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Proteolytic activation of human pancreatitis associated protein is required for peptidoglycan binding and bacterial aggregation

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Abstract

Pancreatitis associated protein (PAP) is a 16 kDa lectin-like protein, which becomes robustly upregulated in the pancreatic juice during acute pancreatitis. Trypsin cleaves the N terminus of PAP, which in turn forms insoluble fibrils. PAP and its paralog the pancreatic stone protein induce bacterial aggregation and, more recently, PAP was shown to bind to the peptidoglycan of Gram positive bacteria and exert a direct bactericidal effect. However, the role of N-terminal processing in the antibacterial function of PAP has remained unclear. In the present study we demonstrate that N-terminal cleavage of PAP by trypsin at the Arg37-Ile38 peptide bond or by elastase at the Ser35-Ala36 peptide bond is a prerequisite for binding to the peptidoglycan of the Gram positive bacterium *Bacillus subtilis*. The tryptic site in PAP was also efficiently cleaved by the neutral metalloprotease nprE secreted from *Bacillus subtilis*. Trypsin-mediated processing of PAP resulted in the formation of the characteristic insoluble PAP species, whereas elastase-processed PAP remained soluble. N-terminally processed PAP induced rapid aggregation of *Bacillus subtilis* without significant bacterial killing. The bacterial aggregating activity of trypsin-processed and elastase-processed PAP was comparable. In contrast to previous reports, the Gram negative *Escherichia coli* bacterium was not aggregated. We conclude that N-terminal processing is necessary for the peptidoglycan binding and bacterial aggregating activity of PAP and that trypsin-processed and elastase-processed forms are functionally equivalent. The observations also extend the complement of proteases capable of PAP processing, which now includes trypsins, pancreatic elastases and bacterial zinc metalloproteases of the thermolysin type.

Keywords

antibacterial protein; innate immunity; lectin-like protein; peptidoglycan binding protein; N-terminal proteolytic processing

Pancreatitis associated protein (PAP) was described by Keim et al. (1984, 1991) as a 16 kDa secretory protein in rat pancreatic juice which appeared upon induction of pancreatic inflammation and represented up to 5% of total protein [1,2]. The human ortholog was isolated in 1992 from the pancreatic juice of diabetic patients who underwent combined kidney and pancreas transplantation [3]. In these patients the donor pancreas developed acute pancreatitis and levels of PAP in the juice reached up to 7.5% of the total secretory protein. Because PAP was hardly detectable in the normal pancreatic juice, but became upregulated in inflammation, it was considered a unique acute phase protein, which was targeted to the exocrine secretions. Subsequent cDNA cloning revealed that PAP exhibited homology to the carbohydrate binding region of Ca²⁺-dependent (C-type) lectins and thus PAP belongs to a larger secretory protein

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family which contain only a single C-type lectin domain [4,5]. Members of this protein family, which include the extensively studied pancreatic stone protein (PSP), have been described in excellent reviews [6-9]. Graf et al. (2001) demonstrated that trypsin cleaved all three isoforms of rat PAP after Arg37 (Arg11 in the mature PAP protein) and the processed PAP became resistant to proteases and formed insoluble, fibrillar structures [10,11]. The authors proposed that insoluble PAP might be the biologically active form.

PAP expression was also detected in the intestines and other extrapancreatic tissues (reviewed in [6-9]), but the physiological function of PAP in the pancreas or elsewhere has remained contentious. Evidence from a large number of studies seemed to indicate that PAP had a variety of activities, which included anti-inflammatory, anti-apoptotic, proliferative, and antibacterial effects [(reviewed in [6-9]). With respect to the antimicrobial activity, PAP and PSP were shown to induce bacterial aggregation without inhibiting growth, and trypsin appeared to stimulate the effect of PSP [4,12]. More recently, Cash et al. (2006a) demonstrated that PAP can bind to the peptidoglycan layer of Gram positive bacteria and exert a direct bactericidal effect [13]. PAP also bound to chitin and the mannose polymer mannan, indicating that PAP recognizes carbohydrate patterns [13,14]. Cash et al. (2006a) proposed that PAP is part of the innate immune system and its intestinal expression plays an important role in the maintenance of the intestinal bacterial flora [13]. By analogy, in the pancreas PAP is probably upregulated during acute pancreatitis to prevent bacterial infection. It remained unclear, however, whether or not the N-terminal processing of PAP by trypsin and the consequent insoluble fibril formation play any role in the antibacterial function. In the present study we set out to answer this question.

MATERIALS AND METHODS

Bacterial strains

Bacillus subtilis subsp. *subtilis* (Ehrenberg) Cohn was obtained from American Type Culture Collection (ATCC #6051); and designated as wild type *Bacillus subtilis* in this study. The protease deficient *Bacillus subtilis* BG2036 (Δ apr-684, Δ nprE522) strain [15] was a generous gift from Evette S. Radisky (Mayo Clinic Cancer Center, Jacksonville, Florida). The *Escherichia coli* K-12 strain α -select [F^- *deoR endA1 recA1 relA1 gyrA96 hsdR17*(r_k^- , m_k^+) *supE44 thi-1 phoA* Δ (*lacZYA argF*)U169 Φ 80*lacZ* Δ M15 λ^-] was purchased from Bioline (Taunton, MA) and the *Escherichia coli* B strain BL21(DE3) [F^- *ompT hsdS_B* (r_B^- m_B^-) *gal dcm* (DE3)] was from Novagen (Madison, WI). All experiments using *Escherichia coli* in the present study included both strains.

Nomenclature

Numbering of amino-acids of human PAP follows the genetic convention; and starts with the initiator methionine (Met1) of the primary translation product. Because the secretory signal-peptide comprising the first 26 amino-acids is removed in the secretory pathway, the first amino-acid of native, mature human PAP is Glu27 (Figure 1).

Plasmid construction and mutagenesis

The cDNA encoding the mature, secreted human PAP (amino-acids Glu27-Asp175) was PCR-amplified from IMAGE clone #5749980 (GenBank accession BC036776) using the PAP NcoI sense primer [5'- AAA TTT TCC ATG GAA CCA CAA AGA GAA CTG CCC TCT-3' (where the NcoI site is underlined)]; and the PAP SacI antisense primer [5'- AAA TTT GAG CTC TAG TCA GTG AAC TTG CAG ACA TAG -3' (where the SacI site is underlined)]. The PCR product was digested with NcoI and SacI and ligated into the pTrapT7_Hu1 plasmid [16,17] in place of the human cationic trypsinogen gene. In the pTrapT7_PAP expression construct the native secretory signal sequence of PAP is deleted and the N-terminal Glu residue of mature

PAP (Glu27) is replaced by a Met residue (see Figure 1). In addition, codons for Pro29-Gln30-Arg31 have been optimized to increase expression levels in *Escherichia coli*, as described by Cash et al. (2006b) [14].

To express PAP with the first 9 amino-acids deleted (Δ 9-PAP, see Figure 1), we fused the N terminus of Δ 9-PAP to the C terminus of a self-splicing mini intein from the cyanobacterium *Synechocystis sp.* Previously, we have used this method successfully for the expression of human cationic trypsinogen with an authentic, native N terminus [18]. The intein fusion construct was generated by overlap extension PCR. First, the ~460 nt DnaB mini-intein gene was amplified from the plasmid pTwin2 (New England Biolabs) using the InteIn NcoI sense primer [5'-CGG GAG TCC ATG GCT ATC TCT GGC GAT AGT CTG ATC AGC-3' (where the NcoI site is underlined)] and the InteIn Δ 9-PAP antisense primer (5'-GAT CCG TGC GTT GTG TAC AAT GAT GTC ATT CGC GAC-3'). Next, the PAP cDNA corresponding to amino-acids Ala36-Asp175 was amplified using the InteIn Δ 9-PAP sense primer (5'-GTA CAC AAC GCA CGG ATC CGC TGT CCC AAA GGC TCC-3') and the PAP SacI antisense primer described above. The underlined sequences in the InteIn Δ 9-PAP sense and antisense primers overlap and thus allow the PCR products obtained in the first round of amplifications to hybridize. PCR products from the first two reactions were gel-purified, mixed, extended and amplified using the InteIn NcoI sense primer and the PAP SacI antisense primer. The resulting PCR product was digested with NcoI and SacI and ligated into the pTrapT7_PAP plasmid.

Expression and refolding of PAP

Human full-length PAP and Δ 9-PAP were expressed in *Escherichia coli* as cytoplasmic inclusion bodies and were subjected to *in vitro* refolding according to the protocol published by Cash et al. (2006b) [14], with minor modifications. *Escherichia coli* BL21 cells were transformed with PAP expression plasmids. Cells were grown in 600 mL Luria-Bertani (LB) medium containing 100 μ g/mL ampicillin at 37 °C until the optical density (OD) of the culture measured at 600 nm reached 0.6-0.9. PAP expression was then induced with 1 mM concentration of isopropyl 1-thio- β -D-galactopyranoside (IPTG) for 3 hours. Cells were harvested by centrifugation at 10,000 rpm for 10 min, and the pellet was re-suspended in 15 mL wash buffer [20 mM Tris-HCl (pH 8.0), 10 mM K-EDTA, 1% Triton X-100, final concentrations]. Cells were divided into 5 mL aliquots and sonicated for 3 $\times$ 1 min using a Heat Systems Ultrasonics cell disruptor, Model W-200R with a microtip probe, at continuous mode on a power-setting of 5. The bacterial cell lysate was centrifuged at 20,000 rpm for 15 min and the pellet containing the inclusion bodies was re-suspended in 35 mL wash buffer and centrifuged again. The washing procedure was repeated two more times. The final inclusion body pellet was re-suspended in 10 mL solubilization buffer [7 M guanidine-HCl, 30 mM DTT, 100 mM Tris-HCl (pH 8.0), 2 mM K-EDTA, final concentrations] and incubated at room temperature with continuous gentle mixing on an Adams Nutator mixer for 2 hours followed by centrifugation at 20,000 rpm for 15 min. The supernatant (~10 mL) was then added to 125 mL refolding buffer [50 mM Tris-HCl (pH 8.0), 10 mM KCl, 240 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 0.5 M guanidine-HCl, 0.4 M sucrose, 1 mM reduced glutathione, 0.1 mM oxidized glutathione and 0.5 M arginine-HCl, final concentrations] pre-equilibrated with argon. The refolding mix was kept under argon at 4 °C for 24 hours, and then dialyzed overnight against 2 changes of 10 liters of 20 mM Na-acetate (pH 5.0) buffer.

Purification of PAP

After dialysis, precipitated proteins were removed by ultracentrifugation (40,000 rpm for 45 min at 4 °C) and re-folded PAP (which typically increased in volume during dialysis to ~200 mL) was loaded onto a MonoS HR 5/5 cation exchange column (Pharmacia) through the pump of an FPLC chromatography system (Pharmacia) at a flow rate of 2 mL/min. The column was then developed with a NaCl gradient of 0-1 M [in 20 mM Na-acetate (pH 5.0)] at a flow rate

of 1 mL/min. Fractions (1 mL) were collected and 15 μ L of each peak fraction was analyzed by 15% SDS-PAGE and Coomassie Blue staining. Pure fractions were pooled, aliquoted and stored at -20 °C until use. Typical yields were 5-10 mg purified PAP per 600 mL initial culture.

Refolding and purification of the intein- Δ 9-PAP fusion followed the same protocol. The self-splicing intein fusion tag is removed during expression, and the liberated Δ 9-PAP can be refolded and purified as described above. Yields were typically lower than those seen with full-length PAP, approximately 2-3 mg protein per 600 mL initial culture.

Expression and purification of human pancreatic protease zymogens

Human trypsinogens and pro-elastase 2A were expressed in *Escherichia coli* as described previously [16,17,19-21]. Pro-elastase 3A and 3B, chymotrypsinogen B1, B2 and C were expressed in transiently transfected human embryonic kidney (HEK) 293T cells as previously reported [22]. Trypsinogens, pro-elastases and chymotrypsinogen C were purified by ecotin affinity chromatography [23], whereas chymotrypsinogen B1 and B2 were purified by Mono S cation exchange chromatography followed by gel filtration on a Superose 6 HR 10/30 column (Pharmacia) [22].

Protein concentrations

Concentrations of the purified protein solutions were calculated from their ultraviolet absorbance at 280 nm, using the following theoretical extinction coefficients (<http://ca.expasy.org/tools/protparam.html>). PAP and Δ 9-PAP, 43,805 M⁻¹ cm⁻¹; cationic trypsinogen, 37,525 M⁻¹ cm⁻¹; anionic trypsinogen, 38,890 M⁻¹ cm⁻¹; mesotrypsinogen, 41,535 M⁻¹ cm⁻¹; pro-elastase 2A, 73,505 M⁻¹ cm⁻¹; pro-elastase 3A, 76,025 M⁻¹ cm⁻¹; proelastase 3B, 74,535 M⁻¹ cm⁻¹, chymotrypsinogen B1 and B2, 47,605 M⁻¹ cm⁻¹ and chymotrypsinogen C, 64,565 M⁻¹ cm⁻¹.

Activation of digestive proenzymes

Chymotrypsinogens and pro-elastases (1-2 μ M concentration) were activated with immobilized bovine trypsin (Pierce) for 60 min at 37 °C. An aliquot (20 μ L) of trypsin beads was pelleted by centrifugation, washed twice with 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and re-suspended in 200 μ L of the same buffer containing the proenzymes. After completion of the activation reaction, the trypsin beads were removed by centrifugation. Trypsinogens (2 μ M concentration) were activated to trypsin with human enteropeptidase (R&D Systems; 28 ng/mL concentration) for 30 min at 37 °C, in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂.

Preparation of peptidoglycan from *Bacillus subtilis*

Cell wall (crude peptidoglycan) was prepared from *Bacillus subtilis* according to Leclerc et al. (1989) [24] with minor modifications. Cells were grown overnight in 3 L of LB medium at 30 °C and harvested by centrifugation at 8,000 rpm for 10 min at 4 °C. Cells were re-suspended in 100 mL 0.1 M Tris-HCl (pH 8.0), kept at 4 °C for 15 min and centrifuged again. Cells were re-suspended in 100 mL 4 % (w/v) SDS solution. The suspension was incubated for 1 h at room temperature with continuous mixing on a rocking platform followed by sonication on ice for 10 min. The extract was then heated to 100 °C for 20 min and centrifuged at 8,000 rpm for 20 min at room temperature. To remove SDS and membranes, the pellet was washed in 100 mL 0.2 % (v/v) Triton X-100 with continuous mixing for 30 min at room temperature, followed by four washes with 80 mL of distilled water. Cell wall was centrifuged at 8,000 rpm for 20 min at 4 °C and freeze-dried. The final yield was approximately 300 mg cell wall powder per 3 L initial culture.

To remove peptidoglycan-associated proteins (including proteases), 2 mg cell wall powder was re-suspended in 1 mL 100 mM Tris-HCl (pH 8.0), 1 mM CaCl₂ and digested with 50 mg/mL proteinase K at 37 °C overnight. Next day 100 mM DTT and 1 % SDS (final concentrations) were added and the suspension was heated to 95 °C for 15 min. DTT and SDS were removed by washing the peptidoglycan preparation 10 times with 2 mL distilled water. Finally, protease-free peptidoglycan was suspended in water at a concentration of 1 mg/mL and stored at 4 °C.

Labeling of bacteria with fluorescein 5(6)-isothiocyanate (FITC)

Bacillus subtilis and *Escherichia coli* were grown to OD (600 nm) of 0.6. One mL of each culture was centrifuged and the bacterial pellets were washed with 50 mM carbonate-bicarbonate buffer (pH 9.0) containing 10 mM NaCl and re-suspended in 1 mL of the same buffer. Fifty µL of FITC [Sigma 46950; dissolved in dimethyl sulfoxide (DMSO) at 10 mg/mL (25.7 mM) concentration] was added (1.2 mM final concentration) and the reaction mixtures were incubated in the dark, at room temperature for 1 h. Labeled cells were centrifuged at 14,000 rpm for 1 min and the bacterial pellets were washed five times with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl to remove residual FITC. Finally, FITC-labeled cells were re-suspended in 1 mL of 100 mM Tris-HCl (pH 8.0), 1 mM CaCl₂.

RESULTS

N-terminal processing of PAP by human trypsins

We have expressed human PAP in *Escherichia coli* according to a published protocol [14] and purified the lectin to homogeneity by cation exchange chromatography. Incubation of PAP with 10 nM concentrations of human cationic or anionic trypsin resulted in the rapid conversion of the PAP band to a lower mobility species on SDS-PAGE (Figure 2A). N-terminal sequencing revealed that trypsin cleaved the Arg37-Ile38 peptide bond and generated an 11 amino-acid shorter PAP form (Δ 11-PAP; see Figure 1), in accordance with published reports [10,11]. In contrast to cationic and anionic trypsin, mesotrypsin did not process PAP to any extent whatsoever. This observation is in agreement with previous results showing that mesotrypsin digests most protein substrates poorly and cannot activate pancreatic zymogens [20].

N-terminal processing of PAP by human pancreatic elastases

Human PAP was first discovered in the pancreatic juice of patients who underwent pancreas transplantation and developed transient acute pancreatitis in the graft pancreas [3]. We have obtained a sample of the pancreatic juice used in these studies (PS19, also see [25] for additional information) and fractionated it by MonoQ anion-exchange chromatography. The fractions were analyzed by SDS-PAGE and Coomassie Blue staining and PAP was tentatively identified as a 17 kDa protein band present in fractions 16-18 (Supplementary Figure S1). This band was transferred from fraction 17 to a PVDF membrane and subjected to Edman degradation, which confirmed the identity of PAP. Surprisingly, however, the N-terminal sequence of PAP present in this fraction lacked 9 amino acids from its N terminus (Δ 9-PAP; see Figure 1), evidently as a result of cleavage at the Ser35-Ala36 peptide bond. To identify the protease responsible for this atypical processing, we digested recombinant PAP with a panel of human pancreatic proteases, which included chymotrypsin B1, chymotrypsin B2, chymotrypsin C, elastase 2A, elastase 3A and elastase 3B. Elastase 2A and 3B processed the N terminus of PAP *in vitro* and protein sequencing confirmed that the site of processing was the Ser35-Ala36 peptide bond, which conforms to the canonical elastase-sensitive peptide bonds (Figure 2B). Chymotrypsin B1, chymotrypsin B2 and elastase 3A did not cleave PAP, whereas chymotrypsin C slowly degraded the lectin (not shown). The results indicate that in the absence of trypsin PAP can be processed N-terminally by an alternative, elastase-dependent mechanism. Although not shown, a commercial preparation of human sputum leukocyte elastase (Elastin Products Company, SE563) degraded PAP without appreciable N-terminal processing.

Addition of trypsin to elastase-processed $\Delta 9$ -PAP converted this species to $\Delta 11$ -PAP, as confirmed by N-terminal sequencing. The trypsin-sensitivity of $\Delta 9$ -PAP posed a technical challenge as the potential existed for unnoticed trypsin contamination in the pancreatic elastase preparations used to generate $\Delta 9$ -PAP. Unwanted tryptic processing of $\Delta 9$ -PAP would not be easily detected, as $\Delta 9$ -PAP and $\Delta 11$ -PAP do not resolve into separate bands on SDS-PAGE. To circumvent this problem, we expressed $\Delta 9$ -PAP in *Escherichia coli* as a fusion protein with a self-splicing mini intein as described in *Materials and Methods*. Upon self-removal of the fusion tag, authentic $\Delta 9$ -PAP could be refolded and purified. In the studies presented below, experiments were performed using both $\Delta 9$ -PAP derived from intein-fusions and $\Delta 9$ -PAP generated through *bona fide* elastase processing, with identical results.

Solubility and protease sensitivity of trypsin-processed $\Delta 11$ -PAP and elastase-processed $\Delta 9$ -PAP

Upon cleavage of the Arg37-Ile38 peptide bond by trypsin, the newly generated $\Delta 11$ -PAP presumably undergoes a conformational change, which results in two well-documented novel properties, (1) formation of insoluble aggregates which can be pelleted by centrifugation [10, 11]; and (2) resistance to proteolytic degradation by pancreatic proteases [10] or by proteinase K [26]. To ascertain whether elastase-processed PAP acquires similar properties, first we compared solubility of $\Delta 11$ -PAP and $\Delta 9$ -PAP. As shown in Figure 3A, intact, unprocessed PAP was soluble, whereas after processing by trypsin, aggregation of $\Delta 11$ -PAP became detectable within 5 min and the greater part of $\Delta 11$ -PAP was precipitated by 30 min at 22 °C. The tendency for aggregation of $\Delta 11$ -PAP was comparable at 22, 30, and 37 °C, whereas aggregation proceeded significantly slower at 4 °C and only minimal precipitate was recovered after 30 min (Figure 3B). In contrast to trypsin-processed PAP, elastase-processed $\Delta 9$ -PAP remained soluble over the same time course and only a small amount of precipitate appeared by 30 min (Figure 3C). As expected, addition of trypsin to elastase processed $\Delta 9$ -PAP resulted in aggregation (not shown).

Next, we digested full-length PAP, $\Delta 11$ -PAP and $\Delta 9$ -PAP with proteinase K. Unprocessed PAP was degraded by the protease over the 60 min time course; whereas trypsin-processed $\Delta 11$ -PAP was completely stable (Figure 4A). Similarly, $\Delta 9$ -PAP showed increased resistance against proteinase K, although some degradation was noticeable by 60 min (Figure 4B). Taken together, these observations suggest that N-terminal processing by elastase generates a PAP isoform that is similar (as judged by increased protease resistance) yet not identical (as shown by lack of aggregation) in conformation to trypsin-processed PAP.

N-terminal proteolytic processing of PAP is essential for peptidoglycan binding

Recently, Cash et al. (2006a) demonstrated that PAP binds to the peptidoglycan of Gram positive bacteria and exerts a direct bactericidal effect [13]. However, the significance of N-terminal processing of PAP in its peptidoglycan binding activity was not addressed. We compared the ability of full-length PAP and its proteolytically processed $\Delta 9$ -PAP and $\Delta 11$ -PAP forms to bind to purified peptidoglycan of the Gram positive bacterium *Bacillus subtilis*. In the binding experiments, peptidoglycan was incubated with PAP at 22 °C for 30 min, the mixture was centrifuged at 4 °C and the distribution of PAP between the pellet (bound PAP) and the supernatant (unbound PAP) was analyzed by SDS-PAGE and Coomassie Blue staining. Figure 5A demonstrates that full-length PAP did not bind to peptidoglycan, whereas both the trypsin processed $\Delta 11$ -PAP and elastase-processed $\Delta 9$ -PAP were fully bound.

Since aggregation and consequent sedimentation of trypsin-processed $\Delta 11$ -PAP at 22 °C might confound the results of the pull-down assay, we measured the time-course of peptidoglycan binding and compared it to the time-course of $\Delta 11$ -PAP precipitation. As shown in Figure 5B, binding of $\Delta 11$ -PAP to peptidoglycan was complete in 1 min at 22 °C, whereas significant

precipitation of $\Delta 11$ -PAP occurred only after 15 min (cf. Figure 3A). Furthermore, experiments performed at 4 °C demonstrated that binding of $\Delta 11$ -PAP to peptidoglycan was complete in 30 min (Figure 5B), while precipitation of $\Delta 11$ -PAP at this temperature was minimal after 30 min incubation (cf. Figure 3B).

N-terminal processing of PAP by the *Bacillus subtilis* nprE protease

The results described above indicate that proteolytic processing of PAP by trypsin or elastase must precede peptidoglycan binding. These observations appear to contradict the results of Cash et al. (2006a), who showed peptidoglycan binding using intact, unprocessed PAP protein. The authors, however, observed that the commercial peptidoglycan preparation they used proteolytically cleaved PAP, which resulted in a mobility shift on SDS-PAGE gels. Therefore, it seems reasonable to assume that secreted bacterial proteases might process PAP in a trypsin-like manner and thus promote peptidoglycan binding. To confirm this notion, we incubated PAP with the culture supernatant from wild-type *Bacillus subtilis* and from strain BG2036, in which the genes for two major secreted proteases are deleted, the so-called extracellular neutral metalloprotease (nprE) and the serine alkaline protease (aprE, subtilisin E) [15]. As shown in Figure 6A, incubation of intact PAP with bacterial supernatant from the wild-type strain resulted in the characteristic mobility shift, whereas no processing was observed by culture supernatants of the protease-deficient BG2036 strain. N-terminal sequencing identified the site of processing as the trypsin-sensitive Arg37-Ile38 peptide bond. Addition of 20 mM EDTA (final concentration) to the bacterial supernatant abolished PAP processing (Figure 6B), suggesting that the bacterial protease responsible for PAP processing is the thermolysin-like neutral zinc metalloprotease (nprE). Consistently with this conclusion, a commercial preparation of thermolysin from *Bacillus thermoproteolyticus* Rokko (R&D Systems 3097-ZN) efficiently processed PAP, whereas subtilisin Carlsberg from *Bacillus licheniformis* (Calbiochem 572909) was ineffective (not shown).

N-terminally processed PAP induces aggregation of *Bacillus subtilis*

To characterize the antibacterial effects of the various PAP forms, we incubated wild-type *Bacillus subtilis* and the BG2036 strain with trypsin-processed $\Delta 11$ -PAP, elastase-processed $\Delta 9$ -PAP or intact, full-length PAP. We labeled *Bacillus subtilis* with fluorescent FITC and monitored the PAP-induced aggregation of bacteria under the microscope. Figure 7 demonstrates that the protease-deficient BG2036 strain was aggregated by both trypsin-processed $\Delta 11$ -PAP and elastase-processed $\Delta 9$ -PAP, but not by unprocessed, full-length PAP. These results indicate that N-terminal processing is necessary for the bacterial aggregating activity of PAP and that trypsin-processed and elastase-processed forms are functionally equivalent. The wild type *Bacillus subtilis* strain was aggregated not only by $\Delta 11$ -PAP and $\Delta 9$ -PAP, but also by treatment with intact, full-length PAP, which was evidently processed by the nprE protease in the incubation medium.

Neither PAP species promoted aggregation of Gram negative *Escherichia coli* strains (not shown). This observation stands in contrast to an earlier report which described PAP-induced aggregation of *Escherichia coli* [4].

PAP exerts no significant bactericidal activity against *Bacillus subtilis*

Cash et al. (2006a) described that PAP has a direct killing effect on Gram positive bacteria, although *Bacillus subtilis* was not studied [13]. To test whether N-terminally processed PAP forms kill *Bacillus subtilis*, first we utilized a dilution-plating assay. The bacteria were incubated with $\Delta 11$ -PAP or $\Delta 9$ -PAP at 22 °C for 60 min and 10-fold serial dilutions were plated on LB-agar plates. After overnight growth at 30 °C, colonies were counted. Surprisingly, the number of colony forming units decreased upon PAP treatment only by ~5-10%; however,

this difference was within experimental error. Trypsin-processed $\Delta 11$ -PAP and elastase-processed $\Delta 9$ -PAP gave essentially identical results (Figure 8A).

To ascertain by an independent method whether PAP-treated bacteria are dead or alive, we stained *Bacillus subtilis* with the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes). This kit contains two DNA-binding fluorescent dyes, the green fluorescent dye SYTO 9, which binds to both dead and live bacteria and the red fluorescent DNA stain propidium iodide, which penetrates only dead bacteria. Importantly, binding of propidium iodide to the DNA of dead bacteria quenches the green fluorescence of SYTO 9. Figure 8B demonstrates fluorescent spectra of wild-type *Bacillus subtilis* treated with trypsin-processed $\Delta 11$ -PAP and then stained with both fluorescent dyes. Remarkably, the green peak of the spectrum exhibited only a 10-20% decrease upon PAP treatment, indicating that the large majority of the bacteria were alive. Identical results were obtained with elastase-processed $\Delta 9$ -PAP (not shown). Inspection of the PAP-treated bacterial suspension under a fluorescent microscope revealed green aggregates of live bacteria containing some red stained dead bacteria (not shown). Therefore, we conclude that N-terminally processed forms of PAP do not kill *Bacillus subtilis* to a significant extent.

DISCUSSION

The biological role of PAP has been in question ever since its discovery in 1984 [1]. Recent studies by Cash et al. (2006a) provided evidence that one of the fundamental functions of human PAP and its mouse ortholog regIII γ is an antibacterial activity [13]. PAP was shown to bind to the peptidoglycan of Gram positive bacteria and exert a direct bactericidal effect. *Escherichia coli* and other Gram negative bacteria, whose peptidoglycan layer is shielded by an outer membrane, were not affected by PAP. The authors proposed that PAP is a pattern recognition molecule, responsible for an “evolutionarily primitive form of lectin-mediated innate immunity”. Earlier reports from 1991 and 1993 also described that rat PAP and the paralog human PSP have antibacterial activity, as both molecules were shown to induce bacterial aggregation although without affecting growth [4,12]. Both PAP and PSP have been known to undergo tryptic cleavage and consequent insoluble fibril formation, however, the significance of the proteolytic processing in the antibacterial activity of PAP has not been studied. Iovanna et al. (1993) noted that trypsin seemed to facilitate the bacterial aggregating activity of human PSP, suggesting that PAP function might be similarly regulated by trypsin [12].

The present study convincingly demonstrates that binding of PAP to peptidoglycan and the consequent induction of bacterial aggregation is entirely dependent on the N-terminal proteolytic processing of PAP. Intact, full-length PAP does not bind to peptidoglycan and has no antibacterial activity whatsoever. We found that among the pancreatic proteases not only trypsins but also elastases can process PAP at the N terminus and the trypsin-processed and elastase-processed PAP species exhibit comparable peptidoglycan binding and bacterial aggregating activities. Furthermore, we demonstrated that bacterial proteases secreted extracellularly can cleave PAP at the trypsin-sensitive peptide bond. This phenomenon was also noted by Cash et al. (2006a) [13]. In case of *Bacillus subtilis*, we tentatively identified the zinc metalloprotease nprE as the enzyme responsible for this activity. This latter observation might explain why intact, full-length PAP appeared to exhibit peptidoglycan binding and bacterial agglutinin activities in previous studies.

Arguably, pancreatic elastase-mediated PAP processing is one of the most interesting observations in the present study. However, the existence of a stable elastase-processed $\Delta 9$ -PAP in the pancreatic juice might seem questionable, as this form should be rapidly processed further by trypsin. We isolated elastase-processed $\Delta 9$ -PAP from the pancreatic juice of a patient

with acute pancreatitis, which confirms that it is a naturally occurring variant. Premature activation of digestive protease zymogens inside the pancreas is a hallmark of acute pancreatitis and we propose that under certain conditions elastase activation might precede trypsin activation, resulting in elastase-mediated PAP processing. In addition to pancreatic elastases, the elastase-sensitive Ser35-Ala36 peptide bond might be processed by certain bacterial proteases as well, although direct evidence for this is lacking. Nonetheless, it is intriguing to hypothesize that the N terminus of PAP represents a protease bait region, which can be attacked by various proteases to generate biologically active, N-terminally processed PAP.

Tryptic cleavage of PAP and PSP result in the formation of insoluble fibrils, which were suggested to be responsible for the biological activity of these lectin-like proteins [10,11, PSP reviewed in 6-9]. The observation that elastase-processed PAP remains soluble but still exhibits peptidoglycan binding and bacterial aggregating activity argues that insoluble fibril formation by PAP is not required for these functions. Both trypsin and elastase processing alters the conformation of PAP in a similar manner, as judged by the increased resistance to proteinase K digestion. Despite this similarity, the unique solubility of elastase processed PAP indicates that this PAP form is in a somewhat different conformation than trypsin-processed PAP is. X-ray crystallographic and NMR structures have been reported for full-length, intact PAP [26, 27; Protein Data Bank ID: 2GO0 and 1UV0], but not for N-terminally processed PAP, which could shed light on the structural changes that occur upon N terminal processing. In this respect, the soluble elastase-processed PAP might be a more promising target for crystallographic analysis than the aggregation prone trypsin-processed form.

In contrast to the results of Cash et al. (2006a) [13], in our studies we did not find significant bactericidal activity of PAP and we concluded that the primary antibacterial activity of PAP was bacterial aggregation and not bacterial killing, at least with respect to *Bacillus subtilis*. Cash et al. (2006a) demonstrated the killing effect of PAP on various Gram positive strains but *Bacillus subtilis* was not tested. Thus, strain specific sensitivity to PAP might explain the differing results. Our conclusion that PAP is not bactericidal is also in agreement with earlier reports indicating that PAP or PSP had no inhibitory effect on bacterial growth [4,12]. On the other hand, our observations contradict the same studies inasmuch as we could not demonstrate any effect of PAP on *Escherichia coli*. Since the peptidoglycan layer is not exposed in *Escherichia coli*, PAP should not bind to this bacterium and we cannot explain why aggregation was observed previously, although we note the long incubation times (3 h) used in these studies.

After this manuscript was completed, a paper was posted online in The Journal of Biological Chemistry [28], which reported that trypsin-mediated processing of PAP was required for its bactericidal activity against the Gram positive bacterium *Listeria monocytogenes*. Using mutagenesis and NMR the study also showed that PAP can undergo a conformational switch between biologically inactive and active states and the negative charges in the propeptide regulate this switch in an inhibitory manner. While our results agree with the major conclusion of this paper, there are notable discrepancies between the two studies on some important points. Thus, Mukherjee et al. [28] claim that (1) N-terminal processing of PAP is not required for peptidoglycan binding; (2) the antibacterial effect of PAP is independent of peptidoglycan binding and (3) the major antibacterial effect of PAP is killing. In contrast, our observations clearly establish a direct relationship between N-terminal processing of PAP, binding to peptidoglycan and induction of bacterial aggregation without significant killing. The inconsistent results between the two studies might be related to the use of different bacterial strains and peptidoglycan preparations, the possible interference of bacterial aggregation with the viability assays and the proteolytic lability of the PAP propeptide, which can result in unwanted processing of PAP preparations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

nprE, *Bacillus subtilis* extracellular neutral metalloprotease; PAP, pancreatitis-associated protein; Δ 11-PAP, trypsin-processed PAP with the N-terminal 11 amino-acids removed; Δ 9-PAP, elastase-processed PAP with the N-terminal 9 amino-acids removed; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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MLPPMALPSVSWMLLSCLMLLSQVQG**EEPQRELPSAR**IRCPK
 GSKAYGSHCYALFLSPKSWTDADLACQKRPSGNLVSVLSGAE
 GSFVSSLVKSIGNSYSYVWIGLHDPTQGTEPNGEWSSSD
 VMNYFAWERNPSTISSPGHCASLSRSTAFRLWKDYN CNVRLP
 YVCKFTD

native	<u>EEPQRELP</u>SAR IR	C
recombinant	<u>MEPQRELP</u>SAR IR	C
Δ11-PAP (trypsin-processed)		IR
Δ9-PAP (elastase-processed)	<u>AR</u>	IR

Figure 1.

N-terminal sequences of proteolytically processed PAP forms. The upper half of the figure shows the primary structure of PAP, with the trypsin-sensitive propeptide (Glu27-Arg37) emboldened and underlined. Note that the sequence between Met1 and Gly26 forms the signal peptide, which is removed in the secretory pathway and the 1st amino-acid of mature, secreted PAP is Glu27. The lower part of the figure indicates the N-terminal sequences of full-length, intact PAP and the trypsin-processed and elastase-processed forms. In the recombinant PAP used in these studies Glu27 is replaced with a Met residue.

Figure 2.

N-terminal processing of PAP by trypsin and pancreatic elastases. PAP (5 μ g, 1.5 μ M final concentration) was digested with (A) human cationic trypsin, human anionic trypsin and mesotrypsin (10 nM) or (B) with human elastase 2A (50 nM) and human elastase 3B (150 nM, final concentrations) at 37 °C in 200 μ L final volume of 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 . Samples were precipitated at the indicated times with 10% trichloroacetic acid (final concentration) and analyzed by 15% SDS-PAGE and Coomassie Blue staining.

Figure 3.

Effect of N-terminal processing on the solubility of PAP. Incubations were carried out with 5 μ g PAP (1.5 μ M final concentration) in 200 μ L of 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 . At the indicated times samples were centrifuged at 10,000g for 10 min at 4 $^{\circ}\text{C}$ and the pellets (P) and supernatants (S) were analyzed 15% SDS-PAGE and Coomassie Blue staining. **(A)** PAP was incubated with 10 nM concentration of human cationic trypsin at 22 $^{\circ}\text{C}$ for the indicated times. **(B)** PAP was incubated with 10 nM concentration of human cationic trypsin for 30 min at the indicated temperatures. **(C)** PAP was pre-incubated with 150 nM concentration of human elastase 3B for 30 min at 37 $^{\circ}\text{C}$ to achieve complete processing (see Figure 2B). Samples were then centrifuged (10,000g for 10 min at 4 $^{\circ}\text{C}$) to remove any precipitate and the time-course of

incubation at 22 °C was started after this centrifugation step. Although not shown, identical results were obtained when $\Delta 9$ -PAP obtained from mini-intein fusions (see *Material and Methods*) was incubated at 22 °C.

Figure 4.

Digestion of N-terminally processed PAP with proteinase K. Five μg of intact PAP, trypsin-processed $\Delta 11$ -PAP or elastase-processed $\Delta 9$ -PAP (1.5 μM final concentrations) were digested with 10 $\mu\text{g}/\text{mL}$ concentration of proteinase K at 37 $^{\circ}\text{C}$ in 200 μL final volume of 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2 . Samples were precipitated at the indicated times with 10% trichloroacetic acid (final concentration) and analyzed by 15% SDS-PAGE and Coomassie Blue staining.

Figure 5.

Binding of N-terminally processed PAP to peptidoglycan from *Bacillus subtilis*. Peptidoglycan was washed with 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and re-suspended in the same buffer. Fifty µg of peptidoglycan (50 µL from a 1 mg/mL suspension) was incubated with 5 µg of the indicated PAP species in a final volume of 200 µL (1.5 µM final concentration). Incubations were performed under constant shaking using an IKA Vibrax VXR vibrating mixer at a setting of 1000. Samples were centrifuged at 16,000g for 5 min at 4 °C, and the supernatant was precipitated with 10% trichloroacetic acid (final concentration). The peptidoglycan pellet was washed three times with 200 µL of 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and PAP was eluted by boiling in 50 µL sample buffer for 5 min. PAP in the supernatants (S) and pellets (P) were

visualized by 15% SDS-PAGE and Coomassie Blue staining. **(A)** Peptidoglycan was incubated with intact PAP, trypsin-processed $\Delta 11$ -PAP or elastase-processed $\Delta 9$ -PAP at 22 °C for 30 min. **(B)** Peptidoglycan was incubated with trypsin-processed $\Delta 11$ -PAP for the indicated times at 22 °C or at 4 °C.

Figure 6.

N-terminal processing of PAP by the *nprE* metalloprotease secreted from *Bacillus subtilis*. Samples were precipitated at the indicated times with 10% trichloroacetic acid (final concentration) and analyzed by 15% SDS-PAGE and Coomassie Blue staining. **(A)** PAP (5 μ g, 1.5 μ M final concentration) was incubated at 37 °C in 200 μ L culture supernatant from wild-type *Bacillus subtilis* or from the protease-deficient strain BG2036, supplemented with 0.1 M Tris-HCl (pH 8.0) and 1 mM CaCl_2 (final concentrations). The supernatants were obtained from cultures grown to an OD (600nm) of 0.4 at 30 °C in LB medium. **(B)** PAP was incubated with culture supernatant from wild-type *Bacillus subtilis* as described above, in the absence or presence of 20 mM K-EDTA (final concentration). The culture supernatant was pre-incubated with EDTA for 10 min before addition of PAP.

Figure 7.

Bacterial aggregation induced by N-terminally processed PAP. Wild-type *Bacillus subtilis* and the protease-deficient BG2036 strain were grown and labeled with FITC as described in *Materials and Methods*. FITC-labeled bacteria (200 μ L) were incubated with 10 μ g of trypsin-processed PAP (Δ 11-PAP), elastase-processed PAP (Δ 9-PAP) or intact PAP (3 μ M final concentrations) for 15 min at 22 $^{\circ}$ C and aggregation was visualized under a fluorescence microscope at 200-fold magnification. Although not shown, *Escherichia coli* strains were not aggregated to a detectable extent under the same conditions.

Figure 8.

Effect of N-terminally processed PAP on bacterial viability. **(A)** Viability of PAP-treated *Bacillus subtilis* assayed by dilution plating. Wild type and BG2036 *Bacillus subtilis* were grown to OD (600 nm) of 0.4 in LB medium at 30 °C. Cells (200 μL) were pelleted by centrifugation and re-suspended in 200 μL of 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂ and incubated with 10 μg trypsin-processed Δ11-PAP (3 μM final concentration) at 22 °C. Cells were gently mixed by flicking the incubation tubes every 10 min. After 1 h incubation, 20 μL of 10-fold serial dilutions were plated on LB agar plates. Cells were dispersed by vigorous vortexing before each diluting step and before plating. Colonies were counted after overnight growth at 30 °C. The error bars indicate the standard error of the mean (n=3). Although not shown, identical results were obtained with elastase-processed Δ9-PAP. **(B)** Fluorescence emission spectra of Δ11-PAP-treated *Bacillus subtilis* labeled with the Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes). Aliquots (200 μL) of wild-type *Bacillus subtilis* culture (OD 0.5 at 600 nm) were centrifuged, and the bacterial pellets were re-suspended in 100 μL 0.85% NaCl (“live control”) or 100 μL 0.85% NaCl with 5 μg trypsin-processed Δ11-PAP (3 μM final concentration). To prepare a “dead control” sample, 200 μL culture aliquots were centrifuged, the cells were re-suspended in 100 μL 70 % isopropanol, incubated at 22 °C for 5 min, pelleted by centrifugation and re-suspended in 100 μL 0.85% NaCl. Live control, dead control and Δ11-PAP-treated cells were incubated for 1 h at 22 °C and subsequently mixed with 100 μL 2× fluorescent dye solution (final concentrations of SYTO 9 and propidium iodide were 6 μM and 30 μM, respectively). The 200 μL mixture was incubated for 15 min at 22 °C in the dark and fluorescent emission spectra were recorded with a SpectraMAX GeminiXS Fluorimeter (Molecular Devices) at an excitation wavelength of 470 nm. Although not shown, similar results were obtained with the BG2036 strain or when elastase-processed Δ9-PAP was used.