Alternative Luminal Activation Mechanisms for Paneth Cell α-Defensins

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Background: Matrix metalloproteinase-7 (MMP7) processes mouse pro-α-defensins to bactericidal forms in Paneth cells, and MMP7(−/−) mice are considered to be α-defensin-null.

Results: Activated α-defensins occur in the colonic lumen of MMP7(−/−) mice.

Conclusion: α-Defensins are activated in MMP7(−/−) large bowel lumen by alternative proteolytic conversion mechanisms.

Significance: MMP7(−/−) mice are unsuitable for studying α-defensin deficiency in large intestine.

Paneth cell α-defensins mediate host defense and homeostasis at the intestinal mucosal surface. In mice, matrix metalloproteinase-7 (MMP7) converts inactive pro-α-defensins (proCrps) to bactericidal forms by proteolysis at specific proregion cleavage sites. MMP7(−/−) mice lack mature α-defensins in Paneth cells, accumulating unprocessed precursors for secretion. To test for activation of secreted pro-α-defensins by host and microbial proteases in the absence of MMP7, we characterized colonic luminal α-defensins. Protein extracts of complete (organ plus luminal contents) ileum, cecum, and colon of MMP7-null and wild-type mice were analyzed by sequential gel permeation chromatography/acid-urea polyacrylamide gel analyses. Mature α-defensins were identified by N-terminal sequencing and mass spectrometry and characterized in bactericidal assays. Abundance of specific bacterial groups was measured by qPCR using group specific 16S rDNA primers. Intact, processed bactericidal peptides by proteolytic separation of inhibitory acidic amino acid proregion residues (18–20). In mouse Paneth cells, pro-α-defensins (proCrps) are processed intracellularly by matrix metalloproteinase-7 (MMP7)2 to produce mature ~4.5 kDa α-defensins (termed Crp in mice) (20, 21). MMP7(−/−) mouse small intestinal tissue lacks mature α-defensin peptides and secretes inactive pro-α-defensins, identifying MMP7 as the mouse Paneth cell pro-α-defensin activating convertase (20). Partial removal or mutagenesis of the proregion is sufficient to generate antimicrobial function. Consequently, the three mouse proCrp4 MMP7 processing intermediates are as bactericidal as mature Crp4 (22, 23). In contrast, human Paneth cells lack MMP7 and accumulate pro-α-defensins, e.g. human prodefensin 5 (proHD5), which is processed by trypsin after secretion (19). The proregions of α-defensin precursors are sensitive to proteolysis, but mature α-defensins resist degradation in vitro by MMP7, trypsin, or neutrophil serine proteases (18, 19, 24).

Because mature α-defensins persist in wild type mouse colonic lumen (17) and pro-α-defensin proregions are suscep-

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2 The abbreviations used are: MMP, matrix metalloproteinase; Crp, cryptdin; AU-PAGE, acid-urea PAGE; Bact, Bacteroides spp.; CFU, colony-forming unit; Erec, Eubacterium rectal-Clostidium cocoides; HD5, human Paneth cell α-defensin-5; Lact, Lactobacillus spp.; MIB, mouse intestinal Bacteroides; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RP-HPLC, reverse phase high performance liquid chromatography; SFB, segmented filamentous bacteria.

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Luminal α-Defensin Activation in MMP7(−/−) Mice

tible to proteolytic degradation (25), we tested whether pro-
tases of host or microbial origin could activate pro-α-de-
frons luminally after secretion by MMP7(−/−) Paneth cells.
Results show that MMP7(−/−) mouse colonic lumen contains
active, mature α-defensins, indicating that luminal proteinases
convert inactive precursors to bactericidal forms in the absence
of the native convertase.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Peptides—Peptides were prepared
from recombinant methods and purified to homogeneity as
described (9, 26). Briefly, peptides were expressed in Esche-
richia coli as N-terminal His6-tagged fusion proteins using the
pET-28a expression vector (Novagen Inc., Madison, WI) and
affinity purified (25–27). Peptide homogeneity was assessed by
analytical RP-HPLC, AU-PAGE (28), and MALDI-TOF mass
spectrometry using a Microflex LRF mass spectroscope (Bruker
Daltonics, Maynard, MA) (22).

Animals—Procedures on mice were performed with
approval and in compliance with the policies of the animal care
and use committees at the Medical College of Wisconsin
(MCW), University of California, Irvine, and University of
Southern California. Animals from MCW were housed under
SPF conditions in the MCW Biomedical Resource Center vivar-
ium. C57BL/6 breeding pairs were obtained from Taconic Lab-
oratories, and MMP7(−/−) breeding pairs originally were pro-
vided by Dr. Carole Wilson. HD5 (DEF5(+/+)) transgenic
mice on a C57BL6 background (29) were intercrossed with
MMP7(−/−) mice on a C57BL6 background to generate
DEF5(+/−)MMP7(−/−) mice. Experimental mice were
obtained from in-house breeding colonies at MCW. Experi-
ments at UC Irvine and the University of Southern California
were performed on MMP7(−/−) and C57BL6/6 mice ordered
from The Jackson Laboratory. Animals were housed under 12 h
cycles of light and dark and had free access to standard mouse
chow and water.

Purification of Enteric α-Defensins—Peptides were isolated
using a modified procedure described previously (16). Seg-
ments of ileum, cecum, and distal large bowel were excised from
mice immediately after euthanasia, placed on dry ice, and
subjected to immediate protein extraction or stored at −80 °C
until processed. Protein extracts were prepared from “com-
plete” organ, consisting of tissues plus luminal contents, except
for the HD5(+/−)MMP7(−/−) mouse distal small intestine
and corresponding MMP7(−/−) controls. For these samples,
the luminal contents were flushed from the organ with phos-
phate-buffered saline. Samples were homogenized in 100 ml
of ice-cold 60% acetonitrile plus 1% trifluoroacetic acid (TFA),
incubated at 4 °C overnight, clarified by centrifugation, and
ice-cold 60% acetonitrile plus 1% trifluoroacetic acid (TFA),
phosphate-buffered saline. Samples were homogenized in 100 ml of
the luminal contents were flushed from the organ with phos-
fesins luminally after secretion by MMP7(−/−) Paneth cells.

Peptide Analysis by Mass Spectrometry—Samples were sepa-
rated by C18 RP-HPLC that was developed with an aqueous
10–45% linear gradient of acetonitrile, 0.1% TFA (30) delivered
in 90 min at 1 ml/min. Samples of HPLC fractions were mixed
with an equal volume of 10 mg/ml α-cyano-4-hydroxycinnamic
acid in 60% acetonitrile, 0.1% TFA, dried, and analyzed by
MALDI-TOF MS.

Peptide Transfer and N-terminal Sequencing—α-Defensin
preparations from wild type and knock-out mouse samples
were run in AU-PAGE and electroblotted to 0.1 µm PVDF
membrane (Millipore Immobilon PSQ) using 5% acetic acid as
the transfer buffer. Individual peptide bands were excised from
Coomassie-stained membranes were subjected to nine rounds
of Edman reactions on an ABI 494-HT Procise Edman
Sequencer by the Molecular Structure Facility at the University
of California, Davis.

Quantitative RT-PCR—Tissue samples from distal small
intestine, cecum, and large intestine from MMP7(−/−) mice
were preserved in RNAlater. RNA was isolated using RNaseasy
minikit (Qiagen), according to kit directions. cDNA was generated
for each sample using iScript (Bio-Rad). Real-time PCR
was performed for mouse α-defensins and GAPDH. Isolated
total RNA was quantified by ultraviolet absorbance at 260 nm
using a Nanodrop ND-1000 spectrophotometer, and reverse
transcribed with reverse transcriptase according to the suppli-
ner’s protocol (iScript, Bio-Rad). Real-time PCR was performed
using the tissue-specific cDNA as a template with specific oli-
gonucleotide primer pairs for Crp1, Crp21, and GAPDH as pre-
viously described (13). Each 82.5 µl of PCR reaction contained
each oligonucleotide primer at the final concentration of 0.42
µM 1× SYBR Green Mix (Bio-Rad) and 50–100 ng of template
dNA. The qRT-PCR was performed using the MyiQ Single-
Color Real-Time PCR Detection System (Bio-Rad). A no-
template reaction was included as a negative control for each qRT-
PCR experiment, and for absolute quantification gene-specific
plasmid standards were included within every set of reactions.
The real time PCR program started with an initial step at 95 °C
for 3 min, followed by 40 cycles of 10 s at 95 °C and 45 s at 60 °C.
Data were acquired in the final step at 60 °C.

Bactericidal Assays—The bactericidal activities of α-defen-
sin preparations were tested against E. coli ML35, Listeria
monocytogenes 10403S, and Citrobacter rodentium DBS100
(31) as described (21, 25). Citrobacter rodentium DBS100
was provided by the late Dr. David B. Schauer (Division of Biological
Engineering, Massachusetts Institute of Technology) and is
available as ATCC 51459. Exponentially growing bacteria (5 × 106/ml) were exposed to peptides for 1 h at 37 °C in 50 µl PIPES-TSB, diluted and plated. Surviving bacteria were quan-
tified as colony-forming units per milliliter (CFU/ml) after
overnight growth. Data were from three independent experi-
ments and expressed as the mean ± standard deviations.

Anti-HDS Western Blot—Ten percent of protein extracted
from DEF5(+/−)MMP7(−/−) and MMP7(−/−) intestinal
samples were resolved by AU-PAGE, transferred to a 0.2 µm
nitrocellulose membrane, blocked with 5% skim milk for 1 h at
ambient temperature, and incubated with agitation at room
temperature overnight with rabbit anti-HDS5 immune serum
(32) diluted 1:2000 in Tris-buffered saline/Tween (250 mM Tris,
1.37 mM NaCl, 26.8 mM KCl, 1% Tween, pH 7.4) containing 5% skim milk. Washed blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (Pierce) diluted 1:20,000 in Tris-buffered saline/Tween for 1 h, washed, and developed using SuperSignal pico chemiluminescent substrate (Pierce).

Pro-α-Defensin Sensitivity to Trypsin, Chymotrypsin, and Neutrophil Elastase Proteolysis—Recombinant α-defensins were digested with trypsin (Sigma, T-8253), α-chymotrypsin (Sigma, C-9135), or neutrophil elastase (Elastin Products Company, Inc.) and analyzed for susceptibility to proteolysis by AU-PAGE and MALDI-TOF MS. Samples (10 μg of proCrp4 or 5 μg Crp4 or (6C/A)-Crp4) were incubated with each proteinase overnight at 37 °C at a substrate to enzyme molar ratio of 50:1 in 50 mM ammonium bicarbonate (pH 8.0). Samples consisting of 85% of each digest were analyzed by AU-PAGE, and the remainder was analyzed using MALDI-TOF MS.

Quantitative Real-time PCR Amplification of 16 S rRNA Gene Sequences—The cecum and large intestine isolated from the experimental animals were weighed and homogenized before bacterial genomic DNA was extracted using the Qiagen Stool Kit (Qiagen) following previously published procedures (34, 35). The abundance of specific intestinal bacterial groups was measured by qPCR using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using group specific 16S rDNA primers (Operon Technologies Huntsville, AL) as previously described (34). Group specific primers were used to detect the following major groups: Eubacterium rectale/Clostridium cocoides (Erec), Lactobacillus sp. (Lact), Bacteroides sp. (Bac), Mouse Intestinal Bacteroides (MIB), Segmented Filamentous Bacteria (SFB), and total bacteria (Eubacteria) (34).

RESULTS

Mature α-Defensins in Cecal and Colonic Lumen of MMP7(−/−) Mice—α-Defensins were apparent in protein extracts from complete (whole organ plus luminal contents) cecum and colon of MMP7-null mice (Fig. 1E and F). Acid-urea polyacrylamide gel electrophoresis (AU-PAGE) analyses of proteins separated by gel permeation chromatography showed that small, cationic peptides characteristic of mouse α-defensins (16, 28) were abundant in complete wild-type C57BL/6 mouse small intestine (Fig. 1A). In contrast, similar MMP7(−/−) mouse protein extracts had markedly lower α-defensin levels as found previously (20) (Fig. 1D). The low but detectable α-defensin levels in MMP7(−/−) complete ileum may have resulted from luminal activation of pro-α-defensins.
**Luminal α-Defensin Activation in MMP7(−/−) Mice**

**A**

| Peptide       | Primary Structure                     | Molecular Mass (A. M. U.) |
|---------------|---------------------------------------|--------------------------|
| Crp3          | LRDLVCYCRKGKKRRMNGTCRKHLYMTCR        | 4279.1                   |
| (des-LR)-Crp3 | DLVCYCRKGKKRRMNGTCRKHLYMTCR         | 4006.3                   |
| Crp20         | LHEKSSRLDLCYCRKKDMGQVYQYQGRFSRLL--CRRRRRH | 4947.9                   |
| (des-LS)-Crp21| RDLICLCNRRCNRFELFYGTG--AGPFLR--CRRRRR | 4313.8                   |
| (des-LR)-Crp24| DLVCYCRAGKGRMNGTCGKLYMLCCR          | 3791.7                   |
| Crp27         | LRDLVCYCRAGKGRMNGTCGKLYMLCCR        | 4084.6                   |
| (des-L)-Crp27 | RDLICLCRAGKGRMNGTCGKLYMLCCR         | 3974.6                   |

**B**

that had been secreted by Paneth cells in duodenum and jejunum. α-Defensins were evident in wild type C57BL/6 complete cecum and colon (Fig. 1, B and C), and protein extracts from complete MMP7(−/−) cecum and colon contained processed α-defensins, unexpectedly at wild type levels (Fig. 1, E and F). It should be noted that Paneth cell α-defensins in wild type mouse large intestine derive from luminal contents, not from tissue of the large bowel (14, 17). qRT-PCR measurements showed that Crp mRNA levels in cecum and colon are 1000-fold lower than in ileum of MMP7(−/−) mice, excluding aberrant α-defensin gene expression in colonicocytes or appearance of ectopic Paneth cells induced by the MMP7-null condition as a source of the peptides (supplemental Fig. S1). Because MMP7-null Paneth cells secrete inactive α-defensin precursors (21), the released pro-α-defensins must have become activated after secretion into the lumen.

*Alternative Processing of Pro-α-defensins in the MMP7(−/−)*

*Intestinal Lumen—* α-Defensins from the MMP7(−/−) colonic lumen included native and N-terminally truncated α-defensins as well as a novel variant that is inferred to be a product of alternative luminal processing. The novel N terminus of sequence one was ESLRDVL_Y with a mass consistent with the retention of two Glu-Ser residues from the proregion C terminus (Fig. 2A). This unique α-defensin sequence represents a processed variant not identified previously with MMP7-mediated activation (21, 22, 25). In the past, native and N-terminally truncated α-defensins also were identified in Outbred Swiss distal large intestine (17). Luminal MMP7(−/−) colonic α-defensins in a single P-60 fraction (Fig. 1F) were characterized further by C18 RP-HPLC and MALDI-TOF MS, which identified experimental masses corresponding to Crps 3, 20, 21, 24, and 27 (Fig. 2B), and Edman sequencing detected the N-terminal sequence DLV_Y_RAR, corresponding to Crp24 or Crp27 (Fig. 2A). N-terminal sequence three, DLV_Y_LI, did not match any known C57BL/6 Paneth cell α-defensin. Perhaps the peptide is coded by a novel gene in one of the unannotated gaps in the current assemblies (36, 37). Nevertheless, fully processed α-defensins were found to be abundant in the lumen of the cecum and colon of MMP7 knockouts, evidence that secreted pro-α-defensins had been activated luminally in the absence of the natural convertase.

MMP7-null colonic α-defensins had *in vitro* bactericidal peptide activities equivalent to those of wild type C57BL/6 mice (Fig. 3, A–C) even though MMP7(−/−) and C57BL/6 luminal α-defensins had minor qualitative differences (Fig. 2). As seen previously (17), combined native α-defensin preparations contained small peptide fragments from non-defensin proteins that reduced bactericidal peptide activity relative to the homogenous control peptide, Crp4. These *in vitro* findings showed that α-defensins in the MMP7-null colonic lumen have the potential to contribute to innate immunity.

*Processing of Human Paneth Cell ProHD5 Is Independent of MMP7 in Mice—* Processing of human HD5 differs from mechanisms of mouse enteric α-defensin activation. Intact proHD5 is the predominant molecular form of HD5 in human Paneth cells with only low levels of detectable mature HD5, and only processed HD5 is detected in the intestinal lumen (12). Also, HD5 transgenic (DEFA5(+/+)) mice express HD5 only in Paneth cells of the small intestine and not in the colon (12). Because human Paneth cells lack MMP7 and trypsin is the HD5 activating convertase, we examined the possibility of a role for MMP7 in HD5 processing in the context of DEFA5(+/+) transgenic mouse Paneth cells.

To test whether MMP7 is required to process HD5 in DEFA5(+/+) mouse Paneth cells, we crossed DEFA5(+/+) mice with MMP7(−/−) mice to obtain DEFA5(+/−)
MMp7(−/−) mice, and proHD5 processing still was found to occur in the MMP7-deficient state (Fig. 4). Protein extracts of DEFA5(+/−) MMP7(−/−) flushed ileum contained unprocessed proHD5 as the major form, although a low level of mature HD5 was detected in ileal tissue (Fig. 4). HD5, but not proHD5, was detected in cecum and colon, showing that secreted proHD5 was activated luminally. Also, protein extracts from DEFA5(+/+) mouse complete cecum and colon contained immunoreactive HD5 (not shown). Thus, HD5 did not require MMP7 for processing in DEFA5(+/+) mouse Paneth cells, and the peptide persisted in the distal bowel.

Alternative in Vitro Mouse Pro-α-Defensin Processing by Host Proteases—The presence of activated Paneth cell α-defensins in MMP7(−/−) colonic lumen suggested that host proteases could activate pro-α-defensins by an alternative mechanism. To test this hypothesis, proCrp4 was exposed in vitro to proteases, and digests were analyzed by AU-PAGE and MALDI-TOF MS to assess proteolysis. Trypsin, chymotrypsin, and neutrophil elastase degraded the proCrp4 proregion extensively, producing native Crp4 and additional peptides as major digestion products (Fig. 5, A and C). Trypsin and chymotrypsin digests contained variants with additional C-terminal proregion residues at the Crp4 N terminus (Fig. 5C), and neutrophil elastase produced (LR)-Crp4 and (R)-Crp4 peptide variants (Fig. 5, A and C), variants of Crp4 with one or two additional amino acids retained at the N terminus. Native Crp4 was resistant to all enzymes, but disulfide-null (6C/A)-Crp4 was degraded extensively by all proteinases (Fig. 5, A and C), consistent with earlier findings (26). Although levels of trypsin, chymotrypsin, and neutrophil elastase in the intestinal lumen are unknown, their in vitro proCrp4 activation showed that host enzymes are capable of mediating luminal processing when the cellular convertase is absent.

Mouse Pro-α-Defensin Processing by Bacterial Proteases—Because pro-α-defensins interact with the microbiota once secreted, we tested whether bacterial proteases can activate proCrp4 in vitro. Enterococcus faecalis, an intestinal commensal and opportunistic pathogen (38, 39), secretes both gelatinase (GelE) and serine protease E (SprE) (40). GelE degrades many substrates including the human cathelicidin, LL-37 (41). Exposure of proCrp4 to culture supernatants of wild-type E. faecalis OG1RF resulted in complete precursor conversion to (LR)-Crp4 (Fig. 5), but supernatant from E. faecalis JRC105, a mutant strain lacking GelE and SprE (33), did not process pro-Crp4 to forms recognizable by AU-PAGE or MS analysis (Fig. 5B). Thus, GelE and/or SprE secreted by E. faecalis mediated in vitro proCrp4 processing (Fig. 5, B and C), and no proteolysis occurred within the mature Crp4 polypeptide backbone, regardless of protease exposure (Fig. 5). Therefore, luminal proteinases of microbial origin may process pro-α-defensins alternatively, including specific proteases secreted by species of the commensal microbiota.

Cecal and Colonic Microbiota Are Not Significantly Affected by MMP7 Deficiency—in MMP7(−/−) mice, defective intracellular Paneth cell pro-α-defensin processing is associated with changes in the ileal microbiota, shifting the relative abundance of Bacteroides species (Bact) and Mouse Intestinal Bacteroides (MIB) significantly (13). In contrast to that outcome, the relative abundance of bacterial groups analyzed did not differ significantly in cecum (Fig. 6A) or colon (Fig. 6B) of...
MMP7(−/−) and C57BL/6 mice. Although additional factors may shape the composition of the large intestinal microbiota, the respective similarities of MMP7(−/−) and C57BL/6 cecal and colonic microbiota are consistent with alternative luminal pro-α-defensin processing to normal α-defensin levels in the distal intestinal lumen under MMP7-null conditions.

**DISCUSSION**

In MMP7(−/−) mice, secreted pro-α-defensins are activated luminally by one or more alternative mechanisms.

Enteric α-defensins are made and secreted almost exclusively by small intestinal Paneth cells, and the mature peptides resist proteolysis of the polypeptide backbone, accumulate in the colonic lumen regardless of MMP7(−/−) status (Figs. 1 and 2), and retain bactericidal peptide activity comparable to α-defensins from wild-type mouse colonic lumen (Fig. 3). α-Defensin-associated differences in the ileal microbiota of C57BL/6 and MMP7(−/−) mice did not occur in cecum or colon, consistent with the similar abundance of luminal α-defensin pep-
tides in the mice (Figs. 1 and 6). Representative host proteases and culture supernatants of E. faecalis converted proCrp4 to active forms in vitro (Fig. 5), and we propose that host or microbial proteinases may activate secreted pro-α-defensins in the lumen, if the natural intracellular convertase is absent.

α-Defensin deficiency in MMP7(−/−) mice is restricted to the small bowel. The MMP7(−/−) and C57BL/6 colonic lumens have similar α-defensin peptide levels (Figs. 1 and 2). Studies of MMP7(−/−) mouse susceptibility to oral infection have focused on E. coli and S. enterica serovar Typhimurium infections of the small intestine and cecum and detected reduced bacterial clearance from distal small intestine (20). In MMP7(−/−) mice infected with Shigella flexneri, increased numbers of S. flexneri were detected in colons of newborn MMP7(−/−) mice, but factors other than MMP7 deficiency or low α-defensin levels also may contribute to Shigella survival when Paneth cell numbers are low as they are in newborn mice (42). Because the colonic microbial burden is several orders of magnitude greater than that of the small bowel and wild type and MMP7(−/−) colonic α-defensins are less abundant than in wild type small intestine, α-defensins may affect the colonic microbiota by means other than direct peptide-mediated cell killing. For example, low peptide concentrations of certain defensins inhibit bacterial peptidoglycan synthesis by lipid II binding (10, 43), and α-defensins may use a lipid II binding mechanism to exert selective antimicrobial effects on the local microbiota in the distal bowel. In view of these considerations, studies of enteric α-defensin deficiency in MMP7(−/−) mice are best limited to the small intestine.

Recovery of α-defensins with heterogeneous N termini from MMP7(−/−) colonic lumen shows that luminal proteinases activate extracellular pro-α-defensins in the lumen. In humans, Paneth cell α-defensins are processed extracellularly by trypsin during or after secretion (19), and transgenic HD5 persists in the mouse colonic lumen and perhaps in human colon as well (Fig. 4). Possibly, a number of proHD5 molecules escape trypsin activation and may be activated by luminal host or bacterial proteinases. Transgenic HD5 activation was independent of MMP7 in the DEFA5(+/−)MMP7(−/−) mice, even though pro-α-defensin processing in mouse Paneth cells requires MMP7. The detection of native Crp3 and Crp27 in MMP7(−/−) colonic lumen shows that one or more luminal proteases can cleave pro-α-defensins at the final MMP7 cleavage site, generating Crps as fully processed as those converted by MMP7 within Paneth cells (Fig. 2). Isolation of luminal (des-Leu-Arg)-Crp and (des-Leu)-Crp variants from MMP7(−/−) mice further suggests involvement of an aminopeptidase to generate these products after pro-α-defensin secretion into the lumen (Fig. 2), consistent with previous findings (17). Finally, one abundant α-defensin processing variant from MMP7(−/−) colonic lumen retained two residues from the proregion C terminus due to alternative processing. α-Defensins that retain proregion residues have not been detected previously in wild-type mice (Fig. 2). Thus, luminal proteases cleave the proregion to produce activated, bactericidal α-defensins in vivo, and luminal host or microbial proteinases could provide alternative activating enzymes in the absence of MMP7, the natural convertase. Because all proCrp4 processing intermediates and full-length proCrp4 molecules with mutations at inhibitory acidic amino acid residue positions have the same bactericidal peptide activity as fully processed Crp4 (22, 23), we speculate that any enzymes that cleave the proregion to remove inhibitory acidic amino acids will yield a bactericidal peptide (23).

Diverse host or microbial proteases in the intestinal lumen may provide alternative pro-α-defensin convertase activity in the extracellular environment. Trypsin, chymotrypsin, and neutrophil elastase all remove the proCrp4 proregion in vitro (Fig. 5), and secreted gelatinase or serine protease from E. faecalis cultures process proCrp4 without degrading the α-defensin moiety (Fig. 5). Because the large bowel hosts an estimated 10^{14} commensal bacteria and is chronically exposed to ingested microbes, including many protease-producers (44), secreted bacterial proteases may be an abundant source of luminal pro-α-defensin activating enzymes. Host proteases are robust pro-α-defensin activators in vitro, so it seems unlikely that bacterial enzymes are essential activators of luminal pro-α-defensins in MMP7(−/−) large intestine. Analyses of luminal α-defensins in germ-free MMP7(−/−) mice could provide estimates of the contribution of enteric bacteria to precursor activation. Because α-defensins shape the composition of the resident ileal microbiota, such alternative convertase activities, exemplified by host enzymes and E. faecalis GelE and SprE, would ensure the luminal presence of α-defensins, their selective effects on the microbiota, and the maintenance of innate immunity of the enteric environment.

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