysis when added singly. Both trypsin and furin activated the toxin, which resulted in hemolysis. These results indicate that although erythrocytes do not contain sufficient protease activity to activate AT^{pro} within the time frame of these experiments, the cleavage site of cell-bound toxin is accessible to exogenously added proteases. The cleavage site of cell-bound AT^{pro} is accessible to both trypsin and furin.

Uptake of propidium iodide by toxin-treated CHO or FD11 cells. Although both CHO and FD11 cells were relatively resistant to the alpha-toxin cleavage site mutant, both were killed by wild-type AT^{pro} (Fig. 2). These data indicate that cells produce a protease(s) other than furin that can activate AT^{pro}. This protease(s) recognizes a site similar to that recognized by furin, because when the site is mutated to SGSR the cells are much less sensitive. In order to characterize this alternative protease, cells were incubated with various protease inhibitors prior to the addition of the toxin in cytotoxicity assays. When no inhibitor successfully inhibited killing, we looked for a similar type of assay that could be performed on a shorter time scale. In propidium iodide uptake assays, fluorescence could be continuously monitored. FD11 cells were 22% less sensitive to AT^{pro} than were wild-type CHO cells after 20 min (Fig. 4).

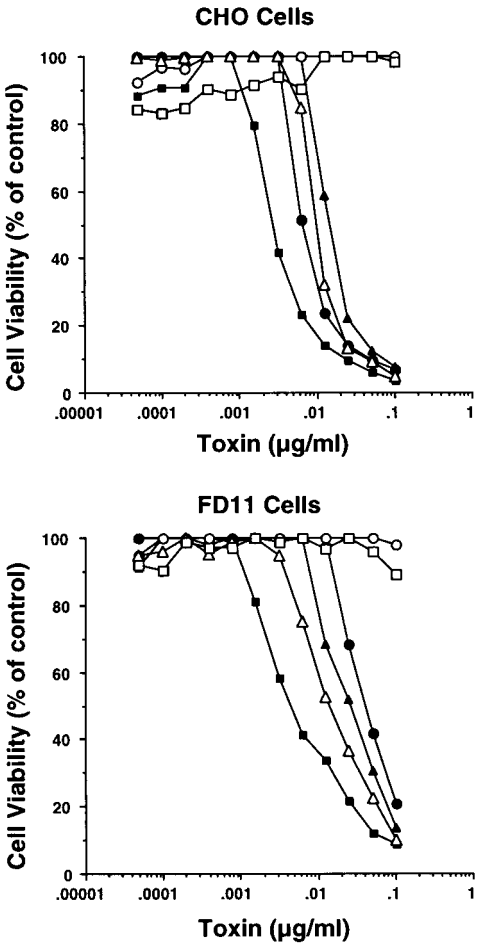

FIG. 2. Cytotoxicity of AT^{pro} or AT^{SGSR} digested with trypsin or furin in CHO or FD11 cells. AT^{pro} or AT^{SGSR} was digested with trypsin or furin as described in the text and then incubated with CHO or FD11 cells for 1 h at 37°C. MTT at 0.5 mg/ml was added, and the cells were incubated at 37°C for an additional hour. The blue formazan crystals were dissolved, and cell viability was determined from reading the A_{540} to A_{650}. AT^{pro}, ●; AT^{pro} plus trypsin, ▲; AT^{pro} plus furin, ■; AT^{SGSR}, ○; AT^{SGSR} plus trypsin, △; AT^{SGSR} plus furin, □.

Leupeptin and antipain inhibited propidium iodide uptake in both CHO and FD11 cells, the inhibition being most dramatic in the FD11 cells (Fig. 5). A number of protease inhibitors, including aprotinin, E64, bestatin, and the salivary leukocyte protease inhibitor were without effect (data not shown). Antipain was more potent than leupeptin, although they both inhibit serine and cysteine proteases. Inhibition by antipain was dose dependent (Fig. 6). At 2.5 mM antipain, propidium iodide uptake caused by AT^{pro} was completely abrogated.

DISCUSSION

C. septicum alpha-toxin belongs to a family of bacterial protein toxins that require proteolytic activation and that have been shown to be substrates for furin (10). Other family members include *Pseudomonas* exotoxin A (PE) (12), diphtheria toxin (DT) (25), Shiga toxin (STx) (8), and anthrax toxin protective antigen (PA) (16). Unlike these toxins, alpha-toxin is not an A-B toxin and therefore its activation does not result in the separation of two distinct functional subunits. Instead, alpha-toxin is converted to an active monomer by proteolytic cleavage near the C terminus. After cleavage, the mature toxin

TABLE 1. Sensitivities of CHO and FD11 cells to trypsin or to the furin-nicked wild-type and furin site mutant *C. septicum* alpha-toxins

Alpha-toxin (cleavage site)[a]	Protease treatment[b]	EC_{50} (ng/ml)[c]	
		CHO cells	FD11 cells
Wild type (RGKR)	None	3 ± 1.5	8.5 ± 3.9
Wild type (RGKR)	Trypsin	4 ± 1.3	30 ± 16
Wild type (RGKR)	Furin	2 ± 0.7	0.9 ± 0.4
Mutant (SGSR)	None	1,830 ± 60	6,700 ± 1,260
Mutant (SGSR)	Trypsin	27 ± 8	37 ± 8
Mutant (SGSR)	Furin	1,770 ± 120	6,500 ± 900

[a] The cleavage site residues were identified as amino acids 395 to 398.
[b] The toxins were incubated with either trypsin for 30 min at 37°C or furin for 60 min at 25°C prior to incubation with cells, as described in Materials and Methods.
[c] EC_{50} refers to the concentration of alpha-toxin that decreased MTT color by 50% from the control color. Each value represents the mean ± standard deviation of three experiments.

can oligomerize into a homo-oligomer on the cell surface to form a prepore complex (21) (AT^{pro} remains as a membrane-bound monomer). This complex is then inserted into the membrane to form a pore (21). In contrast, PE and DT are both cleaved by furin between the domains which confer enzymatic and cell-binding activities. Thus, for PE and DT, cleavage releases the enzymatically active domains, which may then cross cell membranes to reach the cytoplasm. Although both PE and DT are separated into two domains by furin cleavage, the cleavage takes place within distinct cellular compartments, the *trans*-Golgi network and plasma membrane, respectively. STx and the Shiga-like toxins consist of an enzymatically active subunit noncovalently bound to a pentamer of receptor-binding subunits. Furin cleavage releases the polypeptide containing the enzymatic activity from a fragment which remains attached to the cell-bound pentamer. Anthrax toxin is a binary toxin whose components cannot interact prior to the cleavage step. PA, which functions in receptor-binding, is nicked by furin to release a 20-kDa fragment. The remainder of receptor-

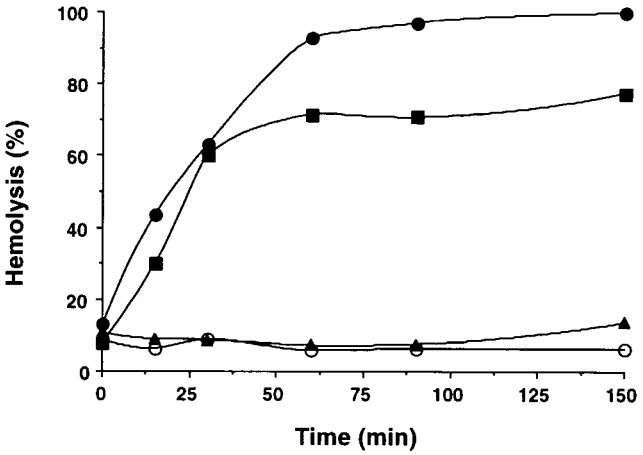

FIG. 3. Proteolytic activation of AT^{pro} bound to erythrocytes. Six micrograms of AT^{pro} was added to 0.5% washed erythrocytes in TBS and incubated for 15 min at 37°C. The cells were washed and then were resuspended in TBS containing 2 mM $CaCl_2$ and trypsin (100 ng) or furin (500 μl). The samples were incubated at 37°C, and aliquots were removed at designated time points. The aliquots were pelleted, and the supernatants were analyzed for hemoglobin content at 540 nm. One hundred percent hemolysis was based on the total release of hemoglobin by the addition of 5 μg of perfringolysin O (21). Trypsin only, ○; AT^{pro}, ▲; AT^{pro} activated with trypsin, ●; AT^{pro} activated with furin, ■.

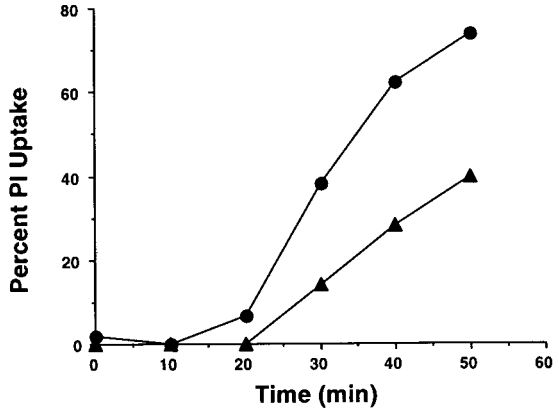

FIG. 4. Propidium iodide (PI) uptake by CHO or FD11 cells incubated with AT^{pro}. CHO (●) or FD11 (▲) cells were incubated with 100 μg of PI per ml and 0.5 μg of alpha-toxin per ml and monitored for increasing fluorescence over time.

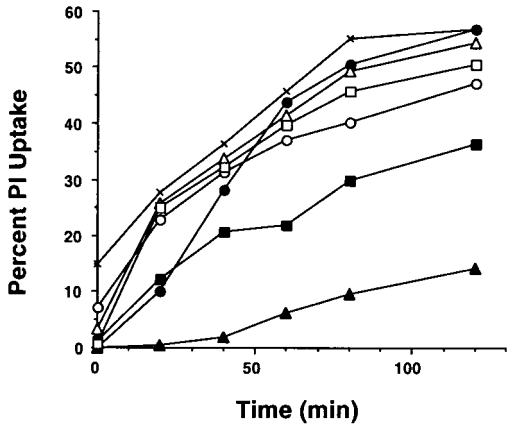

FIG. 6. Dose-dependent effect of antipain on propidium iodide (PI) uptake in FD11 cells treated with alpha-toxin. FD11 cells were incubated with various concentrations of antipain for 30 min at 37°C and then monitored for increasing fluorescence after the addition of PI and alpha-toxin. Results for AT^{pro} (●), AT^{pro} plus 2.5 mM antipain (▲), 1.25 mM antipain (■), AT^{pro} plus 0.625 mM antipain (○), AT^{pro} plus 0.313 mM antipain (△), AT^{pro} plus 0.156 mM antipain (□), and AT^{pro} plus 0.078 mM antipain (×) are indicated.

bound PA can then bind to either LF (lethal factor) or EF (edema factor). The result of the activation of PA is more closely related to that of the activation of alpha-toxin, in that membrane oligomerization of PA and the interaction of PA with LF or EF cannot occur until it is proteolytically activated.

For all but one of the toxins mentioned above, alternate proteases may substitute for furin to cleave them to their active forms. Alpha-toxin activation by proteases other than furin was clearly demonstrated by the nearly complete inhibition of alpha-toxin activity on the furin-deficient FD11 cells by antipain (Fig. 5). Antipain inhibits both serine and cysteine proteases, and therefore one or more of these types of proteases can also activate AT^{pro} in the absence of furin. CHO cells were still able to activate AT^{pro} to about 60% of the maximum rate in the presence of antipain, which demonstrated that furin appears to be the major activating protease for AT^{pro} on CHO cells. For furin activated toxins, the furin recognition site contains the basic sequence RX(K/R)R. Substituting other amino acids within this sequence decreases its ability to become cleaved in vivo. For cases in which the sequence required for furin recognition is altered, furin no longer cleaves the sequence. However, alternate activating proteases cannot cleave toxins when changes are introduced into a dibasic sequence that constitutes only a portion of the furin recognition site (9). Alpha-toxin activation is negatively affected in furin-deficient cells, but activation can still take place at a reasonable rate in the absence of furin in these cells. These results suggest that the alternate proteases may cleave at a site containing dibasic sequences, but the dibasics which are most effectively cleaved are present in the furin consensus site.

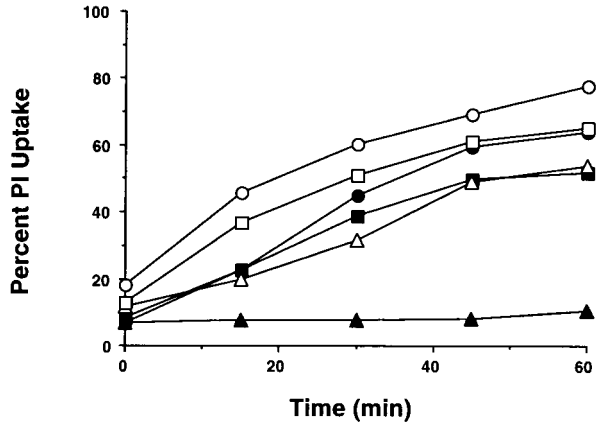

FIG. 5. Propidium iodide (PI) uptake by leupeptin- or antipain-treated CHO or FD11 cells incubated with alpha-toxin. CHO and FD11 cells were incubated with 5 mM leupeptin or 2.5 mM antipain for 30 min at 37°C prior to the addition of 100 μg of PI per ml and alpha-toxin. Fluorescence was monitored over time. Results for CHO cells treated with AT^{pro} (○), AT^{pro} plus antipain (△), and AT^{pro} plus leupeptin (□) and for FD11 cells treated with AT^{pro} (●), AT^{pro} plus antipain (▲), and AT^{pro} plus leupeptin (■) are indicated.

When the furin consensus site was changed from RGKR (AT^{pro}) to SGSR (AT^{SGSR}), alpha-toxin lost several log units of toxicity. Apparently, the furin consensus site is the main target of cellular proteases, whether it is furin or other proteases that target dibasic residues. AT^{SGSR} still retained three juxtaposed upstream basic residues ($\underline{K}_{361}\underline{KR}$SGSR), but these residues apparently did not contribute significantly to the activation of alpha-toxin, at least in the mutant. It is possible that the change in the furin site altered the presentation of the upstream basic residues such that they were not recognized by proteases that recognize dibasic residues. Alternatively, these upstream residues may not normally participate in the activation of alpha-toxin. The fact that trypsin can still activate AT^{SGSR} at a rate similar to that for wild-type toxin probably reflects activation at the downstream Arg_{375}, a residue that we know is recognized by trypsin in the native toxin. However, this residue does not appear to contribute significantly to the in vivo activation of alpha-toxin on CHO cells, since it is also retained in the AT^{SGSR} mutant.

Those toxins for which the crystal structure has been analyzed have been demonstrated to contain an exposed loop of amino acids on which is found the cleavage site (1, 13). Although X-ray crystallography data are not yet available for alpha-toxin, the structure of the related toxin, aerolysin, has been analyzed. The primary sequences of aerolysin and *C. septicum* alpha-toxin share 72% similarity within a region of 387 amino acids (3). Like alpha-toxin, aerolysin requires proteolytic activation at the C terminus, and the sequence at which cleavage takes place is a potential furin recognition site (3). The cleavage site is on a flexible, exposed loop (19), which is hypothesized to occur in alpha-toxin as well. The flexibility of

this loop may be important in the presentation of the cleavage site to cell surface proteases.

We had not previously determined that cellular activation of alpha-toxin took place within the region of alpha-toxin from Asp_{360} to Asp_{370} (DKKRRGKRSVD), although trypsin was able to activate the toxin in vitro at K_{367} (4). The current data clearly show that the sequence RGKR is probably the main site for alpha-toxin activation on the cell. The ability to activate alpha-toxin on the cell surface may be important in vivo. We have found that alpha-toxin that is activated in solution loses a significant amount of its cytolytic activity because alpha-toxin which oligomerizes in solution is cytolytically inactive (4, 22). Oligomerization must take place on the membrane if a pore is to be formed. Therefore, it is reasonable that alpha-toxin has evolved in such a way as to depend on cell surface proteases for activation. This attribute would ensure that alpha-toxin expresses its maximum lethal effect in vivo by preventing premature activation and loss of toxin via solution oligomerization. Therefore, only upon binding to a target cell would the toxin become activated by membrane proteases and oligomerize into functional prepore complex (21, 22), which is inserted to form the transmembrane pore.

Our data also demonstrate that proteases capable of activating AT^{pro} are present on the plasma membrane. Previous experiments designed to characterize eukaryotic proteases that activate bacterial toxins have used toxins that must enter the cytosol in order to manifest toxicity (8, 9). Data obtained from investigating activation of PE, DT, STx, and PA did not clearly distinguish cleavage that took place on the cell surface from cleavage that might have taken place in endosomes, in the Golgi apparatus, or in the cytosol. Since alpha-toxin exerts its effects from the plasma membrane, it is an ideal tool with which to study surface proteases. Our results clearly show that cell surface proteases, including furin, activate alpha-toxin.

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