In Vitro Activation of the Rhesus Macaque Myeloid α-Defensin Precursor proRMAD-4 by Neutrophil Serine Proteinases*1,2

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α-Defensins are mammalian antimicrobial peptides expressed mainly by cells of myeloid lineage or small intestinal Paneth cells. The peptides are converted from inactive 8.5-kDa precursors to membrane-disruptive forms by post-translational proteolytic events. Because rhesus myeloid pro-α-defensin-4 (proRMAD-4(20–94)) lacks bactericidal peptide activity in vitro, we tested whether neutrophil azurophil granule serine proteinases, human neutrophil elastase (NE), cathepsin G (CG), and proteinase-3 (P3) have in vitro convertase activity. Only NE cleaved proRMAD-4(20–94) at the native RMAD-4 N terminus to produce fully processed, bactericidal RMAD-4(62–94). The final CG cleavage product was RMAD-4(55–94), and P3 produced both RMAD-4(55–94) and RMAD-4(57–94). Nevertheless, NE, CG, and P3 digests of proRMAD4 and purified RMAD-4(62–94), RMAD-4(55–94), and RMAD-4(57–94) peptides had equivalent in vitro bactericidal activities. Bactericidal peptide activity assays of proRMAD-4(20–94) variants containing complete charge-neutralizing D/E to N/Q or D/E to A substitutions showed that (DE/NQ)-proRMAD-4(20–94) and (DE/A)-proRMAD-4(20–94) were as active as mature RMAD-4(62–94). Therefore, proregion Asp and Glu side chains inhibit the RMAD-4 component of full-length proRMAD-4(20–94), perhaps by a combination of charge-neutralizing and hydrogen-bonding interactions. Although native RMAD-4(62–94) possesses NE, CG, and P3 proteolysis completely, RMAD-4(62–94) variants with disulfide pairing disruptions or lacking disulfide bonds were degraded extensively, evidence that the disulfide array protects the α-defensin moiety from degradation by the myeloid converting enzymes. These in vitro analyses support the conclusion that rhesus macaque myeloid pro-α-defensins are converted to active forms by serine proteinases that co-localize in azurophil granules.

α-Defensins are effectors of mammalian innate immunity in phagocytic leukocytes of myeloid origin and in small intestine following secretion by epithelial Paneth cells (1). Myeloid and Paneth cell α-defensins are synthesized as ~10-kDa prepropeptides that have canonical signal sequences, acidic proregions, and a 3.5–4.5-kDa mature α-defensin peptide component in the C-terminal moiety of the precursor molecule (1). Biosynthesis of functional α-defensins requires proteinase-mediated conversion of inactive precursors to membrane-disruptive, bactericidal peptides (2, 3). In human small bowel, evidence shows that the Paneth cell pro-α-defensin pro-HD5(20–94) is activated after secretion by anionic and meso trypsin isofoms that cleave the precursor at Arg-62 ↓ Ala-63 to produce HD5(62–94), the predominant form of HD5 in the ileum (4). By contrast, mouse enteric pro-α-defensins (pro-Crps) are converted to active forms by matrix metalloproteinase-7 (MMP-7), which co-localizes with pro-α-defensins in Paneth cell secretory granules (2, 5). Pro-Crps lack in vitro bactericidal activity until the proregion is cleaved by MMP-7 at three sites, including the Crp N terminus (2, 3), and the cleavage event at Ser-43 ↓ Ile-44 enables full bactericidal activity and membrane disruptive behavior (2, 3, 6). Post-translational processing of inactive human neutrophil pro-α-defensin 1 (pro-HNP-1) produces major intermediate forms of 75 and 56 amino acids as well as mature HNP-1 (7), but the convertases that mediate pro-α-defensin activation in primate pro-myelocytes remain unknown.

Rhesus macaque myeloid α-defensins (RMADs)3 are broad spectrum bactericidal peptides (8–10). RMADs 1–3 and 8 have similar primary structures that resemble human neutrophil HNPs 1–3, but RMADs 4/5 and 6/7 are very different from HNP-1, yet differ from each other only by a S28F polymorphism (10). proRMAD primary structures deduced from cDNA sequences predict that RMADs 4 and 5 and RMADs 6 and 7 arise by alternative post-translational processing, consistent with the N-terminal Arg in RMADs 4 and 6. Although RMAD-3 and RMAD-4 are both highly bactericidal in vitro, RMAD-4 has anti-HIV activity that RMAD-3 lacks (8).

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3 The abbreviations used are: RMAD, rhesus myeloid α-defensin; NE, human neutrophil elastase; CG, human cathepsin G; P3, human proteinase 3; 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; PIPES, 1,4-piperazinedithanesulfonic acid; AMU, atomic mass unit.
Proteolytic Activation of Myeloid Pro-α-Defensins

The activating proteinases of human and mouse Paneth cell pro-α-defensins co-localize with their substrates in secretory granules (1, 11), suggesting that rhesus myeloid pro-α-defensins may be activated by proteinases that also occur in neutrophil azurophil granules. Human neutrophil granules form sequentially during myeloid cell differentiation with azurophilic, myeloperoxidase-positive granules being the first to appear at the myeloblast and promyelocyte stage of neutrophil development (12). Myeloid pro-α-defensins are synthesized and accumulate coincidentally with neutrophil elastase (NE), cathepsin G (CG), or proteinase-3 (P3) serine proteinases in myelocytes during myelopoiesis and are localized to the azurophil granules (13) as shown by gene expression profiling and proteomics analyses of neutrophil subcellular fractions (14, 15). Accordingly, we reasoned that the co-localizing serine proteinases could function as convertases for myeloid pro-α-defensins. That hypothesis was tested by exposing recombinant myeloid proRMAD-4(20–94) to human NE, CG, or P3 to test for evidence of proteolysis and in vitro activation of RMAD-4(62–94) bactericidal peptide activity. Each proteinase converted inactive proRMAD-4(20–94) to bactericidal peptides, but only NE cleaved the proRMAD-4(20–94) molecule at the known initiation methionine and as reported previously (3, 17, 19), and all Glu codons were substituted with Gln. Numbering from the initiating methionine and as reported previously (3, 17, 19), and all Glu codons were substituted with Gln. Numbering from the initiating methionine and as reported previously (3, 17, 19), and all Glu codons were substituted with Gln. Numbering from the initiating methionine and as reported previously (3, 17, 19), and all Glu codons were substituted with Gln.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant α-Defensin Peptides and Variants—Recombinant 6x-histidine-tagged fusion proteins from the EcoRI and Sall sites of the pET28a expression vector (Novagen, Inc. Madison, WI) as described (2, 9, 16, 17). The following native and variant rhesus macaque myeloid α-defensins RMAD-4(62–94) (10), (C4/11/31/32A)-RMAD-4(62–94), and (6C/A)-RMAD-4(62–94) were prepared. The natural pET-28a cloning primers for RMAD-4(62–94) are pET-RMAD-4-F (5’-ACA CAC GAA TTC ATG AGA CGC ACC GCA CGT TGC), pET-RMAD-4-C4A-C6A-F (5’-ACA CAC GAA TTC ATG AGA CGC ACC GCA CGT TGC), pET-RMAD-4-C4AC6A-F (5’-ACA CAC GAA TTC ATG AGA CGC ACC GCA CGT GCT), pET-RMAD-4-R (5’-CAG GCC TTC AGG CGT), RMAD-4-C7R (5’-CAT GGC TTC AGG CGT), RMAD-4-C21-R (5’-ATT GAT GTT AGT GCT ACC ATC AAT), RMAD-4-C21A-F (5’-TCT GGG AGT GCT ACC ATC AAT), RMAD-4-C21A-R (5’-ATT GAT GTT AGT GCT ACC ATC AAT), RMAD-4-C21A-R (5’-ATT GAT GTT AGT GCT ACC ATC AAT), pET-RMAD-4-C4A-F (5’-ACA CAC GAA TTC ATG AGA CGC ACC GCA CGT TGC).

To test for the functional role of acidic amino acid positions in the proRMAD-4(20–94) proregion, all Asp codons in proRMAD-4(20–61), wherein residues 20–61 form the proregion of proRMAD-4(20–94) were substituted with Asn codons, and all Glu codons were substituted with Gln.

Preparation of Recombinant RMAD-4(62–94) cDNA clone (16). Primers used to mutagenize RMAD-4(20–94) were prepared as follows: The forward primer, Start F (5’-ATA TAG AAT TCA TGA AGT CAC TCC), the reverse primer, Stop-SalI (5’-AAG TCA CTC CAG GAA ACA GCT GAT GAC GCT GCA ACA). Primer proRMAD-4(20–94) Start F (5’-ATA TAG AAT TCA TGA AGT CAC TCC) was paired with the reverse primer proRMAD-4(20–94) Stop-SalI (5’-AAG TCA CTC CAG GAA ACA GCT GAT GAC GCT). The order of PCR-based mutagenesis reactions and their design followed a scheme used previously to mutagenize Cys residue positions in mouse Crp4 (17). Briefly, in PCR reaction 1, a mutant forward primer containing the mutated codon flanked by three natural codons was paired with a reverse cloning primer, and PCR reaction 2 paired a primer that was the reverse-complement of the mutant forward primer with a forward cloning primer. Samples of purified products from reactions 1 and 2 were combined as templates in PCR reaction 3, using the forward and reverse cloning primers as amplimers, and these putative mutant proRMAD-4 products were cloned in pCR-2.1 TOPO and verified by DNA sequencing. Products were subcloned into Sall and EcoRI sites in pET28a plasmid.
DNA (Novagen, Inc., Madison, WI), and transformed into *E. coli* BL21 (DE3)-CodonPlus-RIL cells (Stratagene) for recombinant expression (3, 16, 17).

**Purification of Recombinant Proteins**—Recombinant proteins were expressed at 37 °C in Terrific Broth medium by induction with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside for 6 h at 37 °C, lysed by sonication in 6 M guanidine-HCl in 100 mM Tris-Cl, pH 8.1, clarified by centrifugation (2, 6, 20), and His-tagged fusion peptides were purified as described (8, 16, 17). After CNBr cleavage of fusions, the recombinant peptides were purified by C18 reverse-phase high performance liquid chromatography (RP-HPLC), and quantitated by absorbance at 230 nm. Molecular masses of purified peptides were determined using matrix-assisted laser desorption ionization mode mass spectrometry (Voyager-DE MALDI-TOF, PE-Biosystems, Foster City, CA) in the UCI Physical Sciences Mass Spectrometry Facility. Peptide homogeneity was confirmed by acid-urea polyacrylamide gel electrophoresis (AU-PAGE), (21) and analytical C18 RP-HPLC at 230 nm.

**Bactericidal Peptide Assays**—Recombinant peptides were tested for microbicidal activity against *E. coli* ML35, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) ΔapoP, wild-type *S. typhimurium* strains CS022, JSG210, and 14082 (from Dr. Samuel I. Miller, University of Washington), *Vibrio cholerae*, *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* 104035 (22). Bacteria growing exponentially in trypticase soy broth (2, 6) were incubated with test peptides in 50 mM Tris-Cl, pH 8.1, clarified by centrifugation (2, 6, 20), and the products of proteolysis were separated and purified by C18 RP-HPLC, quantitated by absorbance 230 nm, and molecular masses of digested peptides were determined using MALDI-TOF MS. Peptide homogeneity was assayed by AU-PAGE (21).

**Purification of Digestion Products of proRMAD-4(20–94)**—40 μg of proRMAD-4(20–94) digested with 1 μg of NE, CG, or P3 and the products of proteolysis were separated and purified using C18-RP HPLC, quantitated by absorbance 230 nm, and molecular masses of digested peptides were determined using MALDI-TOF MS. Peptide homogeneity was assayed by AU-PAGE (21).

**RESULTS**

**In Vitro Activation of the Rhesus Myeloid proRMAD-4(20–94)**

The precursors of mouse Paneth cell α-defensin Crp4 and human neutrophil α-defensins 1–3 (HNP1–3) lack bactericidal activity until enzymatic conversion to their active forms (2, 3, 23–25). To test whether rhesus macaque myeloid pro-α-defensins also require proteolytic conversion from inactive precursors to active forms, the bactericidal peptide activities of recombinant RMAD-4(62–94), and proRMAD-4(20–94) were compared. In contrast to the robust in vitro bactericidal activity of mature RMAD-4(62–94), and consistent with findings for proCrp4 and proHNP-1, equimolar quantities of proRMAD-4(20–94) failed to exhibit detectable bactericidal activity against varied bacterial cell targets (Fig. 1B). Thus, RMAD-4(62–94) bactericidal activity is also dependent on protease-mediated precursor activation.

The mouse and human Paneth cell α-defensin-activating convertases are abundant secretory granule constituents (4, 5, 20). Accordingly, we tested whether neutrophil serine proteinases, which co-localize with α-defensins in azurophil granules, can activate proRMAD-4(20–94) in vitro. Digests of proRMAD-4(20–94) with NE, CG, or P3 were equally bactericidal and as active as native, fully processed RMAD-4(62–94) evidence that each enzyme activated proRMAD-4(20–94) to the same extent under these in vitro conditions (Fig. 1B). None of the proteases cleaved native RMAD-4(62–94) or the α-defensin component of
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**FIGURE 1.** In vitro cleavage and activation of proRMAD-4(20–94) by NE, CG, and P3. In A, samples of proRMAD-4(20–94) (10 μg) were incubated overnight in vitro with (+) or without (−) NE, CG, and P3, and digests were resolved by AU-PAGE and stained with Coomassie Blue (see "Experimental Procedures"). proRMAD-4(20–94) is digested by NE, and the digested product runs alongside native RMAD-4(62–94). CG and P3 digest proRMAD-4(20–94), and the products have lower mobilities in AU-PAGE analysis. In B, bactericidal activities against *S. typhimurium* ΔphoP of recombinant proRMAD-4(20–94), RMAD-4(62–94), and the final serine proteinase cleavage products. Panels A, peptides exposed to NE; B, peptides exposed to CG; C, peptides exposed to P3. Symbols: (\(\Box\)), proRMAD-4(20–94) with no serine protease; (\(-\)), RMAD-4(62–94) with no serine protease; (\(\bigstar\)), RMAD-4(62–94) exposed to serine protease. proRMAD-4(20–94) lacks bactericidal activity, native RMAD-4(62–94) is bactericidal. The three serine proteases convert inactive proRMAD-4(20–94) to a bactericidal molecule.

**FIGURE 2.** The major serine protease cleavage products of proRMAD-4(20–94) have equivalent activity. In A, the final azurophil granule serine protease cleavage sites of proRMAD-4(20–94) deduced by MALDI-TOF MS. The native RMAD-4(62–94) primary structure is underlined. The major products of proRMAD-4(20–94) proteolysis are: NE, RMAD-4(20–94) (\(\bigstar\)); CG, RMAD-4(55–94) (\(\Box\)); P3, RMAD-4(55–94) and RMAD-4(62–94) (\(\bigstar\)). B, bactericidal activity of recombinant proRMAD-4(20–94), RMAD-4(55–94), and the final serine proteinase cleavage products against *E. coli* ML35. Exponentially growing *E. coli* ML35 (A), *S. aureus* (B) and *V. cholerae* (C) were exposed to the peptides at 37 °C in 50 μl of PIPES-TSB buffer for 1 h (see "Experimental Procedures"). Consistent with Fig. 1B, proRMAD-4(20–94) (\(\bigstar\)) lacks bactericidal activity, and native RMAD-4(62–94) (\(\bigstar\)), RMAD-4(62–94) (\(\bigstar\)), RMAD-4(62–94) (\(\bigstar\)) are equally bactericidal.

proRMAD-4(20–94) as is evident from the approximate co-migration of the major cleavage products with native RMAD-4(62–94) in AU-PAGE (Fig. 1A). The major product of proRMAD-4(20–94) cleavage with NE co-migrated with RMAD-4(62–94), showing that it cleaved the proform at the RMAD-4 N terminus (Fig. 1A). On the other hand, the major products of CG and P3 digestion did not, even though NE, CG, and P3 digests of proRMAD-4(20–94) were equally bactericidal (Fig. 1B).

The Major Products of proRMAD-4(20–94) Serine Proteolysis Have Equivalent Activity—Because samples of complete in vitro digests of proRMAD-4(20–94) with NE, CG, or P3 were bactericidal, the active bacterial molecules in those digests were characterized by purification of the major serine proteinase cleavage products using C_{18} RP-HPLC (see "Experimental Procedures"). Based on the known C_{18} elution profiles of proRMAD-4(20–94) and RMAD-4(62–94), the major RMAD-containing fractions were identified using MALDI-TOF MS. To summarize, the major peptide purified from NE digests had a mass of 3964.6 AMU, which corresponds precisely to native RMAD-4(62–94). Similarly, the major CG digestion product was 4627 AMU, and the two main products of P3 cleavage had masses of 4627 and 4396 AMU. Based on these analyses, the final cleavage events mediated by these proteinases occur at Ala-61, Arg-62 for NE, the N terminus of native RMAD-4, at Leu-54, Arg-55 for CG, corresponding to RMAD-4(55–94), and at Leu-54, Arg-55 and Ala-56, Ser-57 for P3, corresponding to RMAD-4(55–94) and RMAD-4(57–94), respectively (Fig. 2A). Although the NE and CG enzymes have endogenous antimicrobial activity, NE and P3 lack activity under the conditions of these bactericidal peptide assays (supplemental Fig. S1). Similarly, CG has modest bactericidal activity at 10 μg/ml, but that concentration is ten times greater than those used in these experiments (supplemental Fig. S1).

Bactericidal Activities of Major proRMAD-4 Serine Protease Cleavage Products—Bactericidal peptide activities of these C_{18}-purified proRMAD-4 cleavage products were assayed against *E. coli* ML35, *S. aureus* and *V. cholerae* (Fig. 2B). The major proRMAD-4 products of in vitro serine protease processing were equivalent in bactericidal activity, comparable to that of
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In Fig. 2B seem unlikely to be of consequence in vivo. Therefore, we conclude that the RMAD-4 variants generated by CG and P3 processing are equal to native RMAD-4(62–94) in activity.

**Functional Effects of Mutagenesis in proRMAD-4(20–94) at Acidic Residue Positions**—In mouse proCrp4(20–92), acidic residue positions in proCrp4(20–43) fully inhibit precursor bactericidal activity (3). To test whether a similar mechanism regulates myeloid pro-α-defensin bactericidal action, Asp and Glu carboxyl groups were eliminated from the proRMAD-4(20–94) proregion by replacements with Asn and Gln amines, respectively or by Ala substitutions at all Asp and Glu positions (Fig. 3A). Equimolar quantities of RMAD-4(62–94), proRMAD-4(20–94), and (DE/NQ)-proRMAD-4(20–94) were tested in bactericidal peptide assays (Fig. 3B). At low peptide levels, (DE/NQ)-proRMAD-4(20–94) showed greater bactericidal activity, than native proRMAD-4(20–94), which lacks activity, but showed attenuated bactericidal activity relative to native RMAD-4(62–94) (Fig. 3B). On the other hand, at concentrations ≥6 μM, (DE/NQ)-proRMAD-4(20–94) and RMAD-4(62–94) were equally active; under the conditions of these assays, proRMAD-4(20–94) lacks activity (Fig. 3B). Thus, removing the anionic charge of the Asp and Glu carboxyl groups was insufficient to override the inhibitory effect of the combined Asp and Glu positions completely. Accordingly, (DE/A)-proRMAD-4(20–94) (Fig. 3A) was compared with RMAD-4(62–94) and proRMAD-4(20–94) in vitro bactericidal peptide assays. Unexpectedly, (DE/A)-proRMAD-4(20–94) displayed greater bacterial cell killing activity than native, fully processed RMAD-4(62–94) —proregion, showing that the mutagenesis abolished all inhibition of proRMAD-4(20–94) activity (Fig. 3B). This finding is consistent with the effects of Gly replacements at Asp and Glu residue positions in the mouse pro-Crp4(20–92) proregion in which the resulting (DE/G)-proCrp4(20–92) molecule and Crp4 had equal activities (3).

As an index of relative membrane disruptive activity, RMAD-4(62–94), proRMAD-4(20–94), and (DE/NQ)-proRMAD-4(20–94) were evaluated by performing permeabilization assays on live E. coli ML35 cells exposed to 0.75 μM to 6.0 μM peptide levels (see “Experimental Procedures”). Consistent with the general behavior of α-defensins and their precursors, exposure to RMAD-4(62–94) induced the greatest level of ONPG conversion, and inactive proRMAD-4(20–94) did not perturb the E. coli cell membranes enough for measurable ONPG to diffuse into the cells (Fig. 4). At every concentration tested, exposure of E. coli ML35 cells to (DE/NQ)-proRMAD-4(20–94) induced greater ONPG conversion than equimolar native proRMAD-4(20–94) but less permeabilization than that caused by RMAD-
Thus, in both bactericidal and membrane permeabilization assays, eliminating Asp and Glu carboxyl groups alone is sufficient to relieve proregion inhibition of proRMAD-4(20–94) activity at $\geq 6 \mu M$ peptide levels, but below 6 $\mu M$, the Asn and Gln replacements in (DE/NQ)-proRMAD-4(20–94) inhibit the precursor RMAD-4 moiety at an intermediate level. Possibly, although Asp and Glu mutagenesis eliminates charge neutralizing effects of carboxyl groups in the proform, hydrogen bonding interactions between Asn and Gln side chains and RMAD-4 basic residues also may contribute to blocking proRMAD-4(20–94) bactericidal activity. Alternatively, proregion amino acids other than Asp and Glu may also inhibit proRMAD-4(20–94) bactericidal action. These collective results suggest that proRMAD-4(20–94) lacks bactericidal activity.

FIGURE 5. Disulfide bonds protect RMAD-4(62–94) from degradation by serine proteases. Panel A shows the primary amino acid sequences of RMAD-4(62–94) and the two disulfide variants (C4/11/31/32/A)-RMAD-4(62–94) and (6C/A)-RMAD-4(62–94) (see “Experimental Procedures”). C1-CV and CIII-CIV disulfide bonds are disrupted in (C4/11/31/32/A)-RMAD-4(62–94) and all disulfide bonds C1-CV, CIII-CIV, and CII-CIV are disrupted in (6C/A)-RMAD-4(62–94). Mutated Cys positions converted to Ala are shown in bold font (right panel). In B, the bactericidal activities of RMAD-4(62–94) disulfide variants are compared. Exponentially growing bacterial cells were exposed to (C4/11/31/32A)-RMAD-4(62–94) (○), (6C/A)-RMAD-4(62–94) (▲), and native RMAD-4(62–94) (●) for 1 h at 37°C (see “Experimental Procedures”). Both disulfide variants have activities comparable with RMAD-4 against S. typhimurium ΔphoP (A), E. coli (B), S. typhimurium JSG 210 (C), L. monocytogenes (D), and V. cholerae (H). Against S. typhimurium CS022 (E) and S. aureus (F), (6C/A)-RMAD-4 has attenuated activity relative to RMAD-4. C, equal quantities ($10 \mu g$) of RMAD-4, (C4/11/28/29A)-RMAD-4(62–94), and (6C/A)-RMAD-4(62–94) were digested for 18 h in the presence of NE, CG, P3, or trypsin, resolved on AU-PAGE, and stained with Coomassie Blue. Disulfide bonds protect α-defensins from proteolysis during conversion to active forms.
because proregion Asp and Glu side chains prevent electrostatic RMAD-4 interactions with bacterial cell targets through a combination of charge neutralizing and hydrogen-bonding interactions (3, 6, 16).

The Disulfide Array Protects RMAD-4(62–94) from NE, CG, and P3 Proteolysis—Analyses of mouse Crp4 have shown that the canonical α-defensin disulfide connectivities are not required for bactericidal activity but confer protection against proteolysis by the activating convertase MMP-7 (17). To test whether that function is a conserved feature of α-defensins, Ala for Cys substitutions were made in RMAD-4(62–94) (Fig. 5A) to retain only the C1Ⅱ-C1Ⅳ pairing ([C4/11/31/32A]-RMAD-4(62–94)) or to disrupt all disulfide bonds ([6C/A]-RMAD-4(62–94)). The effects of mutagenesis on bactericidal peptide activities and on the NE, CG, P3, and trypsin sensitivities of recombinant (C4/11/31/32A)-RMAD-4(62–94) and (6C/A)-RMAD-4(62–94) variants were compared with native RMAD-4(62–94) (10, 16). Consistent with Crp4 disulfide mutagenesis (17), disrupting disulfide bonds in RMAD-4(62–94) did not eliminate in vitro bactericidal activities in relation to the parent molecule (Fig. 5B). Against S. typhimurium CS022, S. aureus, and S. typhimurium 14028S, eliminating RMAD-4(62–94) disulfide bonds attenuated bactericidal activity slightly, and, in contrast to findings with Crp4, none of the disulfide variants had improved activities (Fig. 5B). Ala for Cys substitutions in RMAD-4(62–94) resulted in sensitivity to in vitro proteolysis by NE, CG, and P3, the apparent convertases (Fig. 5C). Because RMAD-4(62–94) is Arg-rich and thus potentially susceptible to trypsin proteolysis, (C4/11/31/32A)-RMAD-4(62–94) and (6C/A)-RMAD-4(62–94) were exposed to trypsin. The disulfide variants were degraded so extensively that the peptides could not be visualized following protease exposure (Fig. 5C). In marked contrast, native RMAD-4(62–94) was completely resistant to trypsin, NE, CG, or P3, which had no effect on peptide mass or migration in AU-PAGE (Fig. 5C) as a function of its disulfide pairings. Based on these analyses of RMAD-4(62–94) and of Crp4 (17), it seems likely that α-defensin disulfide pairings evolved to protect the molecules from proteolysis during post-translational activation, regardless of the activating convertase.

**DISCUSSION**

The three azurophil granule serine proteases, NE, CG, and P3, cleave and activate proRMAD-4(20–94) in vitro. The fact that the human and mouse Paneth cell pro-α-defensin convertases co-localize with their substrates in secretory granules (4, 5), suggested that proRMAD-4(20–94) activation also could be mediated by neutrophil proteinases associated with azurophil granules (13). To test this hypothesis, recombinant proRMAD-4(20–94) molecules were exposed to NE, CG, or P3 to test for evidence of proteolysis and bactericidal peptide activity. Each enzyme cleaved proRMAD-4(20–94) at several different sites within the proregion, at no site within the α-defensin moiety, and processing by all three enzymes resulted in activation. Although only NE cleaved proRMAD-4(20–94) at the known RMAD-4 peptide N terminus to yield RMAD-4(62–94) (Fig. 2A), all final processed products of proRMAD-4(20–94) activation, RMAD-4(55–94), RMAD-4(57–94), or RMAD-4(62–94) have equivalent activities (Fig. 2B). NE and P3 cleave at the N terminus of small aliphatic residues such as Ala, and CG cleaves at the N terminus of Lys. These sites are consistent with their known recognition sites. Unexpectedly, P3 cleaves at Arg in proRMAD-4 perhaps because the tertiary structure of the folded precursor influences sterically an alternative cleavage that P3 is able to recognize (27). Thus, NE, CG, and P3 are individually capable of mediating proRMAD-4(20–94) activation to the same extent in vitro even though CG and P3 do not cleave at the RMAD-4 natural N terminus. Because they co-localize with α-defensins in neutrophil azurophilic granules, it seems likely that the three serine proteinases would participate coincidentally in pro-α-defensin activation with NE catalyzing the final Ala-61↓Arg-62 cleavage event. This suggestion is supported by the fact that CG and P3 intermediates have not been isolated from macaque neutrophils (Tang et al. (10)).

The proregion of proRMAD-4(20–94) maintains the precursor molecule in an inactive state until it is cleaved by the azurophil granule serine proteinases, and activation of pro-α-defensins involves the removal of the proregion acidic residues from covalent association with the RMAD-4(62–94) component of the precursor. The cationic residues in α-defensins play an essential role in α-defensin antibacterial activity as seen in mouse Crp4 (3), and the human enteric α-defensin HD5 (4). Analyses of (DE/NQ)-proRMAD-4(20–94) showed that the variant proregion inhibited bactericidal activity less than native prosegment at precursor concentrations ≤ 6 µM, but ≥ 6 µM (DE/NQ)-proRMAD-4(20–94) and processed RMAD-4(62–94) had equivalent activities (Fig. 3B). Unexpectedly, (DE/A)-proRMAD-4(20–94) showed equivalent or greater activity than native RMAD-4(62–94) (Fig. 3B), perhaps because the Ala substitutions introduced ten moderately hydrophobic side chains to the proregion that may facilitate (DE/A)-proRMAD-4(20–94) interactions with the bacterial cell envelopes. This finding is also consistent with the mouse enteric α-defensin Crp-4 and its precursor variant (DE/G)-proCrp-4, which have equivalent activity (3). These collective results suggest that proRMAD-4(20–94) lacks bactericidal activity because proregion Asp and Glu side chains prevent electrostatic RMAD-4(62–94) interactions with bacterial cell targets through a combination of charge neutralizing and hydrogen-bonding interactions (3, 16, 28).

The three intramolecular disulfide bonds that characterize α-defensins are an invariant structural feature of the peptide family (7, 29–31), but peptide bactericidal activity does not require that the array be intact (17, 32). This conclusion is supported by analyses of site-directed Ala for Cys substitutions in the mouse enteric α-defensin, where each of the Crp-4 disulfide variants were as active as native Crp-4 (17) and also seen in the human β-defensin-3 (33). Similarly, RMAD-4(62–94) variants lacking two or three disulfide bonds were as bactericidal as the native RMAD-4(62–94) against varied bacterial cell targets (Fig. 5B). Consistent with the sensitivity of Crp4 disulfide variants to MMP-7 (17), NE, CG, and P3 degraded RMAD-4(62–94) disulfide variants extensively (Fig. 5C). As myeloid α-defensins are rich in Arg, we tested proRMAD-4(20–94) sensitivity to trypsin proteolysis. Not surprisingly, trypsin cleaved within the prore-
that, we infer that the disulfide array protects RMAD-4(62–94), trypsin, but disulfide variants were degraded (Fig. 5). From that, we infer that the disulfide array protects RMAD-4(62–94), as well as other α-defensins, from degradation during proteolytic activation, in phagolysosomes, and perhaps following release into extracellular environment of the intestinal lumen and at the sites of inflammation.

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