Structural Determinants of Procryptdin Recognition and Cleavage by Matrix Metalloproteinase-7*

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The bactericidal activity of mouse Paneth cell α-defensins, or cryptdins, is dependent on processing of cryptdin precursors (pro-Crps) by matrix metalloproteinase-7 (MMP-7) (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). To investigate the mechanisms of pro-Crp processing by this enzyme, recombinant pro-Crp4, a His-tagged chimeric pro-Crp (pro-CC), and site-directed mutant precursors of each were digested with MMP-7, and the cleavage products were analyzed by NH2-terminal peptide sequencing. Proteolysis of pro-Crp4 with MMP-7 activated in vitro bactericidal activity to the level of the mature Crp4 peptide by cleaving pro-Crp4 at Ser43, Ile44 and Ala53, and Leu64 in the pro-region and near the Crp4 peptide NH2-terminus between Ser58 and Leu89. Because the Crp4 NH2-terminus occurs at Gly89, not Leu99, MMP-7 is necessary but insufficient to complete the processing of pro-Crp4. Crp activating proteolysis at S58 and L59 was unaffected by I44S/I44D or L54S/L54D loss-of-function mutations in pro-Crp4, and a (L58S)-pro-Cr cc mutant was cleaved normally at Ser43, Val44 and Ser53 sites but not at the peptide NH2-terminus. C57BL/6 mice contain an abundant (L58S)-Crp4 mutant peptide with Leu44 at its NH2-terminus resulting from Ala53, Leu44 cleavage and loss-of-function at the Ser58, Ser59 cleavage site. Thus, α-defensins resulting from mutations at MMP-7 cleavage sites exist in mouse populations. A pro-CC substrate containing both L54S and L59S mutations resisted cleavage at Ser43, Val44 completely, showing that cleavage at one or both downstream sites must precede proteolysis at Ser43, Val44. These findings show that MMP-7 activation of pro-Crps can occur without proteolysis of the proregion, and prosegment fragmentation depends, at least in part, on the release of the Crp peptide from the precursor.

The release of endogenous antimicrobial peptides by mammalian epithelial cells contributes to innate mucosal immunity (1, 2). In the small intestine of most mammals, Paneth cells that reside at the base of the crypt synthesize and secrete microbicidal α-defensins, termed cryptdins in mice, as components of apical secretory granules (3–6). The granules are released by Paneth cells in response to cholinergic agonists or when exposed to bacterial stimuli (7–10). Cryptdin peptides constitute ~70% of the bactericidal peptide activity released by mouse Paneth cells, and cryptdin concentration at the point of secretion is at least 1000 times greater than the minimal bactericidal concentration of the peptides (9). The production of functional α-defensins involves proteolytic processing of inactive precursor forms by mechanisms that differ between mice and humans (5, 11).

α-Defensins are processed from inactive proforms by specific proteolytic cleavage steps. Both neutrophil and Paneth cell α-defensins derive from ~10-kDa prepropeptides that contain canonical signal sequences, acidic proregions, and a ~3.5-kDa mature α-defensin peptide in the COOH-terminal portion of the precursor. Most pro-α-defensins are fully processed in mature phagocytic leukocytes (12, 13), and processing of myeloid α-defensin precursors occurs within 4–24 h after synthesis by apparently sequential events that produce major intermediates of 75 and 56 amino acids (12–14). Deletions in the prosegment adjacent to the proregion-defensin junction impaired post-translational processing of human neutrophil pro-α-defensins when expressed heterologously in mouse 32DCL3 cells (13). The anionic propeptide segments also appear to neutralize the cationic COOH-terminal defensin peptides, as suggested by the inhibition of in vitro α-defensin bactericidal activity when intact proregions are added in trans (5, 14, 15). Human Paneth cells store HD-5 α-defensin in precursor form that is converted rapidly by trypsin to the mature HD-5 peptide after secretion (10, 11, 16), but mouse Paneth cell pro-Crps are processed and activated by trypsin in vitro (17), where the enzyme activates all α-defensins from 8.4-kDa proforms (18). Previously, both procryptdin-1 (pro-Crp1) and a COOH-terminal His4 tagged pro-Crp chimera (pro-

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1 The abbreviations used are: MMP-7, matrix metalloproteinase-7; Crp, cryptdin; pro-Crp, procryptdin; MALDI-TOP 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; N-NTA, nickel-nitrilotriacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PIPES, 1,4-piperazinediethanesulfonic acid; CFU, colony forming units; pro-CC, chimeric pro-Crp.

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CC) containing sequence from pro-Crp1, pro-Crp4, and pro-Crp15 were shown to be activated to 3.5 kDa α-defensins in *vitro* by MMP-7-catalyzed cleavage at conserved sites in the proregion and at the junction of the propeptide and the NH2 terminus of the mature cryptdin peptide (5, 18). In those studies, MMP-7 was found to cleave between Ser43 and Val44 in the prosegment and at Ser59 and Leu59, where Leu59 is the NH2-terminal residue for all known mouse cryptdins except Crp4 and Crp5 (5). Additional preliminary evidence showed that MMP-7 cleaved pro-Crp at Ala53 and Leu54 (5), a site that corresponds to processing intermediates isolated from mouse small intestine (19). Thus, mouse pro-Crps contain conserved sites within the precursor proregion that MMP-7 recognizes and cleaves, but their role in pro-Crp activation is uncharacterized.

In this study, cryptdin biosynthesis was investigated by analyzing the processing of recombinant pro-Crps by MMP-7 in *vitro*. We focused on Crp4, because it is the most bactericidal *in vitro* of Crps (19). Thus, mouse pro-Crps contain conserved sites in the mature domain. Mouse pro-Crp4 contains conserved sites between the proregion and at the junction of the propeptide and the NH2-terminal His6 tag, a 28-amino acid spacer, and pro-Crp4, were dialyzed against 5% acetic acid and lyophilized. Lyophilized proteins dissolved in 80% formic acid were adjusted to 10 mg/ml CNBr in 80% formic acid and incubated under N2 overnight at room temperature. Cleavage was terminated by addition of 5 M guanidine HCl, proteolyzed, diafiltered against 10 volumes of H2O, proteins were lyophilized, dissolved in 6 M guanidine HCl, and purified by C-18 RP-HPLC by eluting peptides over 120 min with a 20–40% acetonitrile gradient. The identities of recombinant pro-Crp4 molecules were verified by MALDI-TOF MS at the UCI Biomedical Protein and Mass Spectrometry Resource Facility and by acid-urea PAGE (21).

**Site-directed Mutagenesis of Pro-Crps**—Mutations were introduced into recombinant pro-Crp4 molecules by PCR. In reaction 1, the mutant forward primer, e.g., pc4I44Dfor, containing the mutant codon flanked by three natural codons was paired with SLpMALCrp4R, the normal reverse primer at the 3’-end of the desired sequence. In reaction 2, the mutant reverse primer, e.g., pc4I44Drev, the exact complement of the mutant forward primer with the reverse orientation was paired with the vector primer pETPCr4-F at the 5’-end of the desired sequence, and sequences were amplified from the pET-28a pro-Crp4 construct as described above: 95 °C for 5 min, followed by successive cycles at 60 °C for 1 min, 72 °C for 1 min, 94 °C for 1 min for 40 cycles. Products from reactions 1 and 2 were purified electrothermally, and 0.5–1 µl samples of gel-purified DNA were combined as templates in PCR reaction 3, using normal external primers, SLpMALCrp4R and pETPCr4-F, as amplifiers. The full-length, mutated pro-Crp4 product of reaction 3 was cloned sequentially into the vectors pCR2.1-TOPO and pET-28a as noted above, and all mutations were verified by DNA sequencing prior to expression.

**Purification of Recombinant Mouse Pro-Crp4**—Recombinant pro-Crp4 and pro-Crps variants with mutated MMP-7 recognition sites were prepared by the pET-28a expression system to produce NH2-terminal His6-tagged fusion proteins (Novagen, Madison, WI). By PCR amplification, a Met-coding trideoxynucleotide was incorporated 5′-ATATATGTCGACTGTTCAGCGGCGGGGGCAGCAGTACAA), corresponding to nucleotides 104–110 and 301–327 in prepro-Crp4 cDNA (20). Reactions were performed using the GeneAmp PCR Core Reagents (Applied Biosystems, Foster City, CA) by incubating the reaction mixture at 94 °C for 5 min, followed by successive cycles at 60 °C for 1 min, 72 °C for 1 min, and 94 °C for 1 min for 40 cycles. The amplified products were cloned in pCR2.1-TOPO, sequenced, digested with EcoRI and SalI, and gel-purified EcoRI/SalI inserts were ligated into EcoRI and SalI-digested pET-28a plasmid DNA, and transformed into both *Escherichia coli* XL-2 Blue and BL21(DE3) Codon Plus cells (Strategene Cloning Systems, Inc., La Jolla, CA). Recombinant precursors were expressed for 6 h at 37 °C in *E. coli* BL21(DE3) Codon Plus cells growing exponentially in Terrific Broth medium by induction with 0.2 µM isopropyl-1-thio-β-D-galactopyranoside under kanamycin selection. Bacterial cells were harvested by centrifugation and stored at −20 °C. Cells were lysed in 6 M guanidine HCl, 100 mM sodium phosphate (pH 8), and clarified by centrifugation in a Sorvall SA-60 rotor at 30,000 × g for 30 min at 4 °C. Fusion proteins were purified immediately after lysozyme clarification.

Recombinant precursor fusion proteins were purified by nickel-nitrotriacetic acid (Ni-NTA, Qiagen) resin affinity chromatography and recovered from fusions after CNBr cleavage. His-tagged fusion proteins were eluted from Ni-NTA resin with 2 column volumes of buffer consisting of 6 M guanidine HCl, 1 M imidazole, and 100 mM Tris-HCl (pH 6.0). Fusion proteins, containing an NH2-terminal His6 tag, a 28-amino acid spacer, and pro-Crps, were dialyzed against 5% acetic acid and lyophilized. Lyophilized proteins dissolved in 80% formic acid were adjusted to 10 mg/ml CNBr in 80% formic acid and incubated under N2 overnight at room temperature. Cleavage was terminated by addition of 5 M guanidine HCl, proteolyzed, diafiltered against 10 volumes of H2O, proteins were lyophilized, dissolved in 6 M guanidine HCl, and purified by C-18 RP-HPLC by eluting peptides over 120 min with a 20–40% acetonitrile gradient. The identities of recombinant pro-Crp4 molecules were verified by MALDI-TOF MS at the UCI Biomedical Protein and Mass Spectrometry Resource Facility and by acid-urea PAGE (21).

**Purification of Recombinant Mouse Chimeric Pro-Cc**—The precursor pro-Cc was baculovirus expressed. The unmodified Crp15 precursor was described (18). Briefly, prepro-Crps15 cDNA was amplified using a forward primer derived from sequence encoding the signal peptide and a reverse primer that changed a Met residue in the COOH terminus of the mature peptide to a Thr (characteristic of Crp1). In addition, the COOH-terminus Arg residue of the precursor was converted to Pro-Arg using the *In Vitro* Site-directed Mutagenesis system (Stratagene). The amplified product was cloned into *EcoRI* and *SalI* restriction sites of pET-28a (20). The amplified sequence was cloned into the transfer vector pVL1393 and transfected into SF9 insect cells along with BaculoGold DNA (BD Pharmingen, La Jolla, CA) to produce recombinant baculovirus. Following a 5–8-day infection of HighFive insect cells (Stratagene) with recombinant baculovirus, cells were harvested by centrifugation and stored at −20 °C. Pellets were thawed and lysed in 6 M guanidine HCl, 100 mM sodium phosphate (pH 8), and 10 mM Tris-HCl (pH 8). Lysates were passed several times through an 18-gauge needle and centrifuged at 4 °C in a Sorvall SS-34 rotor at 12,000 × g. Supernatants were incubated batchwise at room temperature with Ni-NTA resin.
Acid Urea-Polyacrylamide Gel Electrophoresis—Peptide samples were lyophilized, dissolved in 20 μl of 5% acetic acid containing 3.0 M urea, and electrophoresed on 12.5% AU-PAGE for 1 h at 100 V and for 3.5 h at 250 V (21). Resolved proteins were visualized by staining with Coomassie R-250 after exposure in formalin-containing acetic acid/methanol 30/70. Peptide samples were analyzed on analytical C-18 RP-HPLC columns (Vydac 218TP54) in aqueous 0.1% trifluoroacetic acid and eluted at ~35 μl/min using a 10-45% acetonitrile gradient developed over 55 min. Protein fractions containing apparent pro-Crp4 were analyzed by acid urea-(AU)-PAGE as described (18, 21), and their identities were deduced from a combination of NH2-terminal peptide sequencing. Mass spectrometry was used to confirm the predicted sequences and to establish cleavage of pro-Crp4 with MMP-7. Samples were subjected to 5 or more cycles of NH2-terminal peptide sequencing to establish the primary structure. DPIQNTDIEEQRPGEDQAVSFVGFDPETSTLQES, was synthesized by Quality Controlled Biochemicals, Inc., (Hopkinton, MA). The composition and properties of the synthetic sequence has been reported previously (5).

Bacterial Peptide Assays of Pro-Crp4 Activated by MMP-7—The activation of pro-Crp4 was assayed by conducting bacterial peptide assays as described for pro-Crps and pro-Crps. Brucella melitensis was used as a model organism for assessing the bactericidal properties of the synthetic prosegment has been reported previously (5).

MMP-7 Cleavage of Mouse Pro-Crps in Vitro—Recombinant pro-Crp4 and pro-CC molecules were digested with MMP-7, analyzed by AU-PAGE and SDS-PAGE, and samples of the proteolytic digest were analyzed by Tricine SDS-PAGE and GelCode Blue (Pierce) staining. If further purification was required, proteins were concentrated to 0.6 ml using FPLC system (Amersham Pharmacia Biotech). Protein concentration and purity were assessed by reducing Tris-Cl/HCl activation of pro-Crp4 was assayed by conducting bactericidal peptide assays as described for pro-Crps and pro-Crps. The native domain (Calbiochem, La Jolla, CA, or Chemicon International, Inc., Temecula, CA) in buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM CaCl2 for 18–24 h at 37 °C. Samples of pro-Crp4 digests were analyzed by AU-PAGE, and ~200 ng quantities of complete digests were subjected to 5 or more cycles of NH2-terminal peptide sequencing at the UCI Biomedical Protein and Mass Spectrometry Resource Facility. Pro-CC digests were analyzed by Tris-Tricine SDS-PAGE (15% polyacrylamide), and bands were visualized using GelCode™ Blue staining reagent (Pierce). For NH2-terminal sequencing by Edman degradation, protein (3 μg) purified from MMP-7 digested by guest on July 24, 2018http://www.jbc.org/Downloaded from
mouse intestinal proteins had identified an apparent pro-Crp processing intermediate with Leu\(^{59}\) as its amino terminus (19). Because Crp4 is the most potent of the mouse α-defensins, and the Crp4 proregion differs from other pro-Crps at several positions at or proximal to the predicted MMP-7 cleavage sites (Fig. 2), we tested whether MMP-7 processed pro-Crp4 in vitro and whether the products would correspond to those produced by hydrolysis of pro-Crp1.

NH\(_2\)-terminal peptide sequence analysis of MMP-7 digests of pro-Crp4 showed that pro-Crp4 contains cleavage sites common to natural pro-Crps (5, 19). Consistently, four NH\(_2\) termini were detected in the digests: DPIQ \(\ldots\), ISFG \(\ldots\), LHEKS, and LRGLL \(\ldots\), where the underscores denote empty sequencing cycles characteristic of Cys residues. These NH\(_2\) termini correspond to Asp\(^{20}\) at the pro-Crp4 NH\(_2\) terminus, Ile\(^{44}\), Leu\(^{54}\), and Leu\(^{59}\) in the polypeptide chain (Fig. 2). These NH\(_2\) termini result from MMP-7 cleavage of pro-Crp4 peptide bonds at Ser\(^{43}\) \(\downarrow\) Ile\(^{44}\) and Ala\(^{53}\) \(\downarrow\) Leu\(^{54}\) in the proregion and at Ser\(^{56}\) \(\downarrow\) Leu\(^{59}\) near the Crp4 peptide NH\(_2\) terminus. When natural pro-Crp6 purified from MMP-7-null mouse small intestine was analyzed similarly after MMP-7 digestion, the NH\(_2\) termini detected were DPIQNT \(\ldots\), VSFGDP \(\ldots\), LQEGES, and LRLDLV \(\ldots\), the same positions identified in pro-Crp4 and in previous reports (19) (Fig. 2) (5). No other MMP-7 cleaved sites were apparent in these studies. Also, MMP-7 digested the 39-residue, synthetic Crp1 proregion at Ser\(^{43}\) \(\downarrow\) Val/Ile\(^{44}\) and Ser/Ala\(^{53}\) \(\downarrow\) Leu\(^{54}\), the same positions cleaved in pro-Crp4 and pro-Crp6 (Fig. 2). This finding shows that the specificity of MMP-7 cleavage of proregion sites is independent of the COOH-terminal presence of an α-defensin. Because the NH\(_2\)-terminal amino acid of natural Crp4 is Gly\(^{61}\), not Leu\(^{59}\), MMP-7 proteolysis is not sufficient to catalyze complete activation of pro-Crp4 to the fully mature Crp4 peptide. The Arg\(^{60}\) \(\downarrow\) Gly\(^{61}\) cleavage step does, however, require prior hydrolysis at Ser\(^{56}\) \(\downarrow\) Leu\(^{59}\) by MMP-7, because MMP-7 null mice lack mature Crp4 with Leu\(^{59}\) or Gly\(^{61}\) NH\(_2\) termini (5, 18). The enzyme that removes the Leu\(^{59}\)-Arg\(^{60}\) dipeptide at Arg\(^{60}\) \(\downarrow\) Gly\(^{61}\) is unknown but may be a trypsin-like aminopeptidase.

MMP-7 Cleavage of Pro-Crp4 Activates Crp4 Bactericidal Activity—To test whether MMP-7 mediated pro-Crp4 proteolysis results in the production of functional Crp4 peptide, bactericidal peptide activity assays were performed on MMP-7-digested pro-Crp4. *Salmonella typhimurium* PhoP(-) cells were combined with 0–3 \(\mu\)M Crp4 or pro-Crp4 that had been digested with a 0.2 mol eq of MMP-7 or incubated without enzyme (“Experimental Procedures”). No bacterial cell killing was detected when cells were exposed to MMP-7 alone (not shown) or to intact pro-Crp4 (Fig. 3). Conversely, quantitative Ser\(^{58}\) \(\downarrow\) Leu\(^{59}\) hydrolysis near the Crp4 peptide NH\(_2\) terminus activated pro-Crp4 to a bactericidal peptide activity level that was equivalent to equimolar quantities of mature Crp4 with the Gly\(^{61}\) NH\(_2\) terminus (Fig. 3). Consistent with the biochemical evidence of pro-Crp4 proteolysis by MMP-7 (Figs. 1 and 2), MMP-7 cleavage produces a functional Crp4 peptide from its inactive precursor. These findings provided rationale for testing whether structural determinants of those cleavage events exist in the precursor molecule.

**Disulfide Bonds Protect the Crp4 Peptide from MMP-7 Proteolysis during Pro-Crp4 Activation**—The formation of disulfide bonds within the pro-Crp4 molecule confers protection of the Crp4 peptide from MMP-7-mediated proteolysis. To determine whether the specificity of MMP-7 recognition and hydrolysis would be modified by disrupting the disulfide array of the Crp4 peptide region in pro-Crp4, the MMP-7 digestion products of native, reduced, and alkylated pro-Crp4 were characterized (“Experimental Procedures”). The extent of Cys alkylation in reduced and alkylated pro-Crp4 was confirmed by MALDI-TOF-MS, which showed that the mass of reduced and alkylated pro-Crp4 was 350.22 atomic mass units greater than that of native pro-Crp4 (theoretical increase = 348.06 atomic mass units), an indication that the 6 Cys residues in pro-Crp4 had been acetylated. The specificity of proteolysis at MMP-7 sites within the prosequence was the same for native pro-Crp4 and reduced and alkylated pro-Crp4 (Fig. 2). However, MMP-7 also cut reduced pro-Crp4 and reduced and alkylated (L54D)-Crp4 substrates at Leu\(^{62}\) \(\downarrow\) Leu\(^{63}\), Cys\(^{64}\) \(\downarrow\) Tyr\(^{65}\), and Phe\(^{67}\) \(\downarrow\) Leu\(^{68}\), all sites within the polypeptide backbone of the Crp4 α-defensin moiety (Fig. 2). From the relative recovery of individual NH\(_2\)-terminal amino acids, these Cys residues in pro-Crp4 were not acetylated. The specificity of MMP-7 proteolysis during pro-Crp4 processing. Effects of Loss-of-function Mutations in Pro-Crp4 and Pro-CC on MMP-7 Processing.—To test whether specific MMP-7-catalyzed proteolysis within the proregion was a requirement for the activating cleavage step at Ser\(^{58}\) \(\downarrow\) Leu\(^{59}\), a series of pro-Crp4 molecules with mutations at Ile\(^{64}\), Leu\(^{54}\), and Leu\(^{59}\) were prepared as substrates for MMP-7 (Fig. 4A, “Experimental Procedures”). Substituting residues 44 or 54 with Asp or Ser (Asp/Ser) ablated MMP-7 cleavage at those positions completely but only at the mutated sites, because cleavage at unaltered Ala\(^{53}\) \(\downarrow\) Leu\(^{54}\) and Ser\(^{56}\) \(\downarrow\) Leu\(^{59}\) sites proceeded normally (Fig. 4B). Curiously, although the I44D/I44S substitutions abrogated cleavage at Ser\(^{43}\) \(\downarrow\) Ile\(^{44}\), MMP-7 cut (I44D/S)-pro-Crp4 variants at an alternative Ser\(^{43}\) \(\downarrow\) Phe\(^{67}\) site that is not cleaved in wild-type pro-Crp4. To eliminate alternative sites for proteolysis at or near Ser\(^{43}\) \(\downarrow\) Ile\(^{44}\) (DEDED)-pro-Crp4 was prepared (“Experimental Procedures”), and MMP-7 cleaved that substrate only at the Ala\(^{53}\) \(\downarrow\) Leu\(^{54}\) and Ser\(^{56}\) \(\downarrow\) Leu\(^{59}\) positions (Fig. 4B). Similarly, the L54D/L54S substitution eliminated cleavage at Ala\(^{53}\) \(\downarrow\) Leu\(^{54}\), but MMP-7 hydrolyzed (L54D/S)-pro-Crp4 normally at Ser\(^{53}\) \(\downarrow\) Ile\(^{44}\) and

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**Fig. 1.** Recognition and cleavage of mouse pro-Crp4 by MMP-7. A 1-μg sample of recombinant pro-Crp4 (“Experimental Procedures”) was incubated overnight without (left lane) or with (right lane) 2 μg of MMP-7. Proteins in the samples were resolved by AU-PAGE and stained with Coomassie Blue (21). Electrophoretic mobilities of individual components are noted at the right.
tested further by analyzing the MMP-7 digestion products of recombinant pro-Crp4, and reduced and alkylated pro-Crp4 were incubated overnight with or without 0.5 mol eq of MMP-7, and digests were analyzed by 5 cycles of NH2-terminal peptide sequencing. Cleavage sites disclosed by protein sequencing are noted by underlined bold number with the initiating Met residue in prepro-Crps as residue position 1. The mature Crp4 and Crp6 peptide sequences are shown as bold underlined text.

Ser58 → Leu59 (Fig. 4B). Note that reduced and alkylated (L54D)-pro-Crp4 also was cleaved at the same sites within the Crp4 moiety as wild-type reduced and alkylated pro-Crp4 (Figs. 2 and 4B).

The activating pro-Crp4 cleavage step at Ser58 → Leu59 does not require cleavage to occur at either MMP-7 recognition site in the proregion. Because MMP-7 processed the Ser58 → Leu59 site when the Ser43 → Ile44 or Ala53 → Leu54 sites were mutagenized individually (Fig. 4B), pro-Crp4 lacking both prosegment cleavage sites was prepared by introducing an L54D mutation in (DEDED)-pro-Crp4. The resulting (DEDED/L54D)-pro-Crp4 molecule was digested quantitatively by MMP-7 at Ser58 → Leu59 (Fig. 4B). Sequencing MMP-7 digests of (DEDED/L54D)-pro-Crp4 failed to detect new NH2 termini that might have been produced by alternative cleavage events associated with the mutagenesis. Thus, even though MMP-7 cleaves the pro-Crp4 proregion with specificity, activation of Crp4 in vitro is independent of the processing steps in the prosegment.

The effects of Ser mutations at Leu54 and Leu59 on MMP-7 recognition of the upstream Val44 site (Ile44 in pro-Crp4) were tested further by analyzing the MMP-7 digestion products of additional chimeric recombinant pro-Crp molecules. For example, pro-CC is a chimeric pro-Crp derived from pro-Crps15, which differs from pro-Crp1 at one amino acid position in the proregion and at 3 residue positions in the mature α-defensin peptide (data not shown). Recombinant pro-Crps containing an L59S mutation, (L59S)-pro-CC, or both L54S and L59S mutations, (L54S/L59S)-pro-CC (Fig. 5A), were produced in the baculovirus expression system and affinity purified from insect cell lysates (“Experimental Procedures”). As shown by SDS-PAGE and NH2-terminal sequencing, MMP-7 cleaved (L59S)-pro-CC at Ser58 → Leu59 but not at Ser58 → Ser59 (Fig. 5B). Furthermore, MALDI-TOF analyses of the digests confirmed cleavage at the Ser58 → Leu59 bond. However, when both Leu54 and Leu59 were converted to Ser in the (L54S/L59S)-pro-CC molecule, the Ser58 → Val44 site no longer was digested by MMP-7 (Fig. 5C). The (L54S/L59S)-pro-CC double mutant resisted cleavage by MMP-7 completely, and no alternate sites to Ser43 → Val44 propeptide could be detected. Consistent with analysis of MMP-7 digested (DEDED/L54D)-pro-Crp4 (Fig. 3D), V44S and L54S mutations in the upstream sites of the pro-CC proregion did not affect the activating cleavage step at Leu59 in pro-CC as determined by NH2-terminal sequencing of MMP-7 digests (data not shown). Thus, MMP-7 hydrolysis of the Ser43 → Val44 site requires both cleavage events at Leu54, Leu59, or both, release a nearly full-length prosegment from pro-CC and (b) that those cleavage steps precede hydrolysis of the Ser43 → Val44 peptide bond.

Mutation of the Ser58 → Leu59 Processing Site in C57BL/6 Mouse Pro-Crps Variants—Paneth cells in C57BL/6 mice contain a Crp4 variant peptide with an L59S mutation that abrogates cleavage at that position by MMP-7. Comparative AU-PAGE analyses of intestinal protein extracts showed that the abundant apparent α-defensins of C57BL/6 mice do not co-migrate with Crps from inbred or outbred strains of mice (Fig. 6A). To investigate the structural basis for the differences, the distinctive C57BL/6 Crp peptides were isolated by RP-HPLC and identified as potential Crps by co-migration with Crp markers in AU-PAGE and by MALDI-TOF MS (Fig. 6B). Comparisons of selected peptides before and after reduction and alkylation with iodoacetamide identified molecules with 6 Cys

Fig. 2. MMP-7 cleavage sites in mouse pro-Crps. Samples consisting of 2 μg each of natural pro-Crp6, synthetic Crp1 prosegment, recombinant pro-Crp4, and reduced and alkylated pro-Crp4 were incubated overnight with 0.5 mol eq of MMP-7, and digests were analyzed by 5 cycles of NH2-terminal peptide sequencing. Cleavage sites disclosed by protein sequencing are noted by downward arrows that interrupt the individual sequences. Numerals below the primary structures refer to residue positions at the beginning of detected NH2-terminal sequences, numbered with the initiating Met residue in prepro-Crps as residue position 1. The mature Crp4 and Crp6 peptide sequences are shown as bold underlined text.
residues as peptides with masses that increased by 344.9 (theoretical value/H11005 348 atomic mass units when acylated).

Two novel Crp4-related peptides purified from C57BL/6 mouse small intestine were characterized by NH2-terminal peptide sequencing. The NH2 terminus of the abundant peptide, Crp4(B6a), was LHEKSSRDLI_Y_RKGG_N-RGEQVYGT_ . . . , where the underscore characters denote deduced Cys residues (Fig. 6C). Analyses of Paneth cell secretory granules partially purified from mouse crypts showed that Crp4(B6a) was present at the same relative concentration as in extracts of intact small bowel, consistent with the localization of all known -defensins in mouse small intestine. In contrast to the abundance of Crp4(B6a), the Crp4(B6b) peptide was recovered only at very low levels from C57BL/6 mouse small bowel, and the NH2-terminal sequence of the peptide was LSRDLI_L_RNR_ . . . (Fig. 6B). A BLASTP search revealed that both sequences corresponded to Crp4-related small intestinal cDNAs in the RIKEN data base (accession numbers AK008107 and AK008266, respectively). Alignment of the peptide sequences, their deduced pro-Crps and pro-Crp4 identified the peptides as Crp4 variants. The Crp4(B6a) and Crp4(B6b) pro-regions resemble the Crp4 prosegment more closely than other Crp prosegments (Fig. 6C), and both peptides lack 3 amino acid residues between the fourth and fifth cysteine residue positions. That 3-codon deletion displaces amino acids that are present in all other -defensins, and the deletion, found only in Crp4, would shorten the loop formed by the Cys2-Cys5 disulfide bond (23–25). Previously, extensive sequencing of intestinal cDNAs and genomic clones from outbred Swiss mice, and C3H/HeJ, BALB/cJ, and 129/SvJ inbred mouse strains had not de-
Loss-of-function Mutation at Ser58

The Crp4(B6b) peptide also harbors an L59S mutation that would abrogate MMP-7-mediated proteolysis at Ser58 → Leu59. In Crp4(B6b), however, an additional S57L mutation appears to have provided an alternative MMP-7 cleavage site at Lys56 → Leu57 that leads to the appearance of Crp4(B6b), although at very low levels. The order in which the S58L and L59S mutations appeared cannot be inferred from these data, but the prospect that the S58L change may have rescued an earlier L59S loss-of-function substitution is an attractive but speculative notion. Whether Crp4(B6b) levels are low because MMP-7 does not cleave pro-Crp4(B6b) efficiently at Lys56 → Leu57 is not known. Prepro-Crp4(B6b) mRNA levels are very low relative to those of other α-defensin mRNAs (data not shown).

Loss-of-function Mutation at Ser58 → Leu59 Is Associated with Attenuated Bacterial Activity—To test whether L59S inactivation of the Ser58 → Leu59 MMP-7 cleavage site has potential effects on innate immunity, the bactericidal activities of Crp4(B6a) and Crp4 were compared against four species of bacteria. Relative to Crp4, Crp4(B6a) was less active against all bacterial species tested (Fig. 7). Against Staphylococcus aureus and Listeria monocytogenes, both peptides had equivalent bacterial cell killing activities at concentrations of 5 μg/ml or greater (Fig. 7B), but Crp4(B6a) had markedly lower activity against Gram-negative E. coli and the defensin-sensitive S. typhimurium PhoP(−) strain (Fig. 7A). Although these experiments show that Crp4(B6a) is less potent than

FIG. 5. Cleavage of pro-CC serine mutants by MMP-7. A, the amino acid sequence of the pro-Crp chimera, pro-CC, is shown compared with mutants (L59S)-pro-CC and (L54/59S)-pro-CC, which contain one and two Leu to Ser substitutions, respectively. Numbering of the residues below the sequence follows the same scheme as in Fig. 3. The residues underlined in the pro-CC sequence are those that differ between pro-CC and pro-Crp1. The arrows indicate the sites of cleavage by MMP-7, with cleavage at the third site in pro-CC liberating the mature peptide (as defined by NH2 termini of small intestinal peptides isolated from outbred Swiss mice (24)). The Ser mutations in (L59S)-pro-CC and (L54/59S)-pro-CC are shown in bold. The arrows in (L59S)-pro-CC indicate the sites of MMP-7 cleavage as determined in B and by protein sequencing. B and C, samples (1 μg) of either pro-CC and (L59S)-pro-CC (B) or (L54/59S)-pro-CC (C) were incubated overnight with (+) or without (−) 2 μg of active human MMP-7, and digests were resolved by SDS-PAGE (15% polyacrylamide) and stained with GelCode Blue. The position of bands corresponding to MMP-7, as well as each precursor (Procryptdin) and its major MMP-7-cleaved form (Cryptdin), are noted at the right. The molecular mass of protein markers is indicated in kDa to the left. Note that the largest prosegment fragment (DPIQ . . . QAVS) derived from MMP-7 cleavage is not visible on these gels because its highly negative charge appears to preclude staining by anionic dyes (5).
Crp4, the differences in activity cannot be attributed solely to the 5 additional amino acids at the peptide NH₂ terminus. Crp4 and Crp4(B6a) are different at 7 residue positions and the composition and length of their COOH termini also differ greatly. Nevertheless, the findings show that mutations at MMP-7 cleavage sites exist in mouse populations and that the mutated processing intermediates can accumulate as abundant peptide variants.
site at Ser53 | Leu54 is completed. Possibly, MMP-7 catalyzed proteolysis in the proregion at Ser26 | Val24 eliminates the ability of the proregion to inhibit Crp bactericidal activity (5) by fragmenting the 39- and 34-amino acid prosegment fragments that are released from pro-Crps by proteolysis at Ser45 | Leu59 or Ser45 | Leu54, respectively. To summarize, proregion fragmentation depends on Crp peptide activation rather than the converse.

In mature human and rabbit phagocytes, α-defensins predominantly exist in the fully processed state, which is mediated by analogous cleavage events (12, 13, 15). Over a 4–24-h period, three primary cleavage events generate HNP-1 major intermediates of 75 and 56 amino acids, as well as the 30-residue mature peptide, in cells of myeloid origin (12, 13, 15). Extensive amino acid deletions from the COOH-terminal region of the pro-HNP-1 propeptide, but not from the NH2-terminal region of the prosegment, impaired pro-HNP-1 processing (13). Both sets of observations are consistent with MMP-7 processing of pro-Crps in that mutations at the COOH terminus affect cleavage upstream. Our results suggest that the Crp prosegment is removed nearly intact then proteolyzed subsequently, whereas pro-HNPs are sequentially truncated at the NH2 terminus; nevertheless, the end result is the same. Although the enzymes that mediate pro-α-defensin processing differ depending on the system, the overall scheme of α-defensin processing is similar in myeloid and epithelial cells. The anionic prosequences of both cryptdins and HNPs inhibit α-defensin bactericidal activity in vitro (5, 15), and thus may confer cytotoxicity during granulogenesis until processing is complete (14).

The molecular details of human Paneth cell α-defensin processing provide interesting contrasts and comparisons with the biology of pro-Crp activation in the mouse. Mouse Paneth cells secrete mature, 3.5-kDa cryptdins as components of secretory granules, because MMP-7-mediated pro-Crp processing takes place intracellularly before secretion (5). On the other hand, human Paneth cells release the α-defensin HD5 precursor, pro-HD5(20–94), into the small intestinal lumen (11), and pro-HD5(20–94) is processed rapidly after secretion by anionic and meso isoforms of trypsin and not by MMP-7, which human Paneth cells lack (10, 11). A tryptic cleavage site in pro-HD5(20–94) at Arg62 | Ala63 gives rise to the major form of the mature peptide found in washes of the small intestinal lumen (11). However, other processing intermediates, resulting from hydrolysis within the prosegment, also have been identified, suggesting that alternative sites may be used. For example, an HD5 peptide with the NH2 terminus Gly94 has been isolated from supernatants of human small intestinal crypts stimulated to release Paneth cell granules with carbamyl choline (10). The extensive differences between the processing of α-defensins by mice and humans suggest that it is the capacity for releasing mature, microbicidal α-defensins that is conserved, and that Paneth cells from mice and humans evolved differing mechanisms to ensure the delivery of functional peptides into the lumen.

The identification of the Crp4(B6a) variant peptide with a loss-of-function L59S mutation (Fig. 6) shows that mouse populations can accumulate defective pro-Crps. These two variants of Crp4 represent the only Crp4 variants identified in inbred populations and revealed that the Crps from the C57BL/6 strain differ markedly from those of 129SvJ, C3H/HeJ, BALB/cJ, and outbred Swiss mice (Fig. 6A, and not shown). This observation should be considered before extrapolating from C57BL/6 genomic DNA and cDNA sequences to other strains of mice.

**DISCUSSION**

In mouse small intestinal Paneth cells, pro-Crp activation is mediated intracellularly by MMP-7, which processes 60–70% of pro-Crps prior to secretion (5). The studies reported here show that the processing reactions include cleavage steps at two conserved sites in the prosegment and a site at or near the Crp NH2 terminus. Despite the specificity of MMP-7 proteolysis at these positions, loss-of-function mutations in the two prosegment cleavage sites had no effect on the activating cleavage event at the Crp peptide NH2 terminus or near that.

**Fig. 7.** Relative bactericidal activities of Crp4 and Crp4(B6a).

Exponentially growing bacterial cells were exposed to the indicated concentrations of Crp4 or Crp4(B6a) for 1 h, and surviving bacteria were quantitated as described in the legend to Fig. 3. In both panels, open symbols denote Crp4 data points, and filled symbols represent Crp4(B6a) data points. In panel A: ▼, Crp4 versus E. coli ML35; ▼, Crp4(B6a) versus E. coli ML35; ◯, Crp4 versus S. typhimurium PhoP–; ○, Crp4(B6a) versus S. typhimurium PhoP–; symbols in panel B: ▼, Crp4 versus L. monocytogenes 104035; ▼, Crp4(B6a) versus L. monocytogenes 104035; ◯, Crp4 versus S. aureus 710a; ○, Crp4(B6a) versus S. aureus 710a.
mice, at least with respect to Paneth cell gene products. One consequence of the L59S mutation \textit{in vivo} is an abundance of the Crp4(B6a) peptide in C57BL/6 small bowel (Fig. 6), a molecule that has less bactericidal peptide activity and is especially attenuated against the two Gram-negative bacterial species tested (Fig. 7). Thus, one possibility is that the production of alternative cryptdin peptides in different strains of mice may contribute to variation in susceptibility to enteric pathogens. If mutations at MMP-7 cleavage sites can persist in mouse populations and if the mutated processing intermediates can accumulate as abundant, attenuated peptide variants, it seems likely that comparable defects could disrupt HD5 or HD6 processing in human Paneth cells. Such mutations could predispose certain individuals to increased susceptibility to enteric bacterial infections, a notion that is speculative but testable.

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