

# The Pore-forming Toxin Proaerolysin Is Activated by Furin\*

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**Aerolysin is secreted as an inactive dimeric precursor by the bacterium *Aeromonas hydrophila*. Proteolytic cleavage within a mobile loop near the C terminus of the protoxin is required for oligomerization and channel formation. This loop contains the sequence KVRAR<sup>432</sup>, which should be recognized by mammalian proprotein convertases such as furin, PACE4, and PC5/6A. Here we show that these three proteases cleave proaerolysin after Arg-432 *in vitro*, yielding active toxin. We also investigated the potential role of these enzymes in the *in vivo* activation of the protoxin. We found that Chinese hamster ovary cells were able to convert the protoxin to aerolysin in the absence of exogenous proteases and that activation did not require internalization of the toxin. The furin inhibitor  $\alpha_1$ -antitrypsin Portland reduced the rate of proaerolysin activation *in vivo*, and proaerolysin processing was even further reduced in furin-deficient FD11 Chinese hamster ovary cells. The cells were also less sensitive to proaerolysin than wild type cells; however, transient transfection of FD11 cells with the cDNA encoding furin conferred normal sensitivity to the protoxin. Together these findings argue that furin catalyzes the cell-surface activation of proaerolysin *in vivo*.**

Many toxins are secreted by pathogenic organisms as inactive precursors, presumably to protect the producing cells from self-destruction or to increase the efficiency of delivery to the target cells. Activation of toxin precursors often involves proteolytic processing by enzymes produced either by the pathogen itself or by the host organism. The identification of these proteases may be crucial to our understanding of the pathogenesis of the organism.

Aerolysin is a virulence factor secreted by the human pathogen *Aeromonas hydrophila* (Refs. 1–3, for review see Refs. 4 and 5). The protein is released as a soluble dimeric precursor (6, 7) that can bind to specific receptors on target cells (8–12). Proaerolysin must be activated by proteolytic cleavage (13), which releases a C-terminal peptide (14) and leads to a change in secondary structure (15). This enables the next step in chan-

nel formation, which is the generation of a heptameric oligomer (16, 17). Being amphipathic (18), the heptamer can insert into the membrane thereby producing well defined channels (19). In the case of erythrocytes, channel formation leads to cell lysis; however, depending upon the toxin concentration, nucleated cells may undergo a number of changes before death occurs. These include loss of small molecules and ions through the aerolysin channels, vacuolation of the endoplasmic reticulum (12), or even apoptosis.<sup>1</sup>

We have shown that activation of proaerolysin with trypsin is due to cleavage at the carboxyl side of Lys-427<sup>2</sup> (20), which is located in an 18-amino acid surface-exposed flexible loop (21). This loop also contains the sequence K<sup>427</sup>VRRAR<sup>432</sup> which corresponds to one of the motifs recognized by furin-like endoproteases, also called proprotein convertases (PC), suggesting that proaerolysin should also be activated by these enzymes. This family of calcium-dependent serine proteinases is presently composed of seven members in mammalian cells as follows: PC1/PC3, PC2, furin/PACE, PC4, PACE4, PC5/6-A and -B, and PC7/LPC (for review see Ref. 22). These enzymes have been shown to be responsible for the proteolytic excision of biologically active polypeptides from diverse precursor substrates, including prohormones (23) and viral proteins (24–28). In addition furin has been shown to convert the protoxins of *Pseudomonas* exotoxin A, diphtheria toxin, anthrax toxin protective antigen, shiga toxin, and *Clostridium septicum*  $\alpha$ -toxin to their active forms (29–35).

In the present work, we show that proaerolysin can be processed *in vitro* by at least three members of the proprotein convertase family. We show that in CHO<sup>3</sup> cells furin is the major convertase involved in proaerolysin processing. Evidence is shown that activation does not require internalization of the toxin. These observations indicate that the encounter between the protoxin and the convertase can occur at the cell surface and is informative on the cycle of these enzymes that are at steady state mainly localized to intracellular compartments (36–39).

## EXPERIMENTAL PROCEDURES

**Cell Culture and Proaerolysin Purification**—The mutant IdF cell line was obtained from M. Krieger (Massachusetts Institute of Technology, Cambridge, MA). IdF cells were grown and maintained as

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<sup>1</sup> K. L. Nelson, R. A. Brodski, and J. T. Buckley, manuscript in preparation.

<sup>2</sup> Amino acids are numbered starting from the first amino acid of proaerolysin, *i.e.* after removal of the signal peptide.

<sup>3</sup> The abbreviations used are: CHO, Chinese hamster ovary cells; PAGE, polyacrylamide gel electrophoresis; PC, proprotein convertase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; BCRD, before the cysteine-rich domain; BTMD, before the transmembrane domain; PNS, post-nuclear supernatant; r-, rat; h-, human; m-, mouse-.

described (40), using F-12 medium (nutrient mixture Ham's F-12 medium, Sigma) containing 5% fetal calf serum (Sera-Tech, St. Salvator, Switzerland). Wild type and furin-deficient (FD11) mutant CHO cells were grown and maintained in a mixture of F-12 medium and Dulbecco's modified Eagle's medium (1:1) (Sigma) complemented with 10% fetal calf serum. Proaerolysin was purified as described previously (41).

**Production and Purification of Proprotein Convertases (PC) and  $\alpha_1$ -Antitrypsin PDX**—Each proprotein convertase was produced by infection of mammalian BSC40 cells with the appropriate recombinant vaccinia virus, and the proteases were partially purified as described previously (26, 27, 42). The human gene was used for PACE4 and furin, the mouse gene for PC5/6-A, and the rat gene for PC7. Two forms of furin were expressed as follows: BTMD furin corresponding to furin truncated before its transmembrane domain (BTMD, before the transmembrane domain), and BCRD furin corresponding to furin truncated before the cysteine-rich domain (BCRD, before the cysteine-rich domain) (26). BTMD-PC7 corresponds to r-PC7 truncated before its transmembrane domain. The enzymatic activities of the various PCs were monitored using a pentapeptide fluorogenic substrate, pERTKR-MCA (100  $\mu$ M final, Peptides International Inc., Louisville, KY) in 50 mM Tris acetate buffer containing 1 mM CaCl<sub>2</sub>, pH 7.5. After 16 h of incubation with the PC, the fluorescence of free 7-amino-4-methylcoumarin was measured using a Photon Technology International fluorometer (excitation wavelength 370 nm, emission wavelength 470 nm). All PCs were found to be able to cleave the fluorogenic substrate.

Preparative amounts of  $\alpha_1$ -PDX were obtained by expressing a His-Flag-tag variant of  $\alpha_1$ -PDX in the cytosol of bacteria (43).

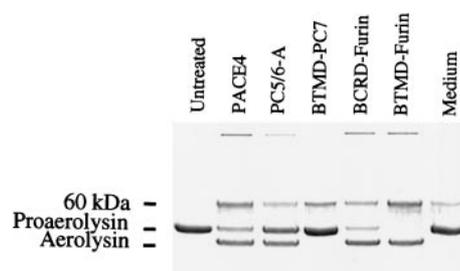
**In Vitro Cleavage by PCs**—Proaerolysin was diluted into a buffer containing 50 mM Tris acetate, 1 mM CaCl<sub>2</sub>, pH 7.4, to a final concentration of 2.8  $\mu$ M and incubated for 60 min at 37 °C with a given volume of partially purified enzyme. The corresponding enzymatic activity on the fluorogenic peptide (see above), expressed in nanomoles release of 7-amino-4-coumarin per 16 h, was 9.6, 1.7, 1.7, and 0.8 for PACE4, PC5/6-A, BCRD furin, and BTMD furin, respectively. Efficiency of cleavage was analyzed by SDS-PAGE followed by Coomassie staining. Similar cleavages were performed in order to test the hemolytic activity as described (44) and determine the exact cleavage site by mass spectrometry as described (20).

**Phase Contrast and Fluorescence Microscopy of Living Cells**—Cells were grown on plastic Petri dishes. After the desired treatment, cells were visualized by phase contrast or by UV fluorescence (to detect the green fluorescent protein) using a Zeiss Axiophot microscope equipped with a cooled CCD camera (Princeton Instruments), controlled by a Power Macintosh. The IPLab Spectrum 3.1 software (Signal Analytics Corp., Vienna, VA) was used for data acquisition.

**Potassium Efflux Measurements**—Confluent CHO monolayers were washed once and incubated in incubation medium (IM) containing Glasgow minimal essential medium (GMEM) buffered with 10 mM HEPES, pH 7.4, 1  $\mu$ g/ml trypsin/chymotrypsin inhibitor, and 0.5% BSA for 30 min at 37 °C, in absence of proaerolysin. Monolayers were then further incubated at 37 °C with proaerolysin in IM for various times. Cells were subsequently washed with ice-cold potassium-free choline medium, pH 7.4, containing 129 mM choline Cl, 0.8 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 5 mM citric acid, 5.6 mM glucose, 10 mM NH<sub>4</sub>Cl, 5 mM H<sub>3</sub>PO<sub>4</sub>, and solubilized with 0.5% Triton X-100 in the same buffer for 20 min at 4 °C. The potassium content of the cell lysates was determined by flame emission photometry using a Philips PYE UNICAM SP9 atomic adsorption spectrophotometer.

**In Vivo Cleavage of Proaerolysin**—Semi-confluent monolayers of CHO cells were washed three times for 5 min with ice-cold PBS containing 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 0.5% BSA (PBS<sup>2+</sup>/BSA) and 1  $\mu$ g/ml trypsin/chymotrypsin inhibitor. Cells were then incubated at 4 °C with either <sup>125</sup>I-proaerolysin or unlabeled proaerolysin in IM. The monolayers were then washed three times for 10 min with PBS<sup>2+</sup>/BSA at 4 °C and incubated for various times at 37 °C. After two washes with PBS, the cells were scraped from the dish, collected by centrifugation at 1500 rpm for 5 min, and gently homogenized in 250 mM sucrose, 3 mM imidazole, pH 7.4, containing the complete mixture of protease inhibitors at the concentration recommended by the manufacturer (Boehringer Mannheim), by passage through a 22-gauge injection needle. A post-nuclear supernatant (PNS) was obtained by centrifugation (2500 rpm) and analyzed for the presence of proaerolysin and aerolysin by SDS-PAGE followed by either radiography or Western blotting. ATP depletion was performed by pretreating cells with 5 mM NaN<sub>3</sub> and 50 mM 2-deoxyglucose in PBS<sup>2+</sup> for 30 min at 37 °C. The efficiency of the ATP depletion was determined by measuring the ATP content of cells using a firefly bioluminescence assay adapted from Kamidate *et al.* (45).

In order to inhibit furin, cells were incubated for 75 min with  $\alpha_1$ -PDX



**FIG. 1. In vitro processing of proaerolysin by proprotein convertases.** Proaerolysin (2.8  $\mu$ M) was incubated with vaccinia virus-produced convertases for 60 min at 37 °C. The digests were performed in 50  $\mu$ l of 50 mM Tris acetate buffer, 1 mM CaCl<sub>2</sub>, pH 7.4. The corresponding enzymatic activity on the fluorogenic peptide (see "Experimental Procedures," expressed in nanomoles release of 7-amino-4-coumarin per 16 h, was 9.6, 1.7, 1.7, and 0.8 for PACE4, PC5/6-A, BCRD furin, and BTMD furin, respectively. The samples were then submitted to SDS-PAGE followed by Coomassie staining. BTMD furin corresponds to h-furin truncated before its transmembrane domain. BCRD furin corresponds to h-furin truncated before the cysteine-rich domain. BTMD-PC7 corresponds to r-PC7 truncate before its transmembrane domain. The protein band migrating at approximately 60 kDa corresponds to an unknown protein present in the culture medium of mammalian BSC40 cells.

(10  $\mu$ M) at 37 °C prior to the proaerolysin treatment. Cells were then processed as described above.

**Transfections**—Transient transfection experiments in CHO FD 11 cells were performed by the CaPO<sub>4</sub>-DNA precipitation procedure described by Graham and van der Eb (46), using a furin expression construct in a plasmid c DNA3 vector. Cells were co-transfected with the DNA of green fluorescent protein (GFP) using GFP expression construct, pBBI25 (Quantum Biotechnologies Inc.).

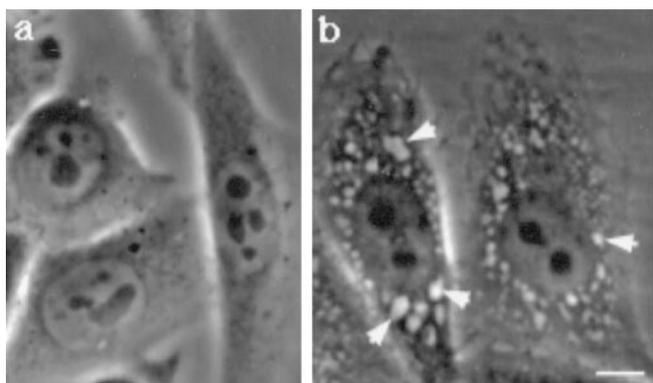
**Other Methods**—SDS-PAGE was performed as described by Laemmli (47). Western blot analysis was carried out using peroxidase-conjugated sheep anti-mouse IgG as a secondary antibody, which was detected by chemiluminescence using Super Signal reagents (Pierce). Protein concentrations of cellular fractions were determined with bicinchoninic acid (BCA, Pierce).

## RESULTS

**Furin and Other Members of the Proprotein Convertase Family Can Process Proaerolysin in Vitro**—Proaerolysin contains the minimal consensus cleavage site (-RXXR-) recognized by furin (30). We first investigated whether furin and other PCs could cleave proaerolysin *in vitro*. As shown in Fig. 1, proaerolysin was processed into a lower molecular weight form by partially purified h-PACE4, m-PC5/6-A, and h-furin. In contrast, the soluble form of r-PC7, although it was able to process the fluorogenic peptide (see "Experimental Procedures"), did not cleave proaerolysin even at 1500 higher convertase to protein ratio and prolonged incubation (21 h) at 37 °C. The approximately 60-kDa band seen in Fig. 1 corresponds to a protein present in the medium used to overexpress the PCs.

The exact cleavage site in proaerolysin for furin, PACE4, and PC5/6-A was deduced from the masses of the aerolysin and the released peptide, which were determined by mass spectrometry. The peptides produced by h-furin, h-PACE4, and m-PC5/6-A were found to have masses of 3835, 3839, and 3838 Da. These are identical within the error of the instrument and indicate a cleavage site before Ser-433 (the calculated mass from this amino acid to the C terminus is 3836 Da). The aerolysins obtained with furin and PACE4 had the expected mass within experimental error (the mass of aerolysin was not determined for PC5/6-A). We could therefore conclude that a single cut had occurred at the carboxyl side of RRAR<sup>432</sup>.

The aerolysins obtained by h-PACE4, m-PC5/6-A, and h-furin treatment were able to oligomerize (Fig. 1) in the same way as aerolysin produced by trypsin. Even though the heptamer is not a covalent complex, it does not disassemble in SDS and therefore can readily be observed by SDS-PAGE. Finally



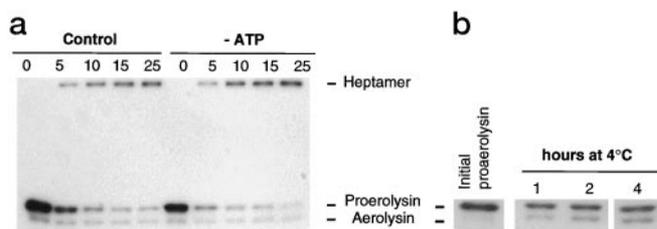
**FIG. 2. Proaerolysin leads to vacuolation of CHO cells.** CHO cells incubated with (b) or without (a) 0.38 nM proaerolysin for 30 min at 37 °C and visualized by phase contrast microscopy. Large translucent vacuoles can be seen in the cytoplasm of proaerolysin-treated cells, some of which have been indicated with *white arrowheads*. Bar, 10.5  $\mu$ m.

aerolysin obtained by cleavage with the various PCs had the same hemolytic activity, measured as described previously (44), as trypsin-treated aerolysin.

**CHO Cells Are Sensitive to Proaerolysin**—Treatment of CHO cells with 0.38 nM proaerolysin led to cell death within a few hours. After 2 h at 37 °C, 20%, and after 3 h, 80% of the cells were no longer able to exclude the DNA intercalating dye, ethidium dimer. The high sensitivity of these cells to the toxin is partly due to the presence of glycosylphosphatidylinositol-anchored receptors at the cell surface that remain to be identified.<sup>4,5</sup>

Within 30 min of exposure to proaerolysin (0.38 nM) and well before cell death, large vacuoles could be observed in the cell cytoplasm (Fig. 2b). Similar vacuolation was previously observed upon treatment of baby hamster kidney cells (12). As shown for baby hamster kidney cells (12), the vacuoles originated from the endoplasmic reticulum since their membranes contained calnexin, a transmembrane lectin present in the endoplasmic reticulum (not shown) (48, 49). The appearance of cytoplasmic vacuoles was used as a simple visual assay for toxin activity.

It has previously been shown that proaerolysin, in contrast to aerolysin, is essentially inactive against erythrocytes (13). The observation that CHO cells are highly sensitive to the protoxin suggested that they express a protease(s) able to convert the protoxin to the active form that is not present or not capable of activating the protoxin in erythrocytes. Activation by the nucleated cells was shown directly by exposing them to proaerolysin at 4 °C and then shifting to 37 °C for various times. As shown in Fig. 3a, upon incubation at 37 °C, proaerolysin was gradually processed into aerolysin. When similar experiments were performed on human erythrocytes, no processing could be detected even after 45 min (not shown). In Fig. 3a, a high molecular weight complex corresponding to the aerolysin heptamer was also formed, which did not form at 4 °C. We could exclude that cleavage was due to contaminating trypsin (e.g. due to low contamination remaining after passaging the cells) since the incubation medium always contained 1  $\mu$ g/ml trypsin/chymotrypsin inhibitor. It is interesting to note that the aerolysin form does not accumulate on cells as function of time (Fig. 3a), in contrast to what is observed *in vitro* (Fig. 1), indicating that oligomerization occurs more rapidly at the cell surface than in solution.



**FIG. 3. Proaerolysin can be processed by host cell proteases at the plasma membrane.** a, CHO cells were depleted or not of their intracellular ATP as described under "Experimental Procedures." Cells were then incubated with 0.38 nM <sup>125</sup>I-proaerolysin for 1 h at 4 °C, thoroughly washed, and incubated with a toxin-free medium at 37 °C. After defined times, cells were homogenized, PNSs were prepared and analyzed by SDS-PAGE (10% gel), followed by autoradiography. Approximately 40% of the <sup>125</sup>I-proaerolysin bound at 4 °C was released into the medium upon 5 min incubation at 37 °C. A further release of 15% was observed after 25 min. b, cells were incubated with 0.38 nM <sup>125</sup>I-proaerolysin for 1 h at 4 °C, thoroughly washed, and incubated with a toxin-free medium again at 4 °C and processed as in a. 25  $\mu$ g of protein were loaded per lane.

As can be seen in Fig. 3, a and b, processing was already apparent at 4 °C suggesting that internalization of the toxin is not required, as membrane transport, including endocytosis, does not occur at this temperature. This conclusion was further supported by the observation that ATP depletion of cells (50) prior to toxin addition did not reduce the conversion of proaerolysin to aerolysin (Fig. 3a). The intracellular ATP concentration was lowered by 98% in these experiments as determined using a firefly bioluminescence assay (not shown).

In order to investigate whether cleavage of proaerolysin into aerolysin is a limiting step in the intoxication process, the kinetics of release of cellular potassium induced by aerolysin, obtained by *in vitro* trypsin cleavage, were compared with those induced by proaerolysin. As shown in Fig. 4, proaerolysin led to channel formation in the plasma membrane of CHO cells thereby leading to a rapid decrease in intracellular potassium. Pre-activation of the toxin with trypsin led to a dramatic increase in the rate of potassium efflux. The half-time was approximately 2.5 min for trypsin-activated aerolysin in contrast to 7.5 min in the absence of pre-activation. The same increase in the rate of potassium efflux was observed when proaerolysin was pre-activated by partially purified furin (not shown).

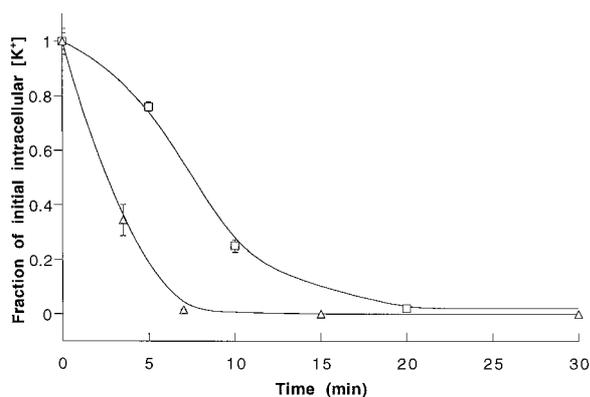
The above experiments suggest that proaerolysin is processed into aerolysin by host cell proteases at the surface of CHO cells and that this cleavage is a limiting step in the channel formation process.

**The Proaerolysin Converting Activity of Id1F CHO Cells Is Inhibited at the Restrictive Temperature**—The experiments shown in Fig. 3 suggest that proaerolysin is processed by host cell proteases. However, in order to rule out the possibility that the processing enzymes came from the culture medium used in these experiments, we have made use of the mutant Id1F CHO cell line (40). These cells have a temperature-sensitive mutation in the gene encoding  $\epsilon$ -COP, one of the components of the COPI coat involved in anterograde and retrograde biosynthetic membrane transport (51). At the restrictive temperature (40 °C),  $\epsilon$ -COP is degraded, and this leads to pleiotropic membrane transport defects in both the biosynthetic and the endocytic pathways (40, 52, 53).

After 45 min exposure to 0.38 nM protoxin, vacuolation of Id1F cells could be observed at the permissive temperature but not at the restrictive temperature (not shown). Vacuolation could, however, be observed independently of the growth temperature upon treatment with trypsin-treated aerolysin (not shown). The differential effect of pro- and mature aerolysin suggested that at 40 °C Id1F cells had a reduced ability to

<sup>4</sup> L. Abrami and F. G. van der Goot, unpublished observations.

<sup>5</sup> V. M. Gordon, K. L. Nelson, J. T. Buckley, V. L. Stevens, R. K. Tweten, and S. H. Leppla, manuscript in preparation.



**FIG. 4. Effect of activation on the toxin-induced potassium efflux from CHO cells.** Cells were incubated with 0.38 nM proaerolysin (□) or trypsin-activated aerolysin (Δ) at 37 °C for various times, and the potassium contents were determined by flame photometry. Experiments were done in triplicate, and the standard deviations were calculated.

process the protoxin. Western blot analysis of cell-associated toxin confirmed that cleavage was impaired in ldlf cells at 40 °C (Fig. 5). These experiments indicate that in ldlf cells grown for 12 h at the restrictive temperature, the number of protease molecules present at the cell surface has significantly decreased. Since biosynthetic membrane transport is impaired in ldlf cells at 40 °C, newly synthesized convertases can no longer reach the plasma membrane (40, 52).

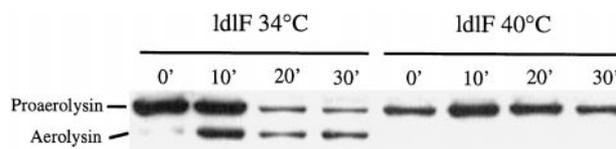
The observation that manipulation of the cells affected the cleavage step rules out the possibility that the proteases are provided by the culture medium under our experimental conditions thereby confirming that the convertases are produced by the target cell.

**Furin Processes Proaerolysin in Vivo**—To investigate whether furin itself could be responsible for proaerolysin processing in CHO cells, we next tested whether  $\alpha_1$ -PDX, a highly selective inhibitor of furin ( $K_i = 600$  pM) (43, 54), would inhibit proaerolysin-induced vacuolation and potassium efflux on CHO cells.  $\alpha_1$ -PDX is a variant of the serpin  $\alpha_1$ -antitrypsin (43, 54) that contains the minimal furin consensus motif in its reactive site loop (-A<sup>355</sup>IPM<sup>358</sup>- was changed to -R<sup>355</sup>IPR<sup>358</sup>-).

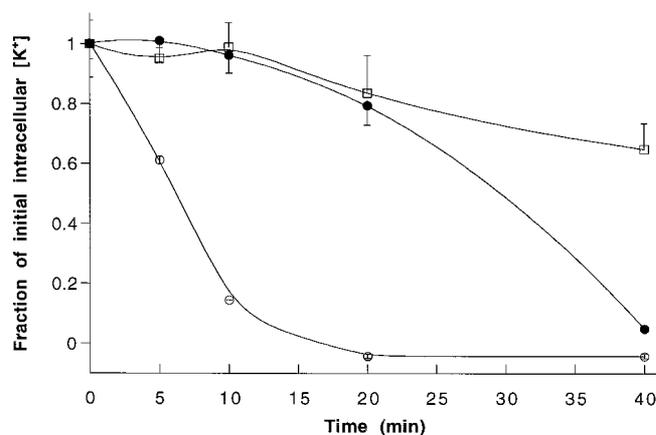
Recombinant  $\alpha_1$ -PDX added to the extracellular medium of the cells significantly inhibited proaerolysin-induced vacuolation (not shown) as well as potassium efflux (Fig. 6). Western blot analysis of CHO-associated toxin confirmed that cleavage was reduced (Fig. 7a). Since  $\alpha_1$ -PDX potently inhibits furin (43), these observations suggest that furin is a major proaerolysin convertase in these cells.

We next studied the effect of proaerolysin on a CHO furin-deficient cell line, FD11 (35, 55, 56). Vacuolation was delayed by more than 90 min when compared with wild type CHO cells (not shown). In contrast, vacuolation upon treatment with trypsin-activated aerolysin was not affected, indicating that only the activation step was impaired in FD11 cells. Potassium efflux induced by 0.38 nM proaerolysin was also dramatically slowed down in FD11 cells when compared with control cells (Fig. 6), whereas efflux induced by aerolysin was not affected (not shown). Fig. 7b shows that precisely proaerolysin cleavage was reduced in FD11 cells.

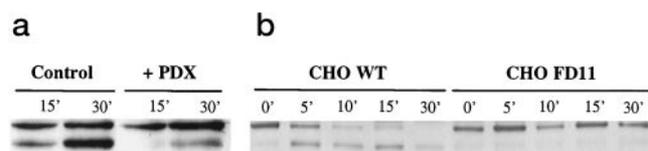
**Furin Restores Proaerolysin Sensitivity in Furin-deficient Cells**—We next tested whether we could restore the proaerolysin sensitivity of FD11 cells to that of wild type cells by transfecting with the cDNA of h-furin. In order to identify the transfected cells, the cDNA of the green fluorescent protein (GFP) was co-transfected. It has been previously observed, by



**FIG. 5. Processing of proaerolysin into aerolysin is inhibited in ldlf cells at the restrictive temperature.** ldlf cells were grown either at the permissive (34 °C) or the restrictive temperature (40 °C) for 12 h. Cells were then incubated with 0.38 nM proaerolysin for 1 h at 4 °C, thoroughly washed, and incubated with a toxin-free medium at 37 °C. After defined times, cells were homogenized, and PNS was prepared and analyzed by SDS-PAGE (10% gel) followed by Western blotting using an anti-proaerolysin antibody (25  $\mu$ g of total protein were loaded per lane). In contrast to Fig. 3, the heptameric form could not be seen in these experiments because this high molecular weight form does not transfer onto the nitrocellulose membrane under our experimental conditions.



**FIG. 6. Furin affects proaerolysin channel forming activity in CHO cells.** Wild type CHO cells were (○) or were not (●) incubated with 10  $\mu$ M  $\alpha_1$ -PDX for 1 h at 37 °C. Proaerolysin (0.38 nM) was then added to the medium. After various times, the potassium content of the cells was determined by flame emission photometry. In parallel FD11 CHO cells (□) were treated with proaerolysin (0.38 nM) at 37 °C, and their potassium content was measured after various times. Experiments were done in triplicate, and the standard deviation was calculated.

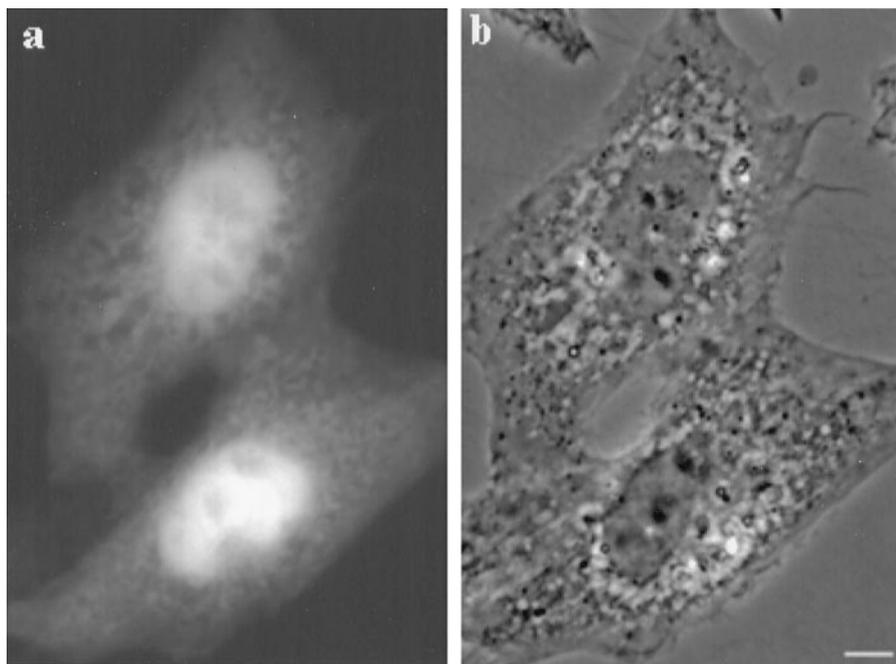


**FIG. 7. Proaerolysin is poorly processed in  $\alpha_1$ -PDX treated or furin-deficient CHO cells.** a, wild type CHO cells were or were not incubated with 10  $\mu$ M  $\alpha_1$ -PDX for 1 h at 37 °C, followed by a 1-h incubation with 0.38 nM proaerolysin at 4 °C and 15 or 30 min at 37 °C (always in the presence of  $\alpha_1$ -PDX). Cells were then homogenized, and PNS was prepared and analyzed by SDS-PAGE (10% gel) followed by Western blotting using an anti-proaerolysin antibody. b, wild type and furin-deficient cells were incubated with 0.38 nM proaerolysin for 1 h at 4 °C, thoroughly washed, and incubated with a toxin-free medium at 37 °C. After defined times, cells were homogenized and analyzed as in a. 25  $\mu$ g of protein were loaded per lane.

us<sup>6</sup> and by others (57), that when cells are transfected simultaneously with two cDNAs, more than 90% of the cells transfected with one of the cDNAs also contain the other. Whereas untransfected FD11 cells did not show any apparent vacuolation within the first 100 min, vacuolation could be seen in furin-transfected cells within 30 min at the proaerolysin concentration used as for wild type CHO cells (Fig. 8). We verified that neither the transfection procedure nor the expression of GFP alone conferred sensitivity to proaerolysin (data not

<sup>6</sup> L. Abrami and F. G. van der Goot, unpublished observations.

**FIG. 8. Transfection of FD11 cells with a cDNA encoding h-furin restores the sensitivity of the cells to proaerolysin.** FD11 CHO cells were co-transfected with the cDNAs encoding for h-furin and GFP as described under "Experimental Procedures." 48 h after transfection cells were treated with 0.38 nM proaerolysin for 50 min. Cells expressing GFP were detected by fluorescence microscopy (a) and analyzed by phase contrast microscopy (b) to assess vacuolation.



shown). The above experiment shows that furin can restore the sensitivity of FD11 cells.

#### DISCUSSION

Proaerolysin absolutely requires proteolytic processing to become active. It has been shown that proaerolysin can be processed by proteases that are produced by *A. hydrophila* itself, by digestive enzymes such as trypsin or chymotrypsin (13, 58), and by members of the PC family (present work).

The present work shows that upon interaction with CHO cells, conversion of proaerolysin into the active toxin occurs primarily via the action of furin. Since we have made similar observations on baby hamster kidney cells<sup>6</sup> and since furin is ubiquitously expressed, it is likely that this observation can be extended to most other cell types. In the absence of furin, however (FD11 cells), cells were not fully protected toward the protoxin suggesting that CHO express other proteases that can process proaerolysin albeit with a far lower efficiency. The other proaerolysin-converting enzymes might also be PCs since we show that several PCs can cleave proaerolysin *in vitro* (Fig. 1). Moreover, we could restore sensitivity of FD11 cells toward proaerolysin to wild type levels, by transfection with the cDNA of PACE4, PC5/6-A, or PC7 (not shown). PACE4 mRNA could not be detected in CHO cells (56) suggesting that these cells do not express PACE4. It is, however, likely that CHO cells express PC7 since this convertase has been shown to be widely expressed (22).

The present work also illustrates that internalization of the protoxin is not required for its processing since endoproteolysis of proaerolysin was observed at 4 °C as well as on ATP-depleted cells (Fig. 3). This observation is not incompatible with the known localization of furin. Indeed although furin localizes to the trans-Golgi network at steady state, it has been shown to cycle with the plasma membrane and endosomes (36, 37, 39). The present data support the hypothesis put forward by Thomas and co-workers (39) that the plasma membrane is a bone fide furin-processing compartment. This hypothesis was based on the observation that furin is tethered to the cell surface by the actin-binding protein ABP-280 that also regulates its internalization. No such mechanism has yet been identified for other proteases. Proaerolysin therefore is an interesting tool to use in the study of the possible cycling of PCs

or other endoproteases with the plasma membrane. Finally, we demonstrate that a specific convertase inhibitor drastically inhibits proaerolysin toxicity. In the future it will be interesting to evaluate convertase inhibitors as antibacterial toxin drugs.

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