A Cellular Metalloproteinase Activates Vibrio cholerae Pro-cytolysin

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Many strains of Vibrio cholerae produce a cytolytin (VCC) that forms oligomeric transmembrane pores in animal cells. The molecule is secreted as a procytolysin (pro-VCC) of 79 kDa that must be cleaved at the N terminus to generate the active 65-kDa toxin. Processing can occur in solution, and previous studies have described the action of mature VCC thus generated. However, little is known about the properties of pro-VCC itself. In this study, it is shown that pro-VCC exist as a monomer in solution and binds as a monomer to eukaryotic cells. Bound pro-VCC can then be activated either by exogenous, extracellular, or by endogenous, cell-bound proteases. In both cases, cleavage generates the 65-kDa VCC that oligomerizes to form transmembrane pores. A wide variety of exogenous proteases can mediate activation. In contrast, the activating cellular protease is selectively inhibited by the hydroxamate inhibitor TAPI, and thus probable candidates are members of the ADAM-metalloprotease family. Furin, MMP-2, MMP-9, and serine proteases were excluded. Cells over-expressing ADAM-17, also known as tumor necrosis factor α converting enzyme, displayed increased activation of VCC, and knockout cells lacking ADAM-17 had a markedly decreased capacity to cleave the protease. The possibility is raised that pro-VCC is targeted to membrane sites that selectively contain or are accessible to cellular ADAM-metalloproteases. Although many microbial toxins are activated by furin, this is the first evidence for processing by a cellular metalloprotease. We identified ADAM-17 as a potent activator of pro-VCC.

Vibrio cholerae El Tor-O1 and non-O1 secrete a membrane-damaging cytotoxic, here designated the V. cholerae cytolytin (VCC), that permeabilizes animal cell membranes (1, 2). The toxin induces lysis of enterocytes and exhibits enterotoxicity in experimental diarrhea models (2, 3). VCC thus contributes to the pathogenesis of gastroenteritis caused by V. cholerae strains, especially with strains not producing cholera toxin (4). Such strains also occasionally cause septicemia (5, 6), and VCC may also be pathogenetically relevant in this setting. Genetic characterization of VCC identified the structural gene hlyA encoding a protein which results, after removal of a signal peptide during secretion, in a protoxin (pro-VCC) of 79 kDa (7, 8). This is the sole form present in supernatants after 8 h of bacterial culture. With time, the protein can undergo N-terminal proteolytic cleavage, and mature 65-kDa VCC is detectable in aged (48 h) culture supernatants (9). Artificial activation of pro-VCC can be achieved in solution by a wide variety of proteases; cleavage then occurs at one of multiple sites between Leu-146 and Asn-158, depending upon the processing agent. In all cases, functionally active 65-kDa toxin is obtained (10). It was shown that the 65-kDa mature VCC binds to lipid membranes in a nonspecific, reversible fashion (11) and assembles into an SDS-resistant oligomer that creates discrete transmembrane pores. First biochemical evidence suggested a pentameric structure (12), but electron microscopic data are in favor of a heptamer (13), as is known to be the case with staphylococcal α-toxin (14, 15) and aerolysin (16). The VCC pore differs from the former pore-forming toxins in being highly anion-specific (17). It is blocked by inhibitors of anion channels and causes formation of vacuoles when it comes to reside in vesicular membranes following endocytosis (18).

Although mature VCC is irreversibly denatured by urea, pro-VCC refolds correctly after the removal of urea, and active VCC is obtained upon trypsinization. This result indicates that the N-terminal pro-peptide acts as an intramolecular chaperone (19). It was also reported that pro-VCC was weakly hemolytic, a finding that was interpreted to indicate that the N-terminal pro-peptide interferes with pore formation (19). As will be detailed in this communication, our data led us to another conclusion.

A theoretical analysis has led to the recognition that VCC shares structural and, probably, functional characteristics with pore-forming toxins of Staphylococcus aureus (α-toxin and leu-kocidin) and with aerolysin (20). VCC is most closely related to staphylococcal cytolsins, and all have a common cytolytic core with a similar overall fold and a similar putative membrane spanning region. In addition, VCC contains a lectin domain that is absent in the other cytolsins.

The properties of pro-VCC have not been described in any detail. Processing may occur in solution, as is also known for aerolysin (21). However, protoxin is the secreted form in both cases, and it is not ad hoc evident that fluid-phase processing should occur in a biological setting. Enteric pathogens generally adhere to target cells, and this intimate contact may shield secreted toxins from the action of soluble host proteases. In septicemia or tissue infections, there would be yet less reason to assume that pro-VCC should mainly be processed prior to contact with target cells. It has remained unclear whether

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25143
Proteolytic Cleavage of V. cholerae Cytolysin

Pro-­VCC itself may bind to cells and be activated thereafter, as has been shown for pro-aerolysin. In the latter case, processing occurs through the action of cell-bound furin (22), the endogenous proteinase that seems to assume a central role in activating many other microbial toxin precursors, including anthrax protective antigen (23, 24), Shiga toxin (25), Pseudomonas exotoxin (26), Clostridium α-toxin (27), and diphtheria toxin (28).

In this study, we report that pro-VCC binds as a monomer to target cells and can subsequently be activated by both exogenous and endogenous proteinases. A proteinase of the ADAM family, rather than furin, was identified as the active principle in the latter case. Processing is followed by oligomerization and transmembrane pore formation.

MATERIALS AND METHODS

Cloning, Recombinant Expression, and Purification of Protein—For expression of the pro-VCC, the hla gene was amplified from V. cholerae El Tor-O1 strain 8731 by PCR and cloned into the expression vector pQE30 (Qiagen) which adds a His tag to the N terminus of proctolyasin. Upon transformation of Escherichia coli M15 with the construct and with plasmid pREP4 to provide an enhanced amount of lac represor, cells were grown at 37 °C in 1 liter of LB broth supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin to an OD600 of 0.6, and protein expression was induced with isopropyl β-D-thiogalactopyranoside (1 mM final concentration) for 3 h. The cells were harvested by centrifugation, resuspended with lysin buffer (20 mM Tris-HCl, 140 mM NaCl, pH 8.0) and disrupted by ultrasonication with a Branson probe sonifier 250 (3 × 1 min, output scale = 30). Inclusion bodies were recovered from the suspension by centrifugation (15 min at 15,000 × g) and washed repeatedly with washing buffer (10 mM Tris, 200 mM NaCl, 2% Triton X-100, pH 8.0). The pellet was finally dissolved in 8 mM urea, 50 mM Tris-HCl, 200 mM NaCl, pH 8.0. The denatured His-tagged fusion protein was purified on Sepharose FF (Amersham Biosciences) preequilibrated with nickel chloride. The column was washed with 3 × 50 ml 2-(N-morpholino)ethanesulfonic acid in 50 mM NaCl, 8 mM urea, pH 6.3, and then eluted with the same buffer adjusted to pH 5.5. For renaturation, the protein (dissolved at 1 mg/ml in buffer containing 8 mM urea) was diluted 50 × in 20 mM HEPES 140 mM NaCl, 50 mM β-­methyl-cyclodextrin, pH 6.8. Samples were processed over a hydroxyapatite column from which the protein was eluted with 70 mM phosphate buffer at pH 6.8. When applicable, proteolytic activation was accomplished by incubation with trypsin (1 μg/ml) at 25 °C for 20 min. Mature non-recombinant VCC was isolated according to published procedures (11).

Native PAGE and Ferguson Plot Analysis—For native PAGE, a continuous buffer system (50 mM Tris, 50 mM glycine, pH 8.9) was used. Polyacrylamide gels were cast with final concentrations of 4, 5, 6, 7, 8, or 9% polyacrylamide, respectively, and loaded with cytolyasin, procytolysin, or procytolysin and with plasmid pREP4 to provide an enhanced amount of procytolysin. Each protein, a primary plot was constructed of the relative electrophoretic mobility versus the polyacrylamide concentration. In a Ferguson plot, the slopes of the primary plots were related to the molecular masses of the protein species (29).

Hemolytic Assay—Serial dilutions of proctolyasin (tryptically activated when indicated) were prepared in duplicate with Hank’s balanced salt solution (HBSS). Rabbit erythrocytes were added to each dilution

![Fig. 1. SDS-PAGE of pro-VCC and mature VCC of 65 kDa.](http://www.jbc.org/)

- **Lane 1**: pro-VCC, lane 2: pro-VCC after trypsin proteolysis; lane 3, mature VCC.

lowed by pre-incubation with protease inhibitors for 5 min at 37 °C when indicated. Cells were then incubated with toxin in HBSS for 60 min at 37 °C, pelleted by centrifugation (5 min at 5000 × g), and washed with HBSS. Cells were finally solubilized with 1% SDS and electrophoresed. Western blots were incubated with rabbit polyclonal antiserum raised against mature V. cholerae cytolyasin (2) and developed with a biotinylated secondary antibody and streptavidin-peroxidase, as described previously (11). Protease inhibitors used were N-ethylmaleimide (1 mM), aprotinin (100 μM), pepstatin (20 μM), phenylmethylsulfonyl fluoride (1 mM) (all from Sigma), PefablocSC (3 mM), and protease inhibitors mixture Complete (Roche), furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (50 μM) (30), 50 μM metalloprotease inhibitor TNP-α protease inhibitor (TAPI) (31) (from Calbiochem-Novabiochem), and 50 μM selective gelatinase inhibitor H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys-OH (32) (from Bachem (Heidelberg, Germany). Trypsin, dispase (protease from Bacillus polymyxa), proteinase K, and proteinase type XVIII (from Rhizopus sp.) were obtained from Sigma.

**Cross-linking Experiments—** Pro-VCC or VCC was incubated with either 0.2–10 mM dimethylsulphoxide in 50 mM triethanolamine, pH 8.5, with 0.06–5.0 mM dithiothreitol in 50 mM HBES, pH 7.0, or with 100 μM dexamethasone-sulphate (DSS) in 50 mM HBES, pH 7.5, for 15 min at 37 °C. The reaction was quenched by the addition of Tris-HCl (pH 8.0) to 100 mM, and samples were analyzed by SDS-PAGE.

Analogous cross-linking experiments were performed on cell-bound toxin. 100 μM DSS (final concentration) was added for 15 min to cells carrying bound pro-VCC. Crosslinking was terminated by adding 100 mM (final concentration) Tris-HCl, pH 8.0, and incubation was done at 37 °C for 5 min. Cell pellets were further washed with 5 mM NaH2PO4, pH 7.0, prior to electrophoresis.

**Genetically Engineered Cell Lines—** Wild-type EC-4 and ADAM-17hrf1/hrf2 EC-2 murine fibroblasts in which ADAM-17 (tumor necrosis factor α converting enzyme) was inactivated (33) were kindly provided by Dr. Roy Black. We produced HEK-293 cells that over-express ADAM-17 as follows. First, a hemagglutinin epitope (encoding YPYDVPDYA) was added to the ADAM-17 gene. The stop codon within the murine ADAM-17 cDNA was deleted by PCR-directed mutagenesis and replaced by an Nrul restriction site, which was used to fuse the ADAM-17 cDNA with the hemagglutinin-epitope. The integrity of the nucleotide sequence was confirmed by DNA sequencing, and C-terminal hemagglutinin-tagged ADAM-17 was expressed using the expression vector pcDNA3 (Invitrogen). Stable transfections of HEK-293 cells with either tagged ADAM-17 or the empty vector were performed by using the calcium phosphate precipitation method. Transfected HEK-293 cells were selected using G418 sulfate (1 mg/ml), and expression was confirmed by Western blotting using hemagglutinin antisera (not shown).

**RESULTS**

**Pro-VCC Is Monomeric in Solution—** Fig. 1 depicts an SDS-PAGE of isolated recombinant pro-VCC (Fig. 1, lane 1) and VCC purified from bacterial culture supernatants (Fig. 1, lane 3). Trypsinized pro-VCC and mature VCC had identical hemolytic activity, and ~40 ng/ml invoked 60% hemolysis of a 5 × 106 cells/ml erythrocyte suspension (data not shown).

The relative mobility of mature VCC and pro-VCC in non-denaturing gel electrophoresis was determined. A Ferguson plot (Fig. 2) demonstrated that the proteins migrated as molecules of 65 and 79 kDa, respectively. When either pro-VCC or...
mature VCC was treated with cross-linking reagents such as dimethylsuberimidate, difluorodinitrobenzene, or disuccinimyd-sulberate in solution, no covalently bonded dimers or multimers were evident by SDS-PAGE and Western blotting (data not shown). These data indicated that both pro-VCC and mature VCC are present in monomeric form in solution.

Protoxin Is Cleaved and Activated After Binding to Cell Membranes—Incubation of nucleated cells with pro-VCC resulted in the reduction of cellular ATP levels, and dose-response curves were obtained that matched those obtained with mature VCC (Fig. 3). Pro-VCC and mature VCC were also found to have essentially identical hemolytic activity, whereby the rate of hemolysis provoked by pro-VCC was retarded. Thus, hemolysis end points were observed after an 2-h incubation with mature VCC and after 4 h with pro-VCC. A 60% hemolysis end point was provoked by ~40 ng/ml of either toxin.

As reported for mature VCC (34), hemolysis by pro-VCC was completely prevented in the presence of polyethylene glycol at osmotically protective concentrations. This observation indicated that protoxysin could form pores of similar size as those of the mature cytolsin.

The above results led us to suspect that pro-VCC could bind to cells and be activated thereafter, possibly by an endogenous proteinase. To corroborate this contention, rabbit erythrocyte ghosts were incubated with pro-VCC or with mature VCC for 60 min at 37 °C, washed, and examined by SDS-PAGE and Western blotting. The results are shown in Fig. 4A. Pro-VCC migrated as a single band of 79 kDa (Fig. 4A, lane 1). After incubation with erythrocyte ghosts, three membrane-bound toxin entities were detected: oligomer, unprocessed pro-VCC, and mature VCC (Fig. 4A, lane 2, unboiled sample). Boiling in SDS resulted in dissociation of the oligomer with predominant strengthening of the mature VCC-band (Fig. 4A, lane 3). When mature VCC (Fig. 4A, lane 4) was applied to the ghosts, membrane-bound toxin was detected predominantly in oligomeric form (Fig. 4A, lane 5), and boiling resulted in dissociation to yield the monomer (Fig. 4A, lane 6). These results indicated that pro-VCC could indeed bind to cells and be cleaved by an endogenous proteinase, and that mature VCC thus formed would assemble into the oligomeric VCC pore.

The kinetics of spontaneous cleavage of cell-bound pro-VCC was investigated (Fig. 4B). Erythrocytes were incubated for 30 min with pro-VCC at 4 °C, and SDS-PAGE/Western blotting led to the detection of bound, unprocessed pro-VCC (Fig. 4B, lane 1). When the cells were post-incubated at 37 °C, it was found that the toxin assembled into SDS-resistant oligomers over time (Fig. 4B, lanes 3–5), and this was accompanied by hemolysis (not shown). After 2 h at 37 °C, the bulk of the toxin was converted to oligomers that were dissociated predomi-

In another experiment, protoxin was first bound to erythrocytes at 4 °C and then exposed to exogenous proteases for only 30 min. Results are shown for trypsin and dispase. In both cases, oligomers formed that, upon dissociation, again yielded the mature 65-kDa band (Fig. 4C). Hemolysis occurred within minutes after exposure to trypsin (not shown). Similar results were obtained with other tested proteases (proteinase K, type XVIII protease).

Additional experiments were conducted with nucleated cells and similar results were obtained. We concluded that pro-VCC can bind both to erythrocytes and intestinal epithelial cells and subsequently can be activated by both endogenous and exogenous proteases.

Inhibition Studies Indicate that a Cell-bound Metalloprotease Cleaves Pro-VCC—To obtain an indication of the nature of the endogenous protease involved in pro-VCC cleavage, specific protease inhibitors were used. In pilot experiments, hemolytic activity of pro-VCC was determined in the presence of various protease inhibitors. Agents that did not inhibit hemolysis were N-ethylmaleimide, an inhibitor of thiol-proteinases; inhibitors of serine-proteinases such as Pefabloc™, aprotinin, and phenylmethylsulfonyl fluoride; pepstatin, which inhibits carboxyl-proteinases; a protease inhibitor mixture (Roche); and gelatinase inhibitor, which inhibits matrix metalloproteinases MMP-2 and MMP-9 (35) but not proteases belonging to the ADAM (A Disintegrin and Metalloprotease) family (36). In contrast, TAPI, which inhibits metalloproteinases (31), including those of the ADAM family, was found to fully suppress hemolysis (not shown).

SDS-PAGE analysis confirmed the specific action of TAPI. As shown in Fig. 5A, phenylmethylsulfonyl fluoride or gelatinase inhibitor did not prevent the formation of oligomers. In contrast, oligomerization was entirely absent in the presence of 50 μM TAPI. The inhibitor had no effect when pre-activated mature VCC was applied (Fig. 5A, lane 6), indicating that suppression of the proteolytic activation step was truly responsible for its protective action. Together, the results led us to conclude that the responsible protease may belong to the ADAM family of metalloproteinases.

Additional experiments were performed using a Chinese hamster ovary cell line, which is known to express furin (22, 26, 27). As shown in Fig. 5B, similar results were obtained. Again, TAPI markedly suppressed cleavage and oligomer formation, whereas the furin inhibitor did not.
ADAM-17 activates pro-VCC—Experiments were conducted with EC-2 cells that lacked ADAM-17 and the respective controls. Fig. 6A depicts the results of Western blots. Cells lacking ADAM-17 bound comparable amounts of pro-toxin at 4 °C (Fig. 6A, lane 2) as control cells (Fig. 6A, lane 3). Samples were washed and subsequently incubated at 37 °C. Oligomerization of VCC (Fig. 6A, compare lanes 4 and 6) and cleavage of pro-VCC (Fig. 6A, compare lanes 5 and 7) was markedly decreased in ADAM-17-deficient cells.

The above findings were corroborated in experiments using transfected HEK-293 cells that over-expressed ADAM-17 (Fig. 6B). Fig. 6B, lane 1 shows the pro-VCC that was applied to the cells at 4 °C, and Fig. 6B, lanes 2 and 3 show further incubation at 37 °C, with oligomerization visible in the unheated sample (Fig. 6B, lane 2) and cleaved toxin visible after heating (Fig. 6B, lane 3). Cleavage and oligomer formation in Fig. 6B, lanes 3 and 2, respectively, was clearly enhanced in the ADAM-17-transfected cells (Fig. 6B, lane 6) compared with control cells (Fig. 6B, lane 5).

Cleavage of Pro-VCC Precedes Oligomerization—Use of TAPI to inhibit processing of pro-VCC rendered it possible to determine which of two possible orders of events underlies pore formation. In the first scenario, pro-VCC would bind as monomer to membranes, undergo proteolytic cleavage, and the mature VCC would oligomerize and form a pore. In the second scenario, cell-bound pro-VCC would first form SDS-labile oligomers, and subsequent proteolytic cleavage would lead to pore formation by SDS-resistant VCC oligomers.

Cells were incubated with pro-VCC in the presence or absence of TAPI, and cross-linking was performed with DSS. In the absence of TAPI, oligomers were detected as expected, and they dissociated after boiling to yield the 65-kDa VCC monomer (Fig. 7, lanes 2 and 3). Cross-linking was found to prevent dissociation of these oligomers (Fig. 7, lanes 4 and 5). In the presence of TAPI, membrane-bound pro-VCC remained monomeric even after exposure to the cross-linker, indicating that pro-VCC oligomers were not formed (Fig. 7, lanes 6-9). Thus, proteolytic cleavage of pro-VCC is apparently required for oligomerization to take place. Fig. 8 depicts events that are proposed to lead to pore formation.
DISCUSSION

Many publications describe the properties and biological activity of mature VCC. In contrast, little is known about pro-VCC, which is the naturally secreted form of the toxin. The present investigation was undertaken to fill this information gap. We employed a recombinant pro-VCC containing a His$_6$ tag at the N-terminal region. This tag has been shown not to interfere with binding and function of other toxins such as staphylococcal leukocidin and clostridial α-toxin (37, 38). Three controls were performed to ascertain that the tag also did not create artifacts in our system. First, binding and hemolytic assays were performed over a pH range of 5.0–8.0 (spanning the pK$_a$ of histidine), with no difference in outcome. Second, recombinant pro-VCC was expressed using a thioredoxin tag instead of His$_6$, and the protein had similar binding and pore-forming properties (not shown). Third, when the His$_6$ tag was added to native 65-kDa VCC, the purified protein entirely lacked binding properties because of irreversible denaturation by guanidine. Thus, the His$_6$ tag itself was devoid of binding properties.

There are striking similarities, but also clear differences, in the mode of action of aerolysin and VCC. Both are secreted as protoxins, both require proteolytic activation, and both build oligomeric transmembrane pores. On the other hand, however, pro-aerolysin exists as a dimer in solution (39, 40), whereas pro-VCC is monomeric. Also, pro-aerolysin binds as a dimer to membranes, whereas cell-bound pro-VCC seems to remain monomeric prior to cleavage. Bound pro-aerolysin is processed by furin, but a metalloproteinase is likely responsible in the case of pro-VCC. In situ activation by furin has been described for other bacterial pore-forming toxins, and the present study is the first to discover a similar role for an endogenous metalloproteinase.

By making use of a cell line in which the metalloproteinase ADAM-17 was overexpressed, we identified this enzyme as an important activator of pro-VCC. In line with this observation, inactivation of ADAM-17 led to decreased processing of pro-VCC. Although several related ADAM proteins are simultaneously expressed in cells, this did not compensate for the absence of ADAM-17. Together, these results directly identify ADAM-17 as a potent activator of pro-VCC.

Why processing of pro-VCC should occur through the action of a cellular metalloproteinase is unclear, especially in view of the fact that no demands on specificity could be discerned when exogenous proteinases were applied to the cell-bound protoxin. We are not aware that this type of experiment has been performed for aerolysin, and future comparison of the results would be of interest. It is tempting to speculate that processing of bound protoxin by an endogenous proteinase may be primarily influenced not by specificity, but instead by the accessibility...
of the substrate to its enzyme. Pro-VCC contains a leucin domain (20) that is absent in aerolysin, and perhaps this domain targets the toxin to membrane sites that contain or are accessible to endogenous metalloproteinases, but not to furin. TAPI-inhibitable metalloproteinases are known to cleave their substrates at sites very close to the surface of the lipid bilayer (41), and we envisage the cleavage site in pro-VCC to become deeply embedded in the extracellular membrane glycocalyx and to be positioned ideally for cleavage by the processing enzyme. Targeting to such sites may simultaneously serve to cluster the toxin molecules, rendering their rapid oligomerization possible after activation.

In sum, pro-VCC seems to be targeted to membranes of mammalian cells, and effective processing can subsequently occur by the action of both exogenous and endogenous proteinases. This would guarantee that the toxin can exert its potent cytotoxic activity in a wide variety of biological settings.

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