Proteolytic processing of defensins is a critical mode of post-translational regulation of peptide activity. Because mouse α-defensin precursors are cleaved and activated by matrix metalloproteinase-7 (MMP-7), we determined if additional defensin molecules, namely human neutrophil defensin pro-HNP-1 and β-defensins, are targets for MMP-7. We found that MMP-7 cleaves within the pro-domain of the HNP-1 precursor, a reaction that does not generate the mature peptide but produces a 59-amino acid intermediate. This intermediate, which retains the carboxy-terminal end of the pro-domain, had antimicrobial activity, indicating that the residues important for masking defensin activity reside in the amino terminus of this domain. Mature HNP-1 was resistant to processing by MMP-7 unless the peptide was reduced and alkylated, demonstrating that only the pro-domain of α-defensins is normally accessible for cleavage by this enzyme. From the 47-residue HBD-1 precursor, MMP-7 catalyzed removal of 6 amino acids from the amino terminus. Neither a 39-residue intermediate form of HBD-1 nor the mature 36-residue form of HBD-1 was cleaved by MMP-7. In addition, both pro-HBD-2, with its shorter amino-terminal extension, and pro-HBD-3 were resistant to MMP-7. However, human and mouse β-defensin precursors that lack disulfide bonding contain a cryptic MMP-7-sensitive site within the mature peptide moiety. These findings support and extend accumulating evidence that the native three-dimensional structure of both α- and β-defensins protects the mature peptides against proteolytic processing by MMP-7. We also conclude that sites for MMP-7 cleavage are more common at the amino termini of α-defensin rather than β-defensin precursors, and that catalysis at these sites in α-defensin pro-domains results in acquisition of defensin activity.

Small, cationic peptides called defensins represent a major class of antimicrobial agent elaborated by cells of the innate immune system. Defensins are synthesized as precursors of 29 to 42 amino acid residues, of which six are conserved cysteine residues that form three intramolecular disulfide linkages (reviewed in Ref. 1). In humans, α-defensins are primarily found in neutrophils (human neutrophil peptides 1–4, or HNP-1 through -4) and small intestinal Paneth cells (human defensins 5 and 6, or HD-5 and -6), although expression of the intestinal forms has been detected in epithelium of the respiratory and female reproductive tract (2–4), as well as inflamed colonic mucosa and cell lines (5–8) and infected urethra (9). Mouse small intestine contains at least 20 distinct mRNAs for α-defensins, called cryptdins. The β-defensins (human β-defensins 1–4, or HBD-1 through -4, and mouse β-defensins 1–6, or mBD-1 through -6) are more widely expressed than the α peptides and are present in a variety of mucosal epithelium and tissues, including the upper airways, kidney and urogenital tract, and the colon (1).

Although both α- and β-defensins assume a β-sheet structure that is stabilized by disulfide bonding, the pattern of linkages between the cysteine residues differs. In addition, α-defensin precursors contain an amino-terminal pro-region that keeps most of them inactive until this region is proteolytically removed. Pro-HD-5 differs in that it has substantial activity against Listeria monocytogenes and Salmonella enterica serovar Typhimurium in vitro as a precursor (10). In contrast to α-defensins, β-defensins generally have very short amino-terminal extensions and all seem to possess various levels of microbicidal activity in their full-length forms (11–19).

We have been studying how defensin activity is regulated by proteolysis. Previously, we demonstrated that cryptdin precursors are activated by matrilysin, or matrix metalloproteinase-7 (MMP-7) (20). MMP-7 recognizes three sites within the 39-amino acid pro-domain (residues 19–58) of cryptdin precursors: the activating site (site 1) at the junction between the pro-segment and the mature peptide (Ser58 → Leu55), a second site (site
MMP-7 Cleavage of α- and β-Defensin Precursors

2) five residues upstream (Ala53 → Leu54), and a third site (site 3) in about the middle of the segment (Ser43 → Val/Ile44) (21, 22). Mutagenesis of these sites revealed that cleavage at site 1 and/or 2 is required for cleavage at site 3, suggesting that the prosegment is released virtually intact and then is further proteolyzed (22). By contrast, HNP precursors are normally sequentially truncated at the amino terminus during neutrophil ontogeny by an as yet unidentified protease(s) (23, 24). For both the cryptdins and HNP-1, at least one function of the fragmentation of the pro-segment may be to eliminate the ability of this segment to inhibit the mature peptides; cryptdin and HNP prosegments can mask defensin microbicidal activity in cis as well as in trans (25, 26). Of the two human intestinal α-defensins, HD-5 and HD-6, activation of HD-5 in the small intestine has been shown to be mediated extracellularly by an intestinal-specific isof orm of trypsin, although MMP-7 can cleave within the pro-domain (10). In other model systems, an intestinal neobladder and carbachol-stimulated small intestinal crypts, multiple processing intermediates of HD-5 and -6 have been detected (5, 27). Multiple forms of HD-5 were also found in cervicovaginal lavage fluid (2), but how these forms are generated has not been established.

As mentioned previously, β-defensins possess antimicrobial activity in their precursor forms, but amino-terminal processing of the constitutively expressed β-defensin HBD-1 produces intermediates with differences in bactericidal potency (11, 28). Although some of the peptide intermediates overlap with those generated by in vitro digestion of the HBD-1 precursor with chymotrypsin (29), it is not clear what proteases actually give rise to all amino-terminal-truncated forms. Furthermore, proteolysis of mouse β-defensins has not been examined. In this study, we have assessed if MMPs target neutrophil α-defensin precursors and β-defensins. We focused primarily on MMP-7, given its established role in processing and activation of mouse α-defensins and its expression in mucosal epithelium, particularly at sites of infection and inflammation.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant forms of the catalytic domain of human MMP-7 and human MMP-14 (MT1-MMP) were purchased from Calbiochem (La Jolla, CA). MMP-7 was also obtained from Chemicon (Temecula, CA) or generated in our laboratory using the bacterial expression plasmid pET23b+ (Novagen, Madison, WI) and purification procedures as described in Ref. 30. In brief, cDNA encoding the catalytic domain of human MMP-7 was cloned into pET23b+ using the HindIII and NdeI sites. Large scale cultures of BL21(DE3) bacteria harboring the pET23b+-MMP-7 plasmid were lysed and MMP-7 purified from inclusion bodies solubilized in 6 M guanidine HCl, refolded by dialysis (50 mM Tris-HCl, pH 7.5, 10 mM CaCl2, 20 μM ZnCl2, and 0.05% Brij-35), and subjected to heparin-agarose affinity chromatography. HNP-1 peptide was either purchased from Peptides International (Louisville, KY) or purified (see below). The 47-amino acid (full-length) form of HBD-1 was purchased from PeproTech (Rocky Hill, NJ) and the 36-amino acid form from Peptides International. The cryptdin precursor procryptdin-His6, (also known as pro-CC) was purified as described in Ref. 22. Complete EDTA-free inhibitor was obtained from Roche and GelCode® Blue Stain Reagent from Pierce.

Purification of Pro-HNP-1 and HNP-1—Recombinant pro-HNP-1 was produced in baculovirus-infected insect cells and purified as described previously (25). HNP-1 was purified from human neutrophil granule lysate as described elsewhere (31).

Expression of Pro-HNP-1 and MMP-7 in RAW264.7 Cells—Pro-HNP-1 cDNA was inserted into the BamHI site of the retroviral plasmid pBMN-IRE-5-Puro (32). The resulting construct was used for transfection (calcium phosphate method) of the Phoenix-A packaging cell line and production of recombinant amphotropic retrovirus as described previously (32). Briefly, retroviral supernatant was harvested and used to infect RAW264.7 cells for 9 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 mM HEPES, and 4 μg/ml Polybrene. Cells were selected and maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml puromycin. To generate the fusion constructs of the human MMP-7 catalytic domain with the pro-domain of MMP-14, we used a PCR-based gene SOEing strategy (33) with MMP-7 and MMP-14 cDNAs as initial templates. The two primers used for amplification of sequences encoding the MMP-14 pro-pre-domain were: forward primer, 5′-GAA TTC TCT CGG TGA TGG CCC GGT GGT CTC-3′ and reverse primer, 5′-GGG CCT CTT CCT TCG AAC ATT GCC GGC-3′. Primers used to amplify for the human MMP-7 sequence were: forward primer, 5′-GGC AAT CTT GGC TGA TGG CCC GTT GGT GCT CTC-3′ and reverse primer, 5′-GGG GTT CCT GCT CCA AAC ATT GCC GGC-3′. Primers used to amplify for the human MMP-7 sequence were: forward primer, 5′-GGC AAT CTT GGC TGA TGG CCC GTT GGT GCT CTC-3′ and reverse primer, 5′-GGG GTT CCT GCT CCA AAC ATT GCC GGC-3′. The resulting PCR products were then used as both templates and primers in a second PCR. The final PCR product was cloned using the TOPO TA pCR2.1 vector (Invitrogen). EcoRI and Sacl restriction sites were used to replace the corresponding sequence in MMP-7 cDNA. To generate the Glu → Gln inactivating mutation, the gene SOEing technique was once again used. Primer set 1 included forward primer, 5′-GGG ATG GCC GAA AAC ATC TTT CCT CGT TGG CTT GAC GCC GGC-3′ and reverse primer, 5′-ATG GCC AAG TTG ATG AGT TGG TTC AAT GAC CCT CTC ACG-3′, whereas primer set 2 included, forward primer, 5′-TGG AAC TCA TCA ACT TGG CCA T-3′ and reverse primer, 5′-GAA TTC TGC GTG TAT GTA ACA TTT ATT GAC ATC-3′ (mutated bases are underlined). The final product yields full-length cDNA (through poly(A) site) with BamHI and EcoRI restriction sites on the 5′ and 3′ end, respectively. Chimeric cDNA constructs were inserted into the pBMN-IRE-5-Blasta vector (with the puromycin resistance cassette replaced with blasticidin resistance cassette) using EcoRI. The vector was used to generate amphotropic virus for infection of RAW264.7 cells expressing pro-HNP-1 as described above. Blasticidin (Invitrogen) was used at 8 μg/ml for selection of MMP-7 positive cells.

Expression and Purification of Recombinant HBD-1 and HBD-2 (His6-tagged and Non-tagged)—HBD-1 and -2 cDNAs in pBacPAK9 (Clontech) were transferred to pGEM-7ZF(+) (Promega, Madison, WI) using BamHI and EcoRI restriction sites. A carboxyl-terminal His6 tag was added to each by PCR.
amplification: for HBD-1, primers were 5′-GGA TCC TGA GTG TTG CCT GCC-3′ (forward) and 5′-GAA TTC TCA ATG ATG ATG GTG CTT CCC GCC CCT-3′ (reverse), and for HBD-2, primers were 5′-GGA TCC GCT CCC AGC CAT CAG-3′ (forward) and 5′-GAA TTC TCA ATG ATG ATG ATG TGG CTT CCT TTG GCC GAA TT-3′ (reverse). PCR products were cloned using the TOPO TA PCR4 vector (Invitrogen). Inserts with and without sequence encoding the His6 tag were excised using BamHI and EcoRI restriction sites and subcloned into baculovirus expression vector pVL1393 (BD Pharmingen, La Jolla, CA). Vector DNA (5 μg) was transfected into Sf9 cells using the BaculoGold™ DNA system from Pharmingen. Recombinant baculovirus was amplified twice and used for a 4–5-day infection of High Five™ insect cells (Stratagene, La Jolla, CA). Cells and conditioned medium were harvested and stored at −20 °C.

Recombinant His6-tagged protein was purified from cell lysates as described previously (22), using a nickel-nitrilotriacetic acid resin (Qiagen Inc., Valencia, CA) to bind and elute the His6 tag under denaturing conditions. Peak fractions were pooled and dialyzed against 10 mM HEPES, pH 7.5, 0.15 M NaCl, 5 mM CaCl2. For purification of non-tagged defensins, High Five cell-conditioned medium was dialyzed against 12 liters of 20 mM Tris-Cl, pH 7.5, several hours at 4 °C and then a fresh 4 liters overnight. Dialysate was filtered (0.22 μm) and applied to an SP-Sepharose column attached to an ÄKTA FPLC system (Amersham Biosciences). Protein was eluted with a NaCl gradient. Fractions were analyzed by Tris-Tricine SDS-PAGE and GelCode® Blue staining. Appropriate fractions were pooled, concentrated using Centricron-3 ultrafiltration units (Millipore, Bedford, MA), and applied to a Superdex 75 HR/10/30 column (Amersham Biosciences) in 20 mM Tris-Cl, pH 7.5, 50 mM NaCl. Fractions were collected by FPLC and purity (>95%) assessed by Tris-Tricine SDS-PAGE and GelCode Blue staining.

Expression and Purification of Recombinant His6-tagged Mouse β-Defensins—By PCR amplification, a His6 tag was added to the carboxyl terminus of mBD-1 cDNA using the L.M.A.G.E. Consortium clone number 517293 (generously provided by A. Ouellette) as a template, forward primer with sequence 5′-GGATCCGGACCCTGGCTGCCACCACTA-3′, and reverse primer with sequence 5′-GGATCCGGACCCTGGCTGCCACCACTA-3′.

Reduction and Alkylation of Defensins—We employed volatile reducing and alkylating agents in a method described by Hale et al. (34). Reducing/alkylating reagent (97.5% acetonitrile, 2% iodoethanol, and 0.5% triethylphosphine) was prepared under nitrogen in an AtmosBag glove bag (Sigma). Peptides in 0.01% acetic acid (22.5–40 μg in 45 μl) were treated with 5 μl of 1 m ammonium carbonate, pH 11, and 50 μl of reducing/alkylating reagent for 1 h at 37 °C. Reagent was allowed to evaporate in a fume hood. Peptides were dissolved in 0.01% acetic acid and analyzed by Tris-Tricine SDS-PAGE and GelCode Blue staining, then used for cleavage reactions.

Cleavage Reactions—Defensin peptides were incubated with an equal mass or less of active recombinant human MMP-7 catalytic domain in buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM CaCl2 for 18–24 h at 37 °C. For calculation of enzyme-to-substrate molar ratios, we used molecular weights provided by the manufacturer (for commercial peptides) or determined from the ExPASy proteomics server (ca.expasy.org). Digests were resolved by Tris-Tricine gel (15% polyacrylamide) electrophoresis and bands were visualized using GelCode Blue staining reagent. For amino-terminal sequencing by Edman degradation, the mass of the defensin was in 2–3-fold excess over that of MMP-7. Digests were separated by Tris-Tricine SDS-PAGE and transferred to mini-ProBlott polyvinylidene difluoride membrane (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions.

Western Blotting—To assess cleavage of HBD-1 or pro-HNP-1, in vitro cleavage reactions or conditioned medium were separated by Tris-Tricine SDS-PAGE (15% polyacrylamide) and transferred to polyvinylidene difluoride membranes using semi-dry electrophoresis (Bio-Rad). To detect pro-HNP-1, membranes were blocked overnight at 4 °C in Tris-buffered saline (25 mM Tris-Cl, pH 7.4, 0.15 M NaCl) containing 0.1% Tween 20 (TBST) plus 2% ECL Advance™ Blocking Agent (GE Healthcare). The primary antibody, a goat polyclonal raised against the carboxyl terminus of HNP (catalogue number sc-22916, Santa Cruz Biotechnology, Santa Cruz, CA), was used at 1:2000 in the same buffer used for blocking. After washing in TBST, blots were incubated with a 1:50,000 dilution of peroxidase-conjugated donkey anti-goat antibody in blocking buffer and developed using ECL Advance reagents (GE Healthcare). For HBD-1, blots were blocked with 10% nonfat milk in TBST and incubated with an anti-HBD-1 antibody we prepared in rabbits and diluted 1:2000 in TBST plus 5% milk.
MMP-7 Cleavage of α- and β-Defensin Precursors

A.

| Pro segment | Mature peptides |
|-------------|-----------------|
| EPLQARAD...| FQVTVS...CCDCF |
|             |                 |

B.

**FIGURE 1. Cleavage of pro-HNP-1 by MMP-7 in vitro.** The primary structure of the HNP-1 precursor, along with known cleavage sites, is depicted in panel A. Major cleavage sites are indicated by the filled arrows, minor sites by the open arrows. Note that cleavage at Met65, Ala66 produces mature HNP-1, whereas an additional cut at Ala66, Cys67 gives rise to mature HNP-2 (23,24). The site recognized by MMP-7 is indicated by the open inverted triangle. The sequence shown in bold is required for appropriate intracellular trafficking of pro-HNP-1 in neutrophil progenitors and myeloid cell lines (62). Numbering in this figure and others is based on the prepro-peptide, with the initiating Met as residue 1. 56 µg of pro-HNP-1 was incubated alone or with 2 µg of active MMP-7 or the catalytic domain of MMP-14 (1:2.4 enzyme-to-substrate molar ratios) for 25 h. Reactions were resolved by Tris-Tricine SDS-PAGE under reducing conditions and stained with GelCode Blue Stain Reagent. The lanes shown are from the same gel but were rearranged for clarity (as denoted by the dashed line).

C.

followed by a 1:5000 dilution of peroxidase-conjugated donkey anti-goat antibody in the same diluent. Blots were developed with enhanced chemiluminescence (1:25 ml Luminol, 0.198 mM p-coumaric acid, 0.1 M Tris-Cl, pH 8.5, 0.00875% H2O2). For MMP-7 immunoblotting, conditioned medium was separated by standard SDS-PAGE (15% polyacrylamide) and transferred to polyvinylidene difluoride membranes. We used a rabbit polyclonal antibody raised against the amino terminus of human MMP-7 (catalogue number AB8118, Chemicon) at 1:5000 and peroxidase-conjugated donkey anti-rabbit at 1:10,000. Phosphate-buffered saline containing 0.1% Tween 20 (PBST) and 5% milk was used as the blocking buffer and antibody diluent, and PBST with 0.5% milk was used for washes. The gels were developed with ECL Advance reagents.

**Gel Overlay Antibacterial Assay—** We used a method described in Lehrer et al. (35) to assess the antimicrobial activity of MMP-7-cleaved defensin precursors. Cleavage reactions were performed as described above, then lyophilized and dissolved in 5 µl of sample buffer containing 3 mM urea, 5% acetic acid, and methyl green as a dye indicator. Mini-gels containing 12.5% polyacrylamide and polymerized with urea and acetic acid were pre-run at 150 V for 1 h in 5% acetic acid. Samples were applied in duplicate to the gels and electrophoresed (in + to − direction) for 45 min under the same conditions. Half of each gel was fixed in 50% methanol, 7% acetic acid, and 5% formaldehyde for 15 min. After washing in water for three times, 5 min each, the gel was stained with GelCode Blue. The other half of each gel was washed twice in 200 ml of 10 mM PIPES, pH 7.4, 15 min each. Although gels were running, the underlay agar was prepared as follows: from a stationary phase liquid culture of the defensin-sensitive strain of Salmonella (MS7953s, a phoP7953::Tn10 derivative of the virulent strain 14028s and a kind gift from Dr. Fred Heffron), 250 µl was used to inoculate 25 ml of tryptic soy broth containing tetracycline (10 µg/ml). Bacteria were grown for 1 h, 15 min to exponential phase (A600 = 0.23), pelleted by centrifugation, washed twice with 10 mM PIPES, pH 7.4, and resuspended in 19 ml of PIPES buffer. Fifty µl of this suspension was added to 10 ml of molten (45°C) overlay agar: 1% agarose (A-6013, Sigma), 0.03% tryptic soy broth, 10 mM PIPES, pH 7.4. This mixture was poured into 100 mm × 100-mm square Petri dishes. Washed gels were placed on solidified underlay agar and incubated for 3 h at 37°C. Gels were then removed and 10 ml of molten overlay agar (1% agarose, 6% tryptic soy broth) added. After solidification, plates were inverted and incubated overnight at 37°C. Gels were stained with 0.002% Coomassie Brilliant Blue R-250, 27% methanol, 5.5% formaldehyde, for 24 h, rinsed 1 h in 10% acetic acid containing 2% dimethyl sulfoxide, and dried between two sheets of cellophane.

**RESULTS**

**Cleavage of Pro-HNP-1 by MMP-7—** One major 56-residue intermediate and several shorter, minor intermediate forms of amino-terminal processed pro-HNP-1, as well as the 29- and 30-residue mature peptides (Fig. 1A), have been identified in peripheral blood neutrophils and promyelocytic cell lines (23,24). To determine whether any of these forms are generated by MMP-7, we incubated active human enzyme with recombinant pro-HNP-1 in vitro. In an overnight incubation, MMP-7 converted the precursor to a single major form (designated clvHNP-1) that migrated between the precursor and mature...
peptides as shown by SDS-PAGE (Fig. 1B). A time course of digestion showed that cleavage is complete within 30 min (data not shown). The catalytic domain of another MMP, MMP-14 (MT1-MMP), produced the same cleavage product (Fig. 1B).

Amino-terminal sequencing confirmed that this 59-amino acid intermediate resulted from cleavage within the pro-domain at Gln35 ↓ Ile36, removing 16 amino acids from the amino terminus (Fig. 1A). To verify that the cleavage was metalloproteinase specific and not due to contaminating proteases in the recombinant preparation, either EDTA or a metalloproteinase-sparing protease inhibitor mixture was added to the reaction; cleavage was prevented only by the addition of EDTA (Fig. 1C).

Because processing of pro-HNPs derived from neutrophils likely occurs in acidic granules, we tested the ability of MMP-7 to produce the same cleavage product at pH 5.0, the lower end of the pH range at which MMP-7 is active (30). The same pro-HNP-1 cleavage intermediate was identified at pH 5.0, 6.0, and 7.5. With extended incubation at pH 5, we found that MMP-7 tended to digest itself (Fig. 2A), suggesting that the proteinase is quite active under weakly acidic conditions and could function within the secretory pathway. A time course analysis showed that cleavage was complete by 30 min at pH 5.0. We also determined that mature HNP-1 was not cleaved by MMP-7 (Fig. 2B), demonstrating that the fully processed peptide was resistant to further proteolysis by MMP-7, as shown before for cryptdins (36). Thus, like the procryptdins, the pro-domain of HNP precursors is a target for MMP cleavage.

Antimicrobial Activity of MMP-7-cleaved Pro-HNP-1—As we reported, pro-HNP-1 and HNP-1 with the pro-segment added in trans are inactive against bacteria (25). Because only a portion of the pro-domain was removed by MMP cleavage of pro-HNP-1, this intermediate allowed us to determine the regions of the pro-segment involved in inhibiting HNP-1 antimicrobial activity. Pro-HNP-1 was digested with MMP-7 and the activity of the intermediate against S. enterica serovar Typhimurium (a defensin-sensitive phoP mutant strain) was assessed using a gel overlay assay (35). As shown by a band of clearing at the position where the intermediate migrates in an acid-urea gel, the intermediate was able to kill bacteria (Fig. 3A). Cleavage of a carboxyl-terminal His6-tagged procryptdin by MMP-7 gave rise to a mature form that was also able to kill bacteria in this assay (Fig. 3B). Like procryptdin-His6 pro-HNP-1 did not produce a band of clearing (data not shown), consistent with earlier findings (25). Quantitative analysis of the antimicrobial activity of MMP-7-cleaved pro-HNP-1 using standard liquid-phase, and radial diffusion assays was not feasible because the conditions optimal for MMP cleavage in vitro (i.e. physiological concentrations of NaCl) do not allow effective bacterial killing by either the intermediate or mature HNP-1.

Regardless, our results demonstrate that in cis, residues in the carboxyl-terminal portion of the pro-domain do not substantially contribute to peptide inhibition. Rather, the residues critical for the inhibitory effect of the pro-domain residue in the amino terminus of the segment. Because of the nature of the gel overlay assay, where peptides are separated by electrophoresis

5 C. L. Wilson, A. P. Schmidt, E. Piirilä, E. V. Valore, N. Ferri, T. Sorsa, T. Ganz, and W. C. Parks, unpublished observations.
and tested in situ against bacteria, it is not possible to determine from our experiments if fragments derived from the amino terminus can inhibit the intermediate when present in trans.

Cleavage of Pro-HNP-1 by MMP-7 in a Cell-based System—Although a significant portion of neutrophil HNP-1 precursor is processed intracellularly, Faurschou et al. (37) recently reported that pro-HNP-1 is also secreted. In addition, other leukocytes and inflamed epithelial cells can synthesize the precursor (38–42), indicating that production of this defensin is not restricted to neutrophils and that activation of the precursor may occur extracellularly. To show that MMP-7 produces clvHNP-1 in a cell-based system, we used a pBMN-IRES-Puro retroviral vector to overexpress the HNP-1 precursor in the mouse macrophage cell line RAW264.7, which does not produce MMP-7 or high levels of other MMPs.6 We took two approaches to assess MMP-7-specific proteolysis of pro-HNP-1. First, active MMP-7 was added directly to the culture medium of puromycin-resistant cells secreting the HNP-1 precursor. For the second approach, we used a vector with a different selectable marker, blasticidin, to co-express MMP-7 with pro-HNP-1. To circumvent the need to activate the enzyme, cDNA sequence encoding the pro-domain of human MMP-14 was fused to the catalytic domain of MMP-7; the pro-segment of MMP-14, which contains a proprotein convertase cleavage site, is removed in the secretory pathway, thus activating the enzyme (43). As a control, an inactive form of MMP-7 containing an Glu → Gln mutation in the catalytic domain was also expressed. Western blotting of conditioned medium from MMP-7 expressing cells confirmed that the recombinant enzymes are processed and secreted as 19-kDa forms (Fig. 4A). Western blotting for HNP-1 demonstrated that, using either approach, pro-HNP-1 is cleaved by MMP-7 to a form that co-migrates with clvHNP-1 produced in vitro (Fig. 4, B and C). The cleavage product was absent when inactive MMP-7 was co-expressed (Fig. 4B) or when the metalloproteinase inhibitor GM6001 was added along with exogenous MMP-7 (data not shown). Taken together, our results suggest that, under conditions in which pro-HNP-1 and MMP-7 are co-expressed in a complex extracellular milieu, MMP-7 is able to cleave pro-HNP-1.

MMP-7 Cleavage of Human β-Defensin Precursors—In contrast to the α-defensins, β-defensins possess short amino-terminal extensions that are not all true pro-domains, because β-defensin precursors possess antimicrobial activity. However, multiple forms of HBD-1 have been detected in various body fluids, including urine, plasma, and bronchial alveolar lavage fluid (11, 28, 29, 44). Most of these forms result from aminoterminal truncation, although one variant in which the carboxyl-terminal Lys residue is removed has been identified (29). To determine whether MMP-7 can cleave the full-length 47-amino acid HBD-1 precursor, we incubated commercial recombinant HBD-1 with enzyme overnight and assessed cleavage by SDS-PAGE and aminoterminal sequencing (Fig. 5, A and B). At this time point, a unique 41-amino acid peptide product was detected, resulting from cleavage between Gly27 and Leu28 (Fig. 5B). We attempted to verify these results using full-length HBD-1 produced in the baculovirus system; however, as we observed previously (11), High Five insect cells secrete the recombinant HBD-1 as a 39-amino acid form (encompassing residues 30 through 68). When purified from insect cell-conditioned medium and incubated with MMP-7, the 39-residue form was not cleaved by the enzyme (Fig. 5C). In addition, neither HBD-2 nor HBD-3 in full-length form was a target for MMP-7 (Fig. 5C and data not shown).

MMP-7 Cleavage of β-Defensins Lacking Disulfide Bonds—As mentioned above, β-defensins are not always secreted in their full-length form from High Five insect cells. Therefore, we placed a His6 tag on the carboxyl terminus of HBD-1 and -2, as well as mouse β-defensin mBD-1 through -3, for purification of recombinant precursors from insect cell lysates. We used this

6 J. Tang, C. L. Wilson, and E. W. Raines, unpublished observations.
MMP-7 Cleavage of α- and β-Defensin Precursors

A.

HBD-1

|       |       | MMP-7 |
|-------|-------|-------|
| 39 aa form | - | + |
| 47 aa form | | |
| 41 aa form (cleaved) | | |

B.

HBD-1

Pro segment

|       | Mature peptide |
|-------|----------------|
| GN FLT LG | HRS DHYNCVSSGGQCLYSACPITFKIQGTCYRGKAKCCK |

C.

HBD-1 39 aa

|       | M | - | + |
|-------|---|---|---|
| 28    |   |   |   |
| 17    |   |   |   |
| 14.2  |   |   |   |
| 6.5   |   |   |   |
| 3.5   |   |   |   |

HBD-2 41 aa

|       | M | - | + |
|-------|---|---|---|
| 47    |   |   |   |
| 28    |   |   |   |
| 17    |   |   |   |
| 14.2  |   |   |   |
| 6.5   |   |   |   |
| 3.5   |   |   |   |

MMP-7

Like human β-defensins, similarly purified His₆-tagged mBD-1 and -2 were hydrolyzed by MMP-7 within the mature peptide sequence (Fig. 7A), but mBD-3 was relatively resistant (data not shown). MMP-7 cleaved at Cys⁴⁴ ↓ Leu⁴⁵ in mBD-1 and at Ala⁴⁷ ↓ Ile⁴⁸ in mBD-2 (Fig. 7B). By contrast, His₆-tagged mBD-2 isolated from cell-conditioned medium was not digested by MMP-7 (data not shown), demonstrating that cleavage of the lysate-derived form is not due to the His tag.

DISCUSSION

In our studies, we have assessed if MMP-7 could mediate the cleavage and activation of α-defensin HNP-1, as well as the generation of amino-terminal variants of β-defensin HBD-1. This work was predicated on our previous findings demonstrating that MMP-7 generates active α-defensins in the mouse small intestine. That HNP and HBD-1 precursors also undergo proteolytic processing has been known for some time, but the physiologic proteases mediating these processing events have not been identified. Here we show that the pro-segment of the

strategy previously for isolation of procrystadins and found that on defensins, the tag is best recognized by the nickel affinity resin under denaturing conditions (20). Following elution from the resin, peptides were renatured by dialysis. We purified the full-length 47-amino acid form of HBD-1 with a carboxyl-terminal His₆ tag using this approach. However, when the peptide was analyzed by Tris-Tricine SDS-PAGE, there was no difference in electrophoretic mobility under reducing and nonreducing conditions (arrow in Fig. 6A). These findings suggested that disulfide bonds are not present in peptides obtained from cell lysates and, furthermore, that these bonds are not spontaneously formed during the dialysis to eliminate protein denaturant. By contrast, HBD-1 secreted into conditioned medium and purified by combination ion exchange and gel filtration chromatography displayed the expected differences in migration under these conditions (Fig. 6A), with reduced peptide migrating ahead of nonreduced (opposite what is normally observed with larger peptides and proteins). In addition, a polyclonal antibody that recognizes only nonreduced HBD-1 (used in Fig. 5A) detects peptide purified from conditioned medium but not peptide from cell lysate when both are analyzed by nonreducing SDS-PAGE and Western blotting (data not shown). Unlike secreted HBD-1, cell lysate-derived peptide, evidently devoid of disulfide bonds, was further cleaved by MMP-7 (Fig. 6B), and this additional cleavage site was mapped to Pro⁶⁰ ↓ Ile⁶¹ within the mature peptide (Fig. 6C). MMP-7 also cleaved His₆-tagged HBD-2 purified from insect cell lysates, but much less efficiently than HBD-1 (Fig. 6B). To validate these findings, we analyzed the cleavage potential of reduced and alkylated forms of HBD-1 and HBD-2, as well as mature HNP-1. All three defensins were indeed digested by MMP-7 when the peptides lacked disulfide bonds (Fig. 6D).

Unlike β-defensins, His₆-tagged procrystadins purified from insect cell lysates spontaneously form the appropriate disulfide linkages. Identical MMP-7 cleavage sites are found in procrystadins obtained from cell lysates and conditioned medium, as well as precursors derived from the small intestine (21, 22). Furthermore, these sites are confined to the pro-segment.

DISCUSSION

In our studies, we have assessed if MMP-7 could mediate the cleavage and activation of α-defensin HNP-1, as well as the generation of amino-terminal variants of β-defensin HBD-1. This work was predicated on our previous findings demonstrating that MMP-7 generates active α-defensins in the mouse small intestine. That HNP and HBD-1 precursors also undergo proteolytic processing has been known for some time, but the physiologic proteases mediating these processing events have not been identified. Here we show that the pro-segment of the
HNP-1 precursor contains an MMP-7 sensitive site, although mature HNP-1 was not produced by MMP-7 cleavage. In addition, MMP-7 was able to generate one of the HBD-1 variants that have been identified in vivo. Taken together with previous results showing MMP-7 cleavage sites in the pro-domains of cryptdins (20–22), as well as at least one site in the pro-segment of the human intestinal α-defensin HD-5 (10), our current data lead us to conclude that MMP-7 recognition of α-defensin pro-segments is a common feature of these antimicrobial peptides. MMP-7 cleavage at susceptible sites within the pro-segments of both cryptdins (45) and HNP-1 precursors produces intermediates with antibacterial activity, suggesting that this protease could mediate a novel, alternative form of pro-NP-1 activation. Our results also demonstrate that the amino terminus of defensin pro-segments, rather than the carboxyl terminus, is critically involved in maintaining the latency of the precursors.

Another common feature among the defensins is that the mature peptides, when folded in their native forms with intact disulfide arrays, are resistant to cleavage by MMP-7. This proteolysis resistance has been shown for cryptdins (36) and now for human myeloid α-defensins and β-defensins.

MMP-7 cleavage within pro-HNP-1 produces an amino-terminal fragment of 16 residues and a 59-residue intermediate comprising 29 amino acids of the 45-residue pro-domain and the mature peptide. The pro-domain contains anionic residues that are believed to interact with and mask or neutralize positively charged amino acids required for efficient killing activity of the mature peptide (46). By MMP-7 cleavage of pro-HNP-1, four negatively charged residues are removed from the amino terminus and four are retained in the pro-domain remnant of the intermediate.

Our results using the gel overlay antimicrobial assay demonstrate that this loss of four anionic residues is sufficient to confer bactericidal properties on the truncated precursor. By comparison, MMP-7-mediated elimination of nine anionic residues of the 11 in the pro-domain of procryptdin-4 produces an intermediate with antimicrobial activity equivalent to mature cryptdin-4 against several, but not all, species of bacteria tested (45). Thus, it appears that for both mouse intestinal and human myeloid α-defensins, the residues important for pro-segment inhibition lie within the amino-terminal region.

Mutagenesis of the pro-domain should help define the role of specific residues in maintaining defensin inactivity.

Could MMP-7, or other enzymes of the family, be involved in intracellular processing of pro-HNPs? Several MMPs are present...
MMP-7 Cleavage of α- and β-Defensin Precursors

Our findings show that, unlike the α-defensins, β-defensin amino termini have limited susceptibility to proteolysis by MMP-7. However, β-defensins do not require the extensive amino-terminal processing that most α-defensins do, given that they have short pro-domains that do not inhibit the antimicrobial activity of the mature peptides. So far, the only β-defensin for which amino-terminal truncation has been truly demonstrated is HBD-1. The full-length form of HBD-1 has 47 amino acids, whereas the form considered to be “mature” has 36 amino acids. Yet multiple forms of HBD-1 with different amino termini have been isolated from urine and vaginal lavage fluid, and these forms appear to have different levels of antibacterial activity under specific conditions (11, 29). In this work, we found that MMP-7 cleaves the 47-amino acid precursor to produce a 41-residue intermediate. This intermediate is a minor component in urine and its effectiveness against bacteria has not been examined. It is possible that this form and other HBD-1 variants may also differ in other properties ascribed to β-defensins, such as the ability to promote chemotaxis of immune cells (56). We cannot rule out that MMP-7 cleaves at other sites within the pro-domain of HBD-1, because we assessed the cleavage reaction products at only one time point. However, lack of cleavage of the 39-residue form secreted by baculovirus-infected High Five insect cells indicates that any other cleavage sites would be restricted to the first 8 residues of the pro-segment.

Whereas procryptdins derived from insect cell lysates can be isolated with native trisulfide arrays, whether His-tagged or not, mouse and human β-defensins purified in a similar way appear to lack disulfide bonding, as evidenced by comparison of their electrophoretic mobilities (see Fig. 6A). At this point, it is not clear if this is because the cysteine residues were modified during the purification procedure and thus unable to form disulfide pairs. Nevertheless, these β-defensin variants were useful in demonstrating that peptides without disulfide linkages are susceptible to MMP-7 cleavage and are hydrolyzed within the mature peptide after the second or third Cys. Similar results were obtained using peptides that had been reduced and alkylated before incubation with MMP-7. Proteolysis that occurs within the mature peptide moiety only when the secondary structure is disrupted is strikingly reminiscent of findings with mouse α-defensins: reduced and alkylated cryptdin-4 contains three MMP-7 cleavage sites that are masked in the disulfide-bonded molecule, and MMP-7 degrades procryptdin-4 forms in which the trisulfide array has been disrupted by mutation of the Cys residues to Ala residues (36). However, we...
have found that not all natively folded β-defensins are protected from proteolysis by all MMPs: MMP-8 and MMP-20 ( enamelysin) cleave within HBD-1, generating a single discrete product by SDS-PAGE. The Proteus mirabilis metalloproteinase ZapA also digests HBD-1, but into multiple fragments, diminishing its antimicrobial activity (57). Interestingly, HBD-2 appears to be resistant to mammalian metalloproteinases as well as ZapA (57). Other classes of proteases can completely degrade human β-defensins, including the cysteine proteases cathepsins B, L, and S (58), as well as the serine proteases tumor-associated trypsin-2 a and trypsin-like protease from Porphyromonas gingivalis (59). The question remains as to whether MMP-7 cleavage of β-defensins without disulfide linkages, as well as MMP-8 and MMP-20 hydrolysis of native HBD-1, gives rise to products that can still kill bacteria. Short peptides derived from the carboxyl terminus of β-defensins exhibit microbicidal activity against specific strains of bacteria (60, 61), suggesting that limited proteolysis might modify but not necessarily eliminate the killing ability. Further experiments will be required to determine the susceptibilities of both α- and β-defensins against a full spectrum of proteases and to explore the effects of proteolysis on all defensin functions.

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