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Inhibition of bactericidal activity is maintained in a mouse α-defensin precursor with proregion truncations

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ABSTRACT

α-Defensin biosynthesis requires the proteolytic conversion of inactive precursors to microbicidal forms. In mouse Paneth cell pro-α-defensin proCrp4$^{(20–92)}$, anionic amino acids positioned near the proregion N-terminus inhibit proCrp4 activity by an apparent charge neutralization mechanism. Because most pro-α-defensins contain proregions of highly conserved chain length, we tested whether decreasing the distance between the inhibitory acidic residues of the proregion and the α-defensin component of the precursor would alter proCrp4 inhibition. Accordingly, two proCrp4 deletion variants, (Δ44–53)-proCrp4 and (Δ44–58)-proCrp4, truncated in a manner corresponding to deletions between MMP-7 cleavage sites, were prepared and assayed for bactericidal peptide activity. Consistent with the properties of full-length proCrp4$^{(20–92)}$, (Δ44–53)-proCrp4 and (Δ44–58)-proCrp4 were processed effectively by MMP-7, lacked bactericidal activity at high peptide levels over a 3 h exposure period, and failed to induce permeabilization of live *Escherichia coli in vitro*. Thus, bringing the inhibitory proregion domain into greater proximity with the Crp4 component of the precursor did not alter the activity of this pro-α-defensin. Therefore, the conserved distance that separates inhibitory acidic proregion residues from the Crp4 peptide is not critical to maintaining proCrp4$^{(20–92)}$ in an inactive state.

1. Introduction

Mammalian defensins are effectors of innate immunity that function in neutrophils and at varied epithelial surfaces, including the skin, oral cavity, airway, and the gastrointestinal tract [19,26]. The mammalian defensins comprise three subfamilies, α-, β-, and θ-defensins, with broad spectrum microbicidal activities [12] that generally are mediated by membrane disruptive mechanisms facilitated by net electropositive charge and amphipathicity [6,12,17]. Specifically, the α-defensins are abundant peptide constituents of neutrophil azurophil granules that mediate non-oxidative killing following microbial phagocytosis, and in dense core granules of Paneth cells at the base of small intestinal crypts which are released into the lumen of the small bowel [14,19]. Regardless of their sites of expression, α-defensins are synthesized as inactive precursors that must undergo proteolytic conversion to give rise to processed, mature peptides with bactericidal activities [4,5].

Events associated with pro-α-defensin activation involve proteolytic cleavage of the proregion by lineage-specific proteinases, which may occur during granulogenesis in the regulated pathway or after secretion in certain instances [22]. After co-translational removal of canonical signal sequences, the inactive ~8.5 kDa pro-α-defensins consist of an acidic prosegment of ~4 kDa that is N-terminal of the 3.5–4.4 kDa α-defensin component of the precursor [4,5]. Most myeloid α-defensin precursors are fully processed in the azurophil granules of neutrophils, and *in vitro* studies implicate the granule serine proteinases elastase, cathepsin G, and proteinase-3 as the activating convertases [2,8]. Human Paneth cells accumulate unprocessed proforms, e.g., pro-HD5, which are activated by anionic and meso-trypsins after secretion [7]. In contrast, mouse Paneth cell α-defensins, termed cryptdins (Crps), are activated by matrix metalloproteinase-7 (MMP-7) intracellularly and prior to secretion [24]. For example, 8.4 kDa mouse pro-cryptdin-4 (proCrp4$^{(20–92)}$) lacks *in vitro* bactericidal and membrane disruptive peptide activities [16] until specific prosegment cleavage events mediated by MMP-7 at

Abbreviations: AU-PAGE, acid-urea polyacrylamide gel electrophoresis; Crps, cryptdins; Crp4, cryptdin-4; HD5, human defensin 5; HNPs, human neutrophil α-defensins; proCrps, pro-cryptdins; proCrp4$^{(20–92)}$, pro-cryptdin-4$^{(20–92)}$; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MMP-7, matrix metalloproteinase-7; PIPES, piperazine-1,4-bis (2-ethanesulfonic acid); RMAD, rhesus myeloid α defensin.

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Ser^{43}↓Ile^{44}, Ala^{53}↓Leu^{54}, and Ser^{58}↓Leu^{59} relieve the inhibitory effects of the proregion on the defensin component of the precursor by severing their covalent association [20,23]. Thus, the proregion maintains proCrp4, as well as additional pro-α-defensins [4,7,8], in an inactive state.

Acidic amino acids that cluster near the N-terminus of the proCrp4$^{(20–43)}$ proregion mediate inhibition of proCrp4 [3]. Site-directed mutagenesis experiments have shown that Arg residues in Crp4 contribute essential electropositive charge that is required for Crp4 bactericidal peptide activity, although the effect of mutation was independent of the actual Arg residue positions in the peptide primary structure [21]. In nearly all pro-α-defensins, the electropositive charge of the α-defensin component is balanced or neutralized by anionic amino acid residues in the prosegment. For example, in mouse proCrp4, cleavage at Ser^{43}↓Ile^{44} catalyzed by MMP-7 removes 24 amino acid residues from the prosegment N-terminus, including nine acidic amino acids [23]. That proteolytic event is sufficient to activate and enable full bactericidal and membrane disruptive behavior of Crp4 [3,23], because all MMP-7 processing intermediates, Crp4$^{(44–92)}$, Crp4$^{(54–92)}$ and Crp4$^{(59–92)}$, are as bactericidal as the fully processed Crp4 molecule [23]. Furthermore, charge neutralizing mutagenesis of proregion Asp and Glu residues eliminated prosegment inhibition of proCrp4 [23]. Replacement and deletional mutagenesis studies showed that those acidic amino acids positioned nearest the proCrp$^{(20–92)}$ N-terminus, between residue positions 20–28, predominate in mediating inhibition of proCrp4 membrane disruptive mechanisms [3].

The role of these N-terminal electronegative residue positions and the highly conserved chain length of α-defensin proregions suggested that the peptide bond distance between these inhibitory elements and the Crp4 moiety of the proform may provide an optimal distance for maintaining proCrp4 in an inactive state. To test this hypothesis, we prepared proCrp4 variants that retain the inhibitory acidic amino acids but have proregion deletions of 10 and 15 amino acids that correspond to proregion fragments produced by MMP-7 proteolysis. The truncated proCrp4 molecules with inhibitory anionic amino acids placed closer to the mature peptide were completely lacking in bactericidal peptide activity, evidence that conserved prosegment chain length is not a critical determinant of its inhibitory activity.

2. Materials and methods

2.1. Preparation of proCrp4 proregion deletion variants

Amino acids between MMP-7 cleavage sites at Ile^{44} and Ala^{53}, inclusively, were deleted from the proregion of proCrp4 by a series of mutagenizing PCR reactions to prepare (Δ44–53)-proCrp4 using a previously described approach [17,20]. PCR site-directed mutagenesis was performed using GeneAMP PCR Core Reagents (Applied Biosystems, Foster City, CA). The cDNA template for the first and second reaction was a pET-28a expression construct for native proCrp4 as previously reported [20]. For the first reaction, the forward primer Δ44–53-PC4-f1 (5′-CAGGC TGTGT CTCTT CATGA AAAA-3′) was paired with reverse primer Crp4-R (5′-TATAT GTCGA CTCAG CGGCG TGGCA-3′. For the second reaction, the forward primer EcoR1-Met-PC4-f (5′-GCGCG AATTC ATGGA TCCTA TCCAA AACAC A-3′) and reverse primer Δ44–53-PC4-R1 (5′-TTTTT CATGA AGAGA CACAG CCTG-3′) were used. Each amplification reaction mixture was incubated at 94 °C for 5 min followed by 5 cycles of 94 °C for 30 s, 38 °C for 30 s, and 72 °C for 30 s followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s and finally an extension reaction for 7 min at 72 °C. A third reaction generated the full-length mutagenized product by using 0.01% of amplification products from the first and second PCR reactions as templates and using a forward cloning primer complementary to the 5′-ends of each template strand [10,23]. Forward primer EcoR1-Met-PC4-f was paired with reverse primer Crp4-R in amplification reactions that were incubated at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 25 cycles, followed by a 7 min extension reaction at 72 °C to prepare the final proCrp4 variant coding sequence.

A longer deletion variant corresponding to residues between the Ile^{44} and Ser^{58} MMP-7 cleavage sites, termed (Δ44–58)-proCrp4, also was prepared to investigate the effect of prosegment length on proCrp4 inhibition. Amino acids Ile^{44} to Ser^{58} of the proCrp4 prosegment were deleted inclusively by iterative mutagenizing PCR reactions as described above. For the first reaction, the forward primer Δ44–58-PC4-f1 (5′-CAGGC TGTGT CTTTG AGAGG TTTG-3′) was paired with the reverse primer Crp4-R. For the second reaction, the forward primer EcoR1-Met-PC4-f was paired with the reverse primer Δ44–58-PC4-R1 (5′-CAAAC CTCTC AAAGA CACAG CCTG-3′). Each amplification reaction and the third PCR reaction was carried out as described in the previous section to give the final proCrp4 variant PCR product. ProCrp4 variant PCR products were cloned in pCR-2.1 TOPO, the mutagenesis verified by DNA sequencing, excised with SalI and EcoRI, subcloned into pET-28a plasmid DNA (Novagen, Inc.), and transformed into *Escherichia coli* BL21(DE3)-pLysS cells (Stratagene, La Jolla, CA) for recombinant expression [17,20].

2.2. Induction and purification of recombinant peptides

ProCrp4 variants were purified using methods previously described [16,17,20]. The recombinant peptides were expressed in *E. coli* BL21(DE3)-pLysS as N-terminal His_6-tagged fusion proteins cloned in-frame between the EcoRI and SalI sites of the pET-28a expression vector polylinker (Novagen, Inc., Madison, WI). Met residues were introduced at the peptide N-termini providing a unique CNBr cleavage site so as to release the peptide from the His_6 tag fusion partner [17,20]. *E. coli* cells were grown at 37 °C in Terrific Broth medium consisting of 12 g of Bacto Tryptone (Becton Dickenson Microbiological Systems, Inc., Sparks, MD), 24 g of Bacto Yeast Extract (Becton Dickenson), 4 ml of glycerol, 900 ml of H_2O, 100 ml of sterile phosphate buffer consisting of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, and 70 μg/ml kanamycin. Recombinant peptide expression was induced in mid-log phase cells by addition of isopropyl-β-D-1-thiogalactopyranoside at a final concentration of 0.1 mM for 4 h at 37 °C [17]. The peptides were purified to homogeneity by HPLC.

Induced bacterial cells were lysed by sonication in 6 M guanidine–HCl in 100 mM Tris–Cl (pH 8.1), and the soluble protein fraction was clarified by centrifugation [16,17,20]. His-tagged fusion peptides were purified using nickel-nitrilotriacetic acid (Qiagen, Valencia, CA) resin affinity chromatography [20]. The peptides were eluted with 1 M imidazole, 6 M guanidine–HCl, 100 mM Tris–HCl and then dialyzed in 5% acetic acid. To cleave the His tag fusion partner, the fusion peptides were lyophilized and reacted with10 mg/ml cyanogen bromide (CNBr) in 80% formic acid for 18 h at 25 °C, diluted with 10 vol of water and lyophilized [23]. After CNBr cleavage, the mutagenized peptides and cleavage products were resuspended in 5% acetic acid and then purified to homogeneity by C18 reverse-phase high performance liquid chromatography (RP-HPLC, data not shown). Homogeneity was assessed by acid-urea (AU)-PAGE analyses as described [13,18]. Peptide concentrations were quantified by UV absorption at 280 nm based on their extinction coefficients. The molecular masses of the purified peptides were determined using MALDI-TOF-MS (Voyager-DE MALDI-TOF, PE-Biosystems, Foster City, CA) at the Mass Spectroscopy Facility, Department of Chemistry, University of California, Irvine, CA, and using a Microflex LRF System spectrometer (Bruker Daltonics, Billerica, MA).