Activation of Paneth Cell $\alpha$-Defensins in Mouse Small Intestine*

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Paneth cells in small intestine crypts secrete microbiocidal $\alpha$-defensins, termed cryptdins, as components of enteric innate immunity. The bactericidal activity of cryptdins requires proteolytic activation of precursors by matrix metalloproteinase-7 (MMP-7; matrilysin) (Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., Lopez-Boado, Y. S., Stratman, J. L., Hultgren, S. J., Matrisian, L. M., and Parks, W. C. (1999) Science 286, 113–117). Here, we report on the intracellular processing of cryptdin proforms in mouse Paneth cells. Peptide sequencing of MMP-7 digests of purified natural proforms identified conserved cleavage sites in the prosegment adjacent to the cryptdin peptide N terminus between Ser58 and Leu59. Immunostaining co-localized precursor prosegments and mature cryptdin peptides to Paneth cell granules, providing evidence of their secretion. Extensive MMP-7-dependent procryptdin processing occurs in Paneth cells, as shown by Western blot analyses of intestinal crypt proteins and proteins from granule-enriched subcellular fractions. The addition of soluble prosegments to in vitro antimicrobial peptide assays inhibited the bactericidal activities of cryptdin-3 and -4 in trans, suggesting possible cytoprotective effects by prosegments prior to secretion. Levels of activated cryptdins were normal in small bowel of germ-free mice and in sterile implants of fetal mouse small intestine grown subcutaneously. Thus, the initiation of procryptdin processing by MMP-7 does not require direct bacterial exposure, and the basal MMP-7 content of germ-free Paneth cells is sufficient to process and activate $\alpha$-defensin precursors. MMP-7-dependent procryptdin activation in vivo provides mouse Paneth cells with functional peptides for apical secretion into the small intestine lumen.

The release of endogenous antimicrobial peptides by mammalian epithelial cells contributes to innate mucosal immunity (1, 2). The crypts of Lieberkuhn in the small intestine of most mammals contain Paneth cells that secrete $\alpha$-defensins (cryptdins), lysozyme, secretory phospholipase A2, xanthine oxidase, CD95 ligand, CD15, and tumor necrosis factor-$\alpha$ as components of apically oriented secretory granules (3–10). Although certain Paneth cell $\alpha$-defensins have been detected in mouse skin and testis (11, 12) and in human oropharyngeal and urogenital mucosa (13, 14), in the small intestine, $\alpha$-defensins are specific to Paneth cells (9). Exposure of Paneth cells to cholinergic agonists or bacterial stimuli elicits granule discharge into the crypt lumen (15), and carbamylcholine mediates secretion via increased cytosolic Ca$^{2+}$ (16). Regardless of how mouse Paneth cell secretion is stimulated, cryptdins constitute $\sim$70% of the released bactericidal activity, and the concentration of cryptdins is estimated to be 25 mM at the point of secretion in the crypt lumen (15).

$\alpha$-Defensins are processed from inactive proforms by specific proteolytic cleavage steps. Both neutrophil and Paneth cell $\alpha$-defensins derive from $\sim$10-kDa prepropeptides that contain canonical signal sequences, acidic proregions, and an $\sim$3.5-kDa mature $\alpha$-defensin peptide in the C-terminal portion of the precursor. For example, maturation of myeloid pro-$\alpha$-defensins appears to involve two primary cleavage steps, and most $\alpha$-defensins in mature phagocyte leukocytes are completely processed (17–20). In a heterologously expressed human neutrophil pro-$\alpha$-defensin, deletions in the prosegment adjacent to the proregion-defensin junction impairs post-translational processing in 32DCL3 cells (19).

In mouse Paneth cells, matrix metalloproteinase-7 (MMP-7; matrilysin) mediates the processing and activation of $\alpha$-defensins from 8.4-kDa proforms (21). MMP-7 gene disruption ablates procryptdin processing, resulting in accumulation of cryptdin precursors and the absence of activated mature cryptdin peptides in the small intestine (21). Lacking functional cryptdin peptides, MMP-7-null mice have a defect in clearance of intestinal infections, and they succumb more rapidly and to lower doses of virulent Salmonella typhimurium compared with control mice (21). Thus, the cryptdin deficiency resulting from defective procryptdin activation is associated with a measurable deficit in mucosal immunity and increased risk of systemic disease.

In this study, cryptdin biosynthesis was investigated by characterizing details of intracellular procryptdin processing in

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† The abbreviations used are: MMP-7, matrix metalloproteinase-7 (matrilysin); PBS, phosphate-buffered saline; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; RP-HPLC, reverse-phase high performance liquid chromatography; AU-PAGE, acid/urea-polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PIPES, 1,4-piperazinediethanesulfonic acid; PT, post-transplantation day.
mouse Paneth cells. The products of in vitro cleavage of pro- 
cryptdin-1 and natural procryptdins by MMP-7, the localiza- 
tion of the cryptdin proregion in the exocytotic pathway, and 
the extent of procryptdin activation in Paneth cells of adult 
mice have now been characterized. Our results show that 

**Experimental Procedures**

**Animals and Tissue Preparation**—All procedures on mice were 
performed in compliance with the policies of the Institutional Animal Care 
and Use Committee of the University of California, Irvine (UCI). 45-
Day-old male outbred Swiss mice (Crl:CD-1(ICR/BR)), 6-week-old 
male BALB/cJ and C57/BL6 mice, 6-week-old female 
BALB/cJ mice, and 6-week-old adult germ-free Swiss mice were 
prepared from Charles River Breeding Laboratories, Inc. (Wilmington, 
MA). Matrigel (MMP-7)-null mice were 6–8-week-old males back-
crossed for 10 generations into the C57/BL6 background. Mice 
were housed under 12-h cycles of light and dark and had free access 
to standard rat chow and water.

For isolation of small intestine implants, pregnant BALB/cJ 
mice were killed at 15–17 days of gestation by injection with 600 μg 
of Avertin (500 mg of tribromoethanol and 250 mg of 2-methyl-2-butanol 
in 39.5 ml of water/v of body weight. Segments (1–2 cm) of proximal 
small intestine from each fetus were implanted aseptically under 
dorsal subcutaneous skin flaps of individual 6-week-old isogenic male 
BALB/cJ mice (22, 23). Approximately 90% of the implants grew and 
were harvested for isolation of RNA or protein, or they were fixed by 
immersion in phosphate-buffered Formalin. Fixed tissue was processed 
to paraffin blocks; sectioned; and stained with hematoxylin and eosin 
by the Histology Laboratory of the Department of Pathology, University 
of California Irvine Medical Center.

**Preparation of Small Intestine Crypts**—Crypts were prepared by 
EDTA treatment of everted small intestine segments as described (15, 
24–29). Segments of adult intestinal bowel were agitated in 
Hanks’ solution under the same conditions. Enteric α-defensins derive exclusively from Paneth cells in crypts (15, 27, 
28). Briefly, segments of adult mouse small bowel were depaразinized with 
sodium citrate-buffered 30% acetic acid, sonicated, and then centrifuged to 
resuspended in ice-cold 30% acetic acid, 15, 24–29. At 10 min, 10,000 rpm in a Sorvall SA-600 rotor, and granules 
were deposited. Supernatants were clarified by centrifugation for 2 h at 28,000 rpm in a Beckman SW 28.1 rotor, and high speed centrifugation was diluted 10-fold and lyophilized (21).

**Preparation of Paneth Cell Secretory Granules**—Subcellular fractions 
enriched in Paneth cell secretory granules were prepared from duodenal 
and ileal crypts. Crypts were isolated by centrifugation at 700 rpm for 
5 min in a Beckman GS-6R centrifuge were centrifuged in 10 ml of 
ice-cold 10 mM CaCl2/150 mM MgCl2–free buffered saline (PBS, Invitrogen) 
at pH 7.5 and placed under N2 at 750 s.p.i. for 15 min in a Model 1019HC 
nitrogen cavitation bomb (Parr Instrument Co., Moline, IL). Cell lysates 
were produced by equilibrium to atmospheric pressure were diluted 2-fold 
with PBS containing 5 mM EDTA and centrifuged at 700 × g for 10 min at 4 °C. Low speed supernatants and the deposited cell debris were washed by resuspension in ice-cold Hanks’ EDTA solution 
(Invitrogen) and centrifugation at 700 × g for 10 min at 4 °C. Brain 
fluids in the combined supernatants were deposited by centrifugation 
at 27,000 × g for 40 min at 4 °C in the Sorvall SA-600 rotor, and granules 
in the high speed pellet were washed two times by resuspension and 
centrifugation in Hanks’ EDTA solution under the same conditions. 
Granules were stored frozen or dissolved immediately in 30% acetic acid 
and extracted as described above.

**Acid-Urea-Polyacrylamide Gel Electrophoresis**—Lyophilized peptide 
samples were dissolved in 20 μl of 5% acetic acid containing 3.0 M urea 
and electrophoresed on 12.5% acid-urea-polyacrylamide gels for 6 h at 
150 V (29). Resolved proteins were visualized by staining with Coomas- 
sie Blue R-250 after fixation in Formalin-containing acetic acid/meth- 

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al. α-Defensins were identified by their rapid comigration with au-
purified to homogeneity by C_{18} RP-HPLC using a 120-min 10–40% acetonitrile gradient, from which cryptdin precursors eluted between 18 and 30% acetonitrile (data not shown).

The identification of the purified proteins as cryptdin precursors was achieved by N-terminal sequencing and MALDI-TOF-MS. Peptide concentrations were determined using the Bradford assay (Bio-Rad), and the molecular masses of purified putative procryptdins were determined by MALDI-TOF-MS, followed by sequencing in the UCI Biomedical Protein and Mass Spectrometry Resource Facility.

**MMP-7 Cleavage of Mouse Procryptdins in Vitro**—Recombinant cryptdin-1 and natural procryptdins were digested with MMP-7 and analyzed by AU-PAGE and SDS-PAGE, and mixtures of proteolytic digests from MMP-7 cleavage were analyzed by N-terminal sequencing. Samples (1 μg) of recombinant procryptdin-1 (21) and of natural procryptdins A–C purified from MMP-7-null mice were incubated with equimolar quantities of activated recombinant human MMP-7 catalytic domain (Chemicon International, Inc., Temecula, CA) in buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, and 5 mM CaCl_{2} for 24 h at 37 °C. Reactions were analyzed on Tris/Tricine/SDS-15% polyacrylamide gels (Bio-Rad). Curiously, proregions or fragments of proregions were not seen by routine gel staining methods after digestion with MMP-7, even though procryptdins and cryptdin peptides stained well (see Fig. 3A). Samples (200 ng) of complete digests were subjected to eight cycles of N-terminal peptide sequencing at the UCI Biomedical Protein and Mass Spectrometry Resource Facility.

**Western Blot Analyses of Paneth Cell α-Defensin Precursors**—Proteins extracted from adult outbred Swiss mouse crypts were resolved by AU-PAGE, transferred to 0.2-μm nitrocellulose membranes, blocked, and incubated with sheep anti-cryptdin-1 prosegment IgG diluted 1:2000 in Tris-buffered saline/Tween containing 5% nonfat milk at room temperature with agitation (21). Washed blots were incubated with peroxidase-conjugated donkey anti-sheep antibody diluted 1:5000 in Tris-buffered saline/Tween for 30 min, washed, and developed using Supersignal chemiluminescent substrate (Pierce) with a 10–15-min exposure (21). In Western blotting using rabbit anti-cryptdin-1 peptide antiserum, goat anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution (15).

**Assays of Bacterial Peptide Activity**—To measure bactericidal activities, 1 × 10^{6} exponentially growing *E. coli* ML35 cells were incubated with 5 μg/ml synthetic cryptdin-3 or recombinant cryptdin-4 in 10 mM PIPES (pH 7.4) with quantities of prosegment, corresponding to preprocryptdin-1 residues 19–58 (see Fig. 1A). After 60 min at 37 °C, 20 μl of each incubation mixture was diluted 1:2000 with 10 mM PIPES (pH 7.4), and 50 μl of the diluted samples was plated on trypticase soy agar using a Spiral Biotech Autoplate 4000 (Spiral Biotech, Inc., Bethesda, MD). Surviving bacteria were quantitated as colony-forming units/ml on plates after incubation at 37 °C for 12 h.

**RESULTS**

**Cryptdin-1 Prosegment in Mouse Paneth Cells**—A sheep polyclonal antibody raised against the full-length synthetic cryptdin-1 prosegment (Fig. 1A) reacted specifically with preprocryptdin-1 and with procryptdins in extracts of mouse small intestine proteins (Fig. 1). Western blot analysis (see “Experimental Procedures”) showed that the antibody was specific for procryptdins in intestinal protein extracts, which comigrated with recombinant procryptdin-1 (21) (Fig. 1B). Because mouse defensin family proregions have extensive sequence similarity (Fig. 1A), these data are probably a measure of immunoreactivity with procryptdin-1 and with the many defensin and defensin-related precursors expressed by mouse Paneth cells (33–35, 37).

In small intestine, cryptdin transcripts and peptides previously have been found only in Paneth cells (9, 15, 27, 28, 38–42); and consistent with those findings, immunoperoxidase detection of the cryptdin-1 prosegment showed that it also is Paneth cell-specific (Fig. 1C). This finding is in agreement with immunolocalization of the related mouse CRS1C prosegment (Fig. 1A) using a rabbit polyclonal antibody to the CRSIC-1 proregion (36). The reactive cryptdin prosegment antigen appeared to be associated with secretory granules, prompting immunolocalization studies at the electron microscopic level.

**Cryptdin Precursors in Paneth Cell Secretory Granules**—Defensin prosegments as well as cryptdin peptides are constituents of murine Paneth cell secretory granules. The subcellular location of cryptdin precursors within Paneth cells of mouse mid-small bowel was determined using appropriate gold-conjugated protein A or second antibodies. As shown in Fig. 2 (A–C), both the anti-prosegment and anti-cryptdin peptide antibodies reacted strongly and specifically with Paneth cell granules. Preimmune negative control sera had very low back-

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2 D. P. Satchell and A. J. Ouellette, unpublished data.
3 A. J. Ouellette, unpublished data.
Fig. 2. Immunostaining of mouse Paneth cells with anti-cryptdin and anti-segment antibodies. A, shown is an electron micrograph of an adult mouse small intestine crypt. Paneth cells (P) reside at the base of the crypt and are surrounded by undifferentiated crypt...
ground staining (Fig. 2C, inset). With both anti-prosegment and anti-cryptdin peptide antisera, the respective antigens first were detected in the trans-Golgi of the Paneth cell exocytotic pathway (Fig. 2B, inset). Cytoplasmic staining was highly specific for the electron-dense region of secretory granules (Fig. 2, B and C). The electron-lucent halos of Paneth cell granules, which contain high levels of O-linked GalNAc glycoconjugates (43), showed very little gold staining (Fig. 2, B and C). All granules were immunoreactive, and staining was uniformly equivalent regardless of subcellular localization, as shown by quantitation of gold particles over apical or supranuclear granules (Fig. 2D). Despite showing that the anti-prosegment antibody reacted with Paneth cell granules, these findings did not distinguish unprocessed procryptdins from soluble proregions generated by MMP-7 proteolysis of cryptdin precursors. To resolve this question, the products of MMP-7 hydrolysis of procryptdins in vitro and the status of procryptdin activation in Paneth cells in vivo were investigated in detail.

**Specificity of in Vitro Procryptdin Cleavage by MMP-7—** Because Paneth cell α-defensin processing intermediates had not been characterized, mouse procryptdins were purified from MMP-7-null mouse small intestine as substrates for analysis of the MMP-7 cleavage products. MMP-7-null mice are an optimal source for cryptdin precursor purification because cryptdin gene expression occurs at wild-type levels, and procryptdins accumulate in MMP-7-deficient Paneth cells (21). Also, studies of natural substrates avoid potential complications of analyzing possibly misfolded recombinant cryptdin precursors.

Putative mouse procryptdins A–C were purified to homogeneity by combined C₄ and C₁₈ RP-HPLC (see “Experimental
The molecular masses of putative natural procryptdin precursors were determined by MALDI-TOF-MS, and N-terminal sequences were determined by Edman degradation of samples before and after digestion with MMP-7 (see “Experimental Procedures”). N-terminal residue assignments are based on the primary structure of preprocryptdin-1 (Fig. 4B), with residue 1 at the initiating Met residue. ND, not determined.

| Protein      | Mass | Undigested Amino-terminal sequences | MMP-7 digests N terminus |
|--------------|------|------------------------------------|--------------------------|
| Procryptdin  | ND   | DPIQNTD                            | Asp<sup>19</sup>          |
| A            | 8543 | DPIQNTD                            | Val<sup>14</sup>          |
| Procryptdin  | 8478 | DPIQNTD                            | Leu<sup>20</sup>          |
| B            | 8277 | DPIQNTD                            | Asp<sup>19</sup>          |
| Procryptdin  | C    | DPIQNTD                            | Val<sup>14</sup>          |
| C            |      | VSFGDPEG                            | Leu<sup>16</sup>          |

The peptide bonds cleaved in procryptdins A–C by MMP-7 were determined by direct N-terminal sequencing of MMP-7 digests of the precursors (Fig. 3B). For each putative procryptdin, only three N termini were detected besides that of the activated MMP-7 enzyme (Fig. 3B and Table I). The first N-terminal sequence was DPIQNTD . . . , the consensus procryptdin N terminus (Table I). The second sequence was VSFGDPEG . . . , an internal cleavage site between Ser<sup>43</sup> and Val<sup>44</sup> in the prosegment (Fig. 3B and Table I). The VS FG sequence flanking the cleavage site (Fig. 3B, asterisk) within the prosegment is conserved in all mouse defensin family precursors (32, 33, 37) (Fig. 1A). Analysis of procryptdins A–C by SDS-PAGE following cleavage with MMP-7 in vitro produced only one evident primary cleavage product of appropriate mobility for mature α-defensin peptides (Fig. 3A).

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**Activated α-Defensins in Mouse Paneth Cell Secretory Granules**—The co-localization of prosegments and cryptdins in secretory granules (Figs. 1C and 2) prompted an evaluation of the processing status of cryptdin precursors in Paneth cell granules. The distribution of cryptdins and procryptdins in Paneth cell secretory granules was determined by AU-PAGE and Western blotting (15, 31). On acid/urea-polyacrylamide gels, activated α-defensins were the most rapidly migrating intestinal peptides (see “Experimental Procedures”), and they are lacking in MMP-7-null mice (21) (Fig. 4A). Previously, only low levels of procryptdins were detected in secretions elicited from Paneth cells by carbamylcholine exposure (15). Partially purified Paneth cell secretory granules contained abundant activated cryptdins at levels equivalent to those in intact crypts (Fig. 4B, lane 2). Inclusion of a complex of potent proteinase inhibitors in all solutions and buffers during crypt isolation, granule sedimentation, and protein extraction (see “Experimental Procedures”) had no effect on the apparent levels of activated cryptdins (Fig. 4C), a fact taken as evidence that procryptdin processing was not caused by experimental manipulation.

The relative distribution of cryptdins to procryptdins was evaluated by Western blot analysis of Paneth cell granule proteins from wild-type and MMP-7-null mice using an anti-cryptdin-1 peptide antibody (15, 28). As predicted from previous analyses of whole mouse small bowel proteins (21), Paneth cell granules from MMP-7-null mice lacked rapidly migrating activated cryptdins, but contained high levels of procryptdins (Fig. 4D, lane 2). In contrast, granule proteins from wild-type C57/BL6 mice gave strong immunoreactivity at the position of cryptdin mobility, where the signal strength was approximately twice that of the procryptdin region (Fig. 4D). From these considerations and because cryptdins gave weaker immunostaining upon Western blotting compared with equimolar quantities of procryptdins, we estimate that 60–70% of the procryptdins in Paneth cells are processed to functional peptides before secretion (see “Discussion”). Because extensive procryptdin processing is intracellular (Fig. 4), prosegments in granules (Fig. 2) are subject to secretion, suggesting that prosegments might inhibit the bactericidal activities of activated cryptdin peptides.

**Soluble Prosegment Neutralizes Cryptdin Bactericidal Activity in Vitro**—The cryptdin-1 prosegment lacks antimicrobial activity, but it inhibited the bactericidal activity of mature cryptdin peptides in trans. The ability of the soluble cryptdin-1 propeptide, corresponding to residues 19–58 in the cryptdin-1 precursor (Fig. 1A), to inhibit the activity of cryptdin-3 and -4 was tested in bactericidal assays against E. coli ML35 cells. In agreement with the inhibition of myeloid α-defensins by human neutrophil proregions (20), prosegment/cryptdin molar ratios of 0.5–1 or greater inhibited both peptides to approxi-
Intracellular Processing of Mouse Procryptdins

Fig. 4. Intracellular processing of mouse Paneth cell α-defensin precursors. A, samples (250 µg) of protein extracts from adult mouse small intestine were resolved by AU-PAGE, and gels were stained with Coomassie Blue (see "Experimental Procedures"). Lanes 1 and 3, extracts from MMP-7-null mice lack activated defensins (boxed); lanes 2 and 4, extracts from wild-type C57/BL6 mice. B, activated cryptdins are shown in proteins extracted from combined duodenal and ileal Paneth cell granules (see "Experimental Procedures") after resolution by AU-PAGE and staining with Coomassie Blue R-250. Lane 1, extract from intact crypts; lane 2, granule extract; lane C1, 1 µg of cryptdin-1; lane C3, 1 µg of cryptdin-3; lane C4, 1 µg of cryptdin-4. C, proteins extracted from secretory granules prepared from adult mouse crypts in the absence (lane 1) or presence (lane 2) of Protease Inhibitor Mixture Set III (see "Experimental Procedures") were subjected to AU-PAGE. Equivalent quantities of protein were electrophoresed, and the gel was stained with Coomassie Blue. Lanes C1, C3, and C4, 1 µg of cryptdin-1, -3, and -4, respectively. D, proteins from Paneth cell granules purified from wild-type (lane 1) or MMP-7-null (lane 2) adult mouse small intestine were subjected to AU-PAGE, Western-blotted, and probed with anti-cryptdin-1 antibody. Lanes C1 and C3, 1 µg of cryptdin-1 and -3, respectively. In all panels, the boxed regions denote the positions at which cryptdin peptides migrated in the acid/urea gel system. The arrow on the right indicates procryptdins.

Fig. 5. Cryptdin-1 prosegment neutralizes cryptdin bactericidal activities in trans. The synthetic prosegment, corresponding to residues 19–58 of preprocryptdin-1 (Fig. 1A), was combined with 5 µg of cryptdin-3 (A) or cryptdin-4 (B) in the molar ratios shown and incubated with ~1 × 10^8 E. coli ML35 cells for 60 min at 37 °C, and surviving bacteria were determined by colony counting after overnight growth on semisolid medium (see "Experimental Procedures"). Bars labeled C denote bacterial survival in the absence of cryptdin peptides, and bars labeled Crp3 (in A) and Crp4 (in B) show viability after exposure to 5 µg of cryptdin-3 or -4, respectively, in the absence of the prosegment. CFU, colony-forming units.

Fig. 6. Activated cryptdins in germ-free mice. Proteins extracted from the small intestines of adult germ-free mice (lanes 1 and 2), mice conventionalized for 1 day (lanes 3 and 7 days (lanes 4 and 5), and a conventionally reared mouse (lane 6) were analyzed on an acid/urea-polyacrylamide gel and stained with Coomassie Blue. The boxed region denotes the position of cryptdin peptides. Lanes C1, C3, and C4, 1 µg of cryptdin-1, -3, and -4, respectively.
ingly, subcutaneous growth of fetal intestinal implants provides conditions that favor epithelial cell differentiation in structures that develop to resemble the morphology of normal adult small intestine (22, 23, 47). In our experiments, 90% of the implants grew, and Paneth cells were evident at the base of crypts by 12 days post-transplantation (PT12) as judged by hematoxylin/eosin staining. Paneth cell granules increased in number and size between PT12 and PT19 (Fig. 7A). Reverse transcriptase PCR amplification assays for Paneth cell-specific mRNAs in PT7 to PT28 implant RNAs detected lysozyme; MMP-7; and cryptdin-1, -4, and -5 mRNAs at all time points (Fig. 7B). AU-PAGE analysis of proteins extracted from implants removed on PT7 to PT19 showed that activated cryptdins were evident from PT12 onward, resembling adult levels by PT19. Protein extracts from PT7 implants and MMP-7-null mice lacked activated cryptdins (Fig. 7). Because Paneth cells in implants PT12 or older contain processed cryptdins, luminal exposure to bacterial antigens cannot be required to initiate procryptdin processing. Furthermore, Paneth cells that are naive to luminal bacterial antigen exposure contain MMP-7 in adequate quantities to provide functional cryptdins for secretion.

**DISCUSSION**

In mouse small intestine, a substantial fraction of procryptdin activation occurs in Paneth cells and prior to secretion. This conclusion is supported by evidence from electrophoretic and Western blot analyses of proteins extracted from subcellular fractions enriched in secretory granules, where 60–70% of procryptdins exist already activated by MMP-7-dependent proteolytic cleavage (Fig. 3). This value for the fraction of processed precursors represents an overall average for all cryptdin precursors, with the exception of cryptdin-4 and -5, which do not react with the anti-cryptdin-1 antibody. Interpretation of these data is complicated, though, by the dynamics of crypt cell biology and by the ongoing processes of Paneth cell differentiation and granule biogenesis in the regulated secretory pathway. For example, our findings do not distinguish between the procryptdin activation state in mature granules poised for vesicular fusion at the Paneth cell apical membrane from that in nascent granules that are forming in the trans-Golgi. In addition, we cannot discount the possibility of post-secretory activation of the procryptdin molecules that are secreted. Also, Paneth cells differentiate in crypts over 8 days as they emerge from the stem cell zone and descend to the base of the crypt (25). The extent of procryptdin processing in granules of maturing Paneth cells may differ relative to that in fully differentiated cells at the crypt base.

Questions remain regarding the biology of cryptdin prosegments. For example, the inhibition of cryptdin bactericidal activity by addition of the complete prosegment in trans (Fig. 5) is consistent with comparable dose-dependent inhibition of HNP-1 activity by the recombinant HNP-1 prosegment (pro-HNP-1(20–64)) (20), but paradoxical in view of the bactericidal activity of Paneth cell secretions (15). Perhaps, as suggested for myeloid defensin prosegments (20), cryptdin propeptides may interact with additional chaperones to neu-

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**FIG. 7.** Activated cryptdins in fetal mouse intestinal implants grown subcutaneously. A, implanted tissue removed 5–28 days after implantation (PT5 to PT28) was fixed in buffered Formalin, processed, and stained with hematoxylin and eosin (see “Experimental Procedures”). Arrows indicate granule-containing Paneth cells in crypts of developed implants. B, RNAs from PT7 to PT28 implants were amplified by reverse transcriptase PCR using primers specific for lysozyme; MMP-7; and cryptdin-1, -4, and -5 as reported previously (48, 54). As in neonatal small bowel (48), the Paneth cell marker mRNAs were present in the implanted tissues prior to the appearance of recognizable Paneth cells. The Ad lanes contained products amplified from total RNA from adult mouse small bowel, and the W lanes contained equivalent samples of amplification reactions in which water was substituted for template RNA. C, samples (700 µg) of implant protein extracts were analyzed by AU-PAGE as described in the legend to Fig. 6. Lanes contained proteins from implants taken 7 to 19 days after implantation (PT7 to PT19), intestinal protein extracts from MMP-7-null (−/−) and control wild-type (+/+) mice, and 1 µg of cryptdin-3 (C3) and cryptdin-1 (C1) as noted. The boxed region of the gel shows the position of activated cryptdin peptides.
tralize the potential membrane-disruptive activities of mouse α-defensins as they traverse the Paneth cell Golgi stack during granulogenesis. Because secretory granules containing activated cryptdins also react with anti-prosegrome antibodies (36) (Figs. 1 and 2), the processed proregions or proregion fragments (Fig. 3B) are likely to be released along with activated cryptdins as Paneth cells degranulate. The high bactericidal peptide activity in Paneth cell secretions (15) suggests, however, that proregion inhibitory activity may be neutralized before or during secretion. Possibly, MMP-7-catalyzed proteolysis of proregions between Ser244 and Val444, Ser526 and Leu544 (44), and Ser58 and Leu59 during precursor activation may eliminate the inhibitory capabilities of the complete 39-amino acid proregion tested in our studies. Also, MMP-7 cleaved cryptdins A–C reproducibly, but additional cleavage steps may exist, as suggested by the isolation of apparent procryptdin processing intermediates with LQEESLRDLV N termini from mouse small intestine (44). Those intermediates may be MMP-7 cleavage products that our sequencing experiments did not detect, or they may be procryptdin cleavage products of an endogenous enteric prosegment(s) capable of cleaving the precursors in vivo. Interestingly, preliminary studies of MMP-7 digests of recombinant procryptdin-4 have not detected the proregion cleavage site between Ser42 and Ile43. Instead, an abundant LHEKS N-terminal sequence was found, showing that MMP-7 cleaves procryptdin-4 between Ala66 and Leu544,55, a site that corresponds to the intermediates purified by Putse et al. (44). Thus, in vitro, MMP-7 appears to be capable of generating all known procryptdin processing intermediates.

Paneth cells in germ-free mice have almost undetectable MMP-7 levels, as previously determined immunohistochemically (36). Nevertheless, the base-line level of MMP-7 suffices to ensure normal cryptdin activation (Figs. 6 and 7). Similarly, MMP-7-dependent enteric protease(s) capable of cleaving the precursors between Ser244 and Ile43 exist, as suggested by the isolation of apparent procryptdin processing intermediates with LQEESLRDLV N termini from mouse small intestine (44). However, that proregion inhibitory activity may be neutralized before or during secretion. Possibly, MMP-7-catalyzed proteolysis of proregions between Ser244 and Val444, Ser526 and Leu544 (44), and Ser58 and Leu59 during precursor activation may eliminate the inhibitory capabilities of the complete 39-amino acid proregion tested in our studies. Also, MMP-7 cleaved cryptdins A–C reproducibly, but additional cleavage steps may exist, as suggested by the isolation of apparent procryptdin processing intermediates with LQEESLRDLV N termini from mouse small intestine (44). Those intermediates may be MMP-7 cleavage products that our sequencing experiments did not detect, or they may be procryptdin cleavage products of an endogenous enteric prosegment(s) capable of cleaving the precursors in vivo. Interestingly, preliminary studies of MMP-7 digests of recombinant procryptdin-4 have not detected the proregion cleavage site between Ser42 and Ile43. Instead, an abundant LHEKS N-terminal sequence was found, showing that MMP-7 cleaves procryptdin-4 between Ala66 and Leu544,55, a site that corresponds to the intermediates purified by Putse et al. (44). Thus, in vitro, MMP-7 appears to be capable of generating all known procryptdin processing intermediates.

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