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Molecular determinants for the interaction of human neutrophil α defensin 1 with its pro peptide

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Summary

Human neutrophil α-defensins (HNPs) are cationic antimicrobial peptides that are synthesized *in vivo* as inactive precursors (proHNPs). Activation requires proteolytic excision of their anionic N-terminal inhibitory pro peptide. The pro peptide of proHNP1 also specifically interacts with and inhibits the antimicrobial activity of HNP1 intermolecularly. In the light of the opposite net charges segregated in proHNP1, functional inhibition of the C-terminal defensin domain by its pro peptide is generally thought to be of electrostatic nature. Using a battery of analogs of the pro peptide and of proHNP1, we identified residues in the pro peptide region important for HNP1 binding and inhibition. Only three anionic residues in the pro peptide, Glu^{15}, Asp^{20} and Glu^{23}, were modestly important for interactions with HNP1. By contrast, the hydrophobic residues in the central part of the pro peptide, and the conserved hydrophobic motif $Val^{24}Val^{25}Val^{26}Leu^{28}$ in particular, were critical for HNP1 binding and inhibition. Neutralization of all negative charges in the pro peptide only partially activated the bactericidal activity of proHNP1. Our data indicate that hydrophobic forces play a dominant role in mediating the interactions between HNP1 and its pro peptide – a finding largely contrasting the commonly held view that the interactions are of electrostatic nature.

Keywords

defensin; HNP; antimicrobial peptide; pro peptide; electrostatic interaction; hydrophobic interaction

Introduction

Defensins are small cationic antimicrobial peptides commonly found in leukocytes and epithelial cells of mammals[1; 2; 3; 4]. These molecules directly kill microbes through microbial membrane disruption, constituting the first line of innate immune defense against foreign pathogens. Defensins also act as immunomodulators by inducing expression of cytokines as well as chemoattracting and activating immune cells, presumably through receptor-mediated signaling events[5]. Based on the pattern of three disulfide linkages, defensins are categorized intoα, β and θ families. Alpha- and β-defensins form a three-stranded beta-sheet structure

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stabilized by disulfide bridges, whereas θ-defensins adopt a close-end, two-stranded beta-sheet conformation with a ladder pattern of disulfides.

To date, six human α-defensins have been identified. The first four, also known as human neutrophil peptides 1–4 or HNPs 1–4, are abundant in the azurophilic granules of neutrophils[6; 7], while human α defensins 5 and 6, also known as HD5 and HD6, are mainly expressed in intestinal Paneth cells[8; 9]. Human α defensins are synthesized *in vivo* as inactive precursors and activated in a sequence of posttranslational proteolytic events[10]. Unlike HD5 and HD6, which are released as prodefensins and processed extracellularly[4; 11; 12; 13], HNPs 1–4 are processed intracellularly[14].

PreproHNP1, consisting of a 19-residue N-terminal signal peptide, a 45-residue anionic pro sequence and a 30-residue C-terminal mature domain[10], is synthesized in neutrophil precursor cells in bone marrow[14], and proteolytically processed in ER, Golgi complex, and azurophilic granules before its final maturation and storage[10]. Liu and Ganz (1995) first discovered that the pro peptide facilitates sub-cellular trafficking and sorting of proHNP1[14]. Deletion of the N-terminal two fifths of the propiece – a stretch of 18 amino acid residues – had little effect on defensin biosynthesis. However, deletion of the succeeding 13 amino acid residues, encompassing a conserved hydrophobic region, was detrimental to correct defensin trafficking and sorting. This hydrophobic region was therefore considered essential for the biosynthesis and transport of HNP1[14].

ProHNP1 itself is functionally inactive against bacteria[15]. Further, an exogenously added anionic pro peptide can specifically interact with and inhibit the antimicrobial function of HNP1 in a dose-dependent manner[15; 16]. Ouellette and colleagues demonstrated in a recent study that replacement of the anionic residues (Asp/Glu) in the pro peptide region of a mouse α-defensin precursor, proCryptdin-4, by either Gly or Asn/Gln activated proCryptdin-4 with respect to its membrane and bactericidal activity[17]. In the light of opposing net charges generally observed in pro-region and mature defensin, electrostatic forces are thought to be critical for inter-molecular interactions between cationic α-defensins and their anionic pro peptides, and for the intra-molecular functional inhibition seen in proHNPs and proCryptdins as well[15; 18].

In studying the impact of the pro region on the folding and function of HNP1, however, we found that a charge-reversing (cationic) variant of the proHNP1 pro peptide, where Arg/Lys residues were changed to Asp and Asp/Glu residues to Lys, was still capable of binding HNP1 effectively[19]. This finding raises an intriguing question about the precise nature of the molecular determinants for the recognition of HNP1 by its pro peptide. Here we report on the binding of mature HNP1 to a series of synthetic analogs of its pro peptide using surface plasmon resonance. In addition, we determined the inhibition of the bactericidal activity of HNP1 by these pro peptide analogs using a virtual colony counting (vCC) assay. Selected analogs of full-length proHNP1 were also prepared and functionally characterized.

Results

Design of pro peptide analogs

To evaluate the effects of length, charge and hydrophobicity of the pro peptide on its binding to HNP1, we designed three different types of modifications: N-terminal truncation, substitution of acidic residues, and replacement of hydrophobic residues. First, we truncated the N-terminal 9 and 19 residues in the 45-residue pro peptide, generating two analogs designated as Δ9 and Δ19. Second, we replaced with Ala each of eight acidic residues (Asp and Glu) individually, obtaining eight single-mutation pro peptides. In addition, all Asp and Glu residues were simultaneously substituted for Asn and Gln, respectively, yielding one octal-

mutation pro peptide termed DE/NQ. Third, we changed the aromatic residue Trp^{30} to Ala, and an adjacent hydrophobic motif ($Val^{24}Val^{25}Val^{26}Leu^{28}$) conserved in many mammalian alpha-defensins to $Ala^{24}Ala^{25}Ala^{26}Ala^{28}$, resulting in two analogs W30A and VVVL/4A, respectively. The wild type pro peptide, all 13 pro peptide analogs, and folded HNP1 were purified by HPLC to homogeneity, and their molecular masses were ascertained by ESI-MS (data not shown). The amino acid sequence of the proHNP1 pro peptide aligned with 33 unique pro peptide sequences of other mammalian species is listed in Figure 1.

To study the mutational effects of length, charge and hydrophobicity in the context of proHNP1, we chemically synthesized and folded wild type proHNP1 and a selected group of proHNP1 analogs using a published protocol[16; 20], yielding Δ9-, DE/NQ-,VVVL/4A-, and W30A-proHNP1. Immediately following a Met residue in the amino acid sequence, the C-terminal defensin domain was conveniently released by CNBr cleavage of proHNP1 and its folded analogs. Comparison of CNBr-released HNP1 with a previously characterized synthetic HNP1 using analytic RP-HPLC, ESI-MS and antibacterial activity assay indicated that the four proHNP1 analogs all folded correctly (data not shown). We failed, however, to produce correctly folded Δ19-proHNP1 due to massive precipitation of the polypeptide during the oxidative folding process.

Binding versus inhibition

Surface plasmon resonance (SPR) was used to measure the binding affinity of the pro peptides for HNP1. Shown in Figure 2 are four representative association and dissociation kinetics (sensorgrams) of the wild type pro peptide, Δ19, E15A, and DE/NQ on immobilized HNP1. To alleviate subjectivity often associated with SPR data analysis, we also quantified solution interactions between HNP1 and the pro peptide analogs using a previously described, SPR-based competition assay[21]. Representative competition binding curves for the wild type pro peptide, Δ19, E15A, and DE/NQ are shown in Figure 3. The dissociation equilibrium constants (K_d) derived from direct binding assays (on a low-density chip) and IC_{50} values obtained from competition binding assays are summarized in Table 1. K_d and IC_{50} values for VVVL/4A are missing due to lack of HNP1 binding. In addition, the K_d value for W30A could not be reliably determined because the kinetic data did not fit to a 1:1 kinetic binding model. Otherwise, the K_d values are in general 2–10 fold lower than the corresponding IC_{50} values. With W30A and VVVL/4A excluded, relative binding affinities for HNP1 of the remaining 11 pro peptide analogs versus the wild type pro peptide, determined by the two different techniques, correlated reasonably well as shown in Figure 4.

In addition to the SPR-based binding measurements, antibacterial assays were performed to evaluate the ability of various pro peptide analogs (0.4–100 μM) to inhibit the bactericidal activity of 10 μM mature HNP1 against *E. coli* and *S. aureus*. Shown in Figure 5 is a representative dose-dependent defensin inhibition by the wild type pro peptide, Δ19, E15A, DE/NQ, W30A, and VVVL/4A. HNP1 alone at 10 μM killed about 99% of input *E. coli* cells (a 2-log reduction in survival) and approximately 99.9% input *S. aureus* cells (a 3-log reduction in survival). The pro peptides, except for DE/NQ, alone, showed no bactericidal activity against either *E. coli* or *S. aureus* (data not shown). However, DE/NQ was bactericidal only against *E. coli* presumably due to its gained cationicity. When added to 10 μM HNP1, DE/NQ caused a dose-dependent enhancement in *E. coli* killing, as evidenced by a downward survival curve shown in Figure 5A. By contrast, addition of the wild type pro peptide or any other pro peptide analog to 10 μM HNP1 resulted in a dose-dependent increase in bacterial survival due to defensin inhibition. To facilitate quantitative comparison, we defined EC_{90} as the effective concentration of pro peptide at which 90% of input cells were killed by 10 μM HNP1 - equivalent to a one-log reduction in bacterial survival. Tabulated in Table 2 are EC_{90} values generated from the vCC assays for the wild type pro peptide and all 13 pro peptide analogs.

The higher the EC_{90} value of a pro peptide is, the weaker its inhibition of HNP1. Not surprisingly, the wild type pro peptide, with the lowest EC_{90} values for both *E. coli* and *S. aureus*, is among the strongest inhibitors of HNP1.

Effects of truncation

Deletion of the first nine amino acid residues (**E**PLQARA**DE**) of the pro peptide had little impact on its ability to bind and inhibit HNP1. The K_d and IC_{50} values of Δ9 for HNP1, 0.17 μM and 0.72 μM, were similar to those of the wild type pro peptide, 0.20 μM and 0.57 μM, respectively. The Δ9 peptide and the wild type pro peptide also showed indistinguishable potency with respect to their inhibition of the bactericidal activity of HNP1, as reflected by similar EC_{90} values for either *E. coli* or *S. aureus* (Table 2). These results suggest that the first nine residues of the pro peptide, including one Asp and two Glu, are unlikely to be involved in inter-molecular interactions with the defensin.

Further truncation by ten amino acid residues (VAAAP**E**QIAA), however, had markedly different outcomes. The affinity of Δ19 for HNP1 decreased by almost two orders of magnitude compared with the full-length pro peptide (Table 1). The ability of Δ19 to inhibit HNP1 in bactericidal activity assays also significantly weakened, as evidenced by an approximately one-order-of-magnitude increase in EC_{90} for *S. aureus* and *E. coli* (Table 2). Since the ten amino acid residues deleted are largely of hydrophobic nature, these findings suggest that hydrophobic interactions between the pro peptide and HNP1 are important. Additional support to this conclusion comes from charge neutralization studies detailed below.

Effects of charge

Based on the ratios of K_d and IC_{50} values of the pro peptide analogs to those of the wild type, acidic residues Asp and Glu can be divided into three groups: (1) residues with little impact on HNP1 binding ($0.5 < K_d$ and IC_{50} ratios < 2); (2) residues with modest impact on HNP1 binding ($2 < K_d$ and IC_{50} ratios < 10); and (3) residues that significantly impact HNP1 binding (K_d and IC_{50} ratios > 10). Five acidic residues out of a total of eight, Glu^1, Asp^8, Glu^9, Asp^{31} and Glu^{32}, belong to the first group (Table 1), and likely make no direct contact with the defensin. Consistent with this finding is the result on the Δ9 pro peptide, where deletion of the first nine amino acid residues, including Glu^1, Asp^8 and Glu^9, caused virtually no change in the ability of Δ9 to bind or inhibit HNP1.

Mutation to Ala of the remaining three acidic residues in the pro peptide, Glu^{15}, Asp^{20} and Glu^{23}, showed modest effects on HNP1 binding. Ala-substitutions at those positions caused, on average, a 3-fold (by direct binding) or a 5-fold (by competition binding) decrease in binding affinity for HNP1, suggesting that Glu^{15}, Asp^{20} and Glu^{23} may be involved in specific electrostatic interactions with cationic residues in HNP1. Although HNP1 contains four cationic Arg residues, since one Arg forms a structurally important salt bridge within the molecule with a Glu $residue^{20}$, only three are available for potential charge-charge interactions with Glu^{15}, Asp^{20} and Glu^{23} of the pro peptide. It is worth noting that the moderately deleterious effect of an E15A mutation was insufficient to account for the huge difference in K_d and IC_{50} between Δ19 and the wild type pro peptide, underscoring the importance of the hydrophobic residues in the vicinity of Glu^{15} for HNP1 binding.

Neutralizing all eight negative charges (DE/NQ) decreased the binding affinity of the pro peptide for HNP1 roughly by 15-fold. The weakened binding could be fully accounted for by individual mutations of the three moderately important anionic residues Glu^{15}, Asp^{20} and Glu^{23} combined, further supporting that the other five acidic residues, Glu^1, Asp^8, Glu^9, Asp^{31} and Glu^{32}, are functionally dispensable. This finding also indicates that although

important, electrostatic interactions are not a dominant force dictating the binding of the pro peptide to HNP1.

The results from SPR-based binding assays are largely consistent with those from vCC-based functional inhibition assays. On the basis of their respective EC_{90} values (Table 2), the relative potencies of the pro peptides inhibiting HNP1 are: (wild type, Δ9, E1A, D8A, E9A, D31, E32A) ~ (E15A, D20A, E23A) ~ W30A > DE/NQ > Δ19 > VVVL/4A. However, the mutational effects seen from SPR-based binding studies were significantly attenuated in antibacterial activity assays. The ratios of EC_{90} values of all eight singly charge-mutated pro peptides to that of wild type ranged from 1.1 to 2.2 (Table 2), making it hard to differentiate E15A, D20A and E23A from the remaining five acidic residues. Nevertheless, the DE/NQ pro peptide displayed an EC_{90} value for *S. aureus* consistent with the conclusions drawn from the binding studies.

Effects of hydrophobicity

The central region (residues 10–30) of the pro peptide is mostly hydrophobic, containing 16 aliphatic residues, one Gln, one Ser, and the three anionic residues Glu^{15}, Asp^{20} and Glu^{23}. Results from Δ9 and Δ19 suggested that the hydrophobic residues surrounding Glu^{15} of the pro peptide are important for HNP1 binding. Perhaps more strikingly, replacement of the conserved hydrophobic motif $Val^{24}Val^{25}Val^{26}Leu^{28}$ by four Ala residues largely abolished HNP1 binding. The loss of binding of VVVL/4A to HNP1 in SPR-based direct and competition binding assays is consistent with a significantly reduced inhibition of the bactericidal activity of the defensin by the pro peptide analog. As shown in Table 2, for both *E. coli* and *S. aureus* the EC_{90} values of VVVL/4A increased approximately 18-fold compared with the wild type pro peptide. In fact, VVVL/4A was the weakest pro peptide inhibitor of HNP1 in the panel tested. These results further demonstrate the importance of hydrophobic interactions between HNP1 and its pro peptide.

Direct binding assays to obtain a reliable K_d value for W30A were not possible because the data, for unknown reasons, did not fit to a 1:1 kinetic binding model. The result from competition binding assays indicated that the only Trp residue in the pro peptide region is modestly important for HNP1 binding, as evidenced by a 4-fold increase in IC_{50} as a result of the Trp-to-Ala mutation. Antimicrobial activity assays showed that while the W30A mutation increased the EC_{90} value of the pro peptide by a factor of 2 for *E. coli*, the increase in EC_{90} for *S. aureus* was marginal, confirming that the importance of Trp30 for HNP1 binding is likely on par with one of the three moderately important anionic residues (Glu^{15}, Asp^{20} and Glu^{23}).

The standard buffer used in SPR experiments contained 150 mM NaCl, which is generally required to disrupt potential non-specific interactions on the chip surface. A high salt concentration may unnecessarily weaken electrostatic interactions between HNP1 and its pro peptide due to increased charge-shielding (or masking) of cationic and anionic side chains by counter ions in solution. To evaluate the effects of salt on the pro peptide binding to HNP1, we also performed SPR measurements at different NaCl concentrations (25, 50, 100, 150 mM) for the wild type pro peptide using both direct binding (on a high-density chip) and competition binding assays. The results are shown in Figure 6 and Figure 7. The IC_{50} values obtained from competition binding assays, averaging at 0.53 ± 0.10 μM, are largely independent of salt concentration (Figure 6). However, the slope became steeper with increased salt concentration, indicative of a salt-enhanced stronger binding. Salt-enhanced interactions between the pro peptide and HNP1 were more evident in direct binding assays (Figure 7). The K_d value increased by a factor of 2 (from 0.27 μM to 0.5 μM) as the NaCl concentration decreased from 150 mM to 50 mM. A further decrease in NaCl to 25 mM weakened the interaction by an additional 4-fold, attributable mainly to a slower association rate k_{on}. High salt generally weakens electrostatic interactions and strengthens hydrophobic interactions[22; 23; 24]. This is

because desalvation, required for two attracting protein surfaces, becomes more favorable at higher salt concentration for non-polar surface but less favorable for polar surfac[22]. The fact that an increase in ionic strength enhances binding provides strong evidence supporting hydrophobic rather than electrostatic interactions as the dominant force mediating defensin recognition by its pro peptide. Opposite results would otherwise have ensued[22; 23; 24]. It should be pointed out, though, that antibacterial activity assays were performed at low salt concentration as high salt is known to inhibit the killing of bacteria by defensins – a process dictated largely by electrostatic interactions between peptide and microbe[1; 25; 26; 27].

It is generally accepted that hydrophobic interactions are the dominant force in protein folding and stability[28; 29]. Due to a large difference in heat capacity between the folded and unfolded states of a protein, the enthalpy and entropy are strong functions of temperature, and the free energy of folding is temperature dependent[30]. Hydrophobic interactions increase in strength with increasing temperature – a process largely driven by entropy at ambient temperature[30; 31]. We also measured the binding affinity of the pro peptide for HNP1 at 4 °C and 37 °C using the direct SPR binding assay, and found that the K_d value did decrease with temperature, albeit a small difference (0.24 μM at 4 °C and 0.18 μM at 37 °C). It is plausible that the enhancement in binding affinity of the pro peptide for HNP1 was largely cancelled out by an entropy penalty imposed by the disordering of the pro peptide at higher temperatures.

Effects of truncation, charge and hydrophobicity in the context of proHNP1

To evaluate the effects of truncation, charge and hydrophobicity of the pro peptide in the context of proHNP1, we compared the ability of wild type proHNP1, Δ9-proHNP1, DE/NQ-proHNP1, W30A-proHNP1 and VVVL/4A-proHNP1 to kill *E. coli* and *S. aureus*. Wild type HNP1 was used as a positive control, and the data are plotted in Figure 8. As was previously reported[15; 16; 19], the wild type proHNP1 was inactive against both strains. Interestingly, none of the four proHNP1 analogs showed any bactericidal activity against *S. aureus*, and none, except for DE/NQ-proHNP1, was effective against *E. coli* within the concentration range used. The marginal bactericidal activity of DE/NQ-proHNP1 was likely a result of the *E. coli*-killing activity of the pro peptide itself due to gained cationicity. However, the activity of DE/NQ-proHNP1 was significantly less pronounced than that of either HNP1 or the DE/NQ pro peptide, indicative of a reciprocal intra-molecular functional inhibition.

Discussion

Defensins kill bacteria through membrane disruption – a process dictated by the interplay of cationicity and hydrophobicity[26; 32; 33]. The pro regions of α-defensins inhibit the antimicrobial activity of defensins both intra- and inter-molecularly[15; 16; 18]. This report provides us with a rare glimpse at the molecular basis for the recognition of HNP1 by its pro peptide. Several conclusions can be drawn from functional characterizations of a series of pro peptide analogs interacting with HNP1. First, the majority of negatively charged residues in the pro peptide region are not directly involved in interactions with HNP1. The three acidic residues in the pro region, Glu^{15}, Asp^{20} and Glu^{23}, are likely candidate partners of three free Arg residues from HNP1. However, electrostatic interactions, while moderately important energetically, are not a dominant force. Second, the first 9 amino acid residues at the N-terminus are functionally dispensable, whereas the succeeding 10 residues of hydrophobic nature are critical for HNP1 binding. Nevertheless, the more important recognition determinants appear to be the conserved hydrophobic motif $Val^{24}Val^{25}Val^{26}Leu^{28}$ and, to a significantly lesser extent, the adjacent aromatic residue Trp30. Third, disruption of intra-molecular interactions between the pro peptide and the C-terminal defensin domain has limited impact on the bactericidal activity, or lack thereof, of proHNP1. Neutralization of all negative charges in the pro peptide region only partially activates defensin activity – a finding in partial accord with

the results reported by Ouellette and colleagues on proCryptdin-4[17]. Taken together, our findings demonstrate that hydrophobic rather than electrostatic interactions are the dominant force mediating the interactions between HNP1 and its pro peptide – a premise perhaps relevant to the observations made by Ganz and colleagues that the hydrophobic residues in the pro region are important for correct proHNP1 trafficking and sorting in neutrophils[14].

The conclusions from our study are counter-intuitive, contrasting the commonly held view that electrostatic interactions between the oppositely charged N-terminal pro peptide and C-terminal mature defensin domain result in charge neutralization and inhibition of defensin function. Ouellette and colleagues recently demonstrated with mouse proCryptdin-4 that replacement of all acidic residues by Gly in the pro region converted the functionally inactive pro α-defensin into a fully active molecule with respect to bacterial killing and membrane permeabilization, suggesting that electrostatic force-mediated charge neutralization within proCryptdin-4 is responsible for its lack of activity[17]. Similarly, the interactions between HNP1 and its pro peptide are considered to be of electrostatic nature[15], although definitive biochemical and/or structural evidence is still lacking.

The interacting mode for pro peptide and defensin appears different between proCryptdin-4 and proHNP1. The proCryptdin-4 pro peptide is extremely polar and highly anionic, comprising 4 Gly, 26 polar residues including 11 anionic residues and 2 cationic residues, and only 9 hydrophobic residues sparsely distributed across the entire sequence. By contrast, the proHNP1 pro peptide contains 25 hydrophobic residues, 1 Gly, and 19 polar residues including 8 anionic residues and 4 cationic residues. The difference in amino acid composition between cryptdin-4 and HNP1 is equally striking. While the highly cationic cryptdin-4 consists of 8 Arg, 2 Lys and 1 Glu, HNP1 is composed of 1 Glu and only 4 Arg. On the basis of the amino acid compositions of proCryptdin-4 and proHNP1, it is conceivable that electrostatic forces play a major role in mediating both inter- and intra-molecular interactions between cryptdin-4 and its pro peptide. Replacement of Asp and Glu residues in the pro peptide region should disrupt such interactions, exposing the previously sequestered cationic residues in the C-terminal cryptdin-4 domain for microbial killing[17; 34].

We recently demonstrated that the proHNP1 pro peptide catalyzes defensin oxidative folding *in vitro*, either intra- or inter-molecularly, through two independent mechanisms: solubilization of and interaction with HNP1[19]. Surprisingly, however, a charge-reversing mutant of the pro peptide, where Arg/Lys residues were changed to Asp, and Asp/Glu residues to Lys, showed similar catalytic efficiency. Further, the negatively charged wild type pro peptide and its cationic, charge-reversing analog bound to HNP1 with similar affinities. This report provides an important clue and explanation for the previous observations – that is, hydrophobic interactions between HNP1 and its pro peptide are more important than charge-charge interactions. Our findings reported here also shed light on the propensity of misfolding/aggregation of Δ19-proHNP1 – likely resulting from a combination of weakened intramolecular hydrophobic interactions and poor solubility of the polypeptide.

But, how can we reconcile with the Ouellette's report on proCryptdin-4 our finding that Δ9-proHNP1, W30A-proHNP1 and VVVL/4A-proHNP1 were totally ineffective in the killing of *E. coli* and *S. aureus*, whereas DE/NQ-proHNP1 was only partially active against *E. coli*? First, disruption of intra-molecular interactions is always strongly disfavored entropically. Therefore, it is plausible that none of the mutations constructed in the context of proHNP1, including the most promising candidate VVVL/4A-proHNP1, was sufficiently disruptive to intra-molecular pro peptide-defensin interactions. Second, the lack of bactericidal activity of proHNP1 is more complex than previously thought. We have shown that charge neutralization is not a prerequisite for defensin inactivation[19]. For example, covalent attachment of a 10-residue poly Ser sequence to the N-terminus of HNP1 was sufficient to fully inactivate its

bactericidal activity against both *E. coli* and *S. aureus*[19]. We suspect that in some pro α-defensins specific charge-charge or hydrohobic interactions are not critical for keeping the C-terminal defensin domain inactive because a covalently attached pro segment itself, depending on its chemical nature, can exert greater influence on defensin oligomerization and defensin-membrane interactions and, ultimately, defensin activity. Third, cryptdin-4 – a much more potent bactericidal peptide than HNP1 due to its significantly higher number of cationic residues – may be more resilient than HNP1 to functionally deleterious modifications.

Finally, the hydrophobic mode of interaction for HNP1 and its pro peptide is consistent with known structural features of human α-defensins. Due to small size, α-defensins are structurally stabilized primarily by three disulfides with little or no hydrophobic packing in the core. As a result, most non-polar residues including some disulfide bonds are either fully exposed or partially solvent-accessible. This should provide sufficiently large hydrophobic surfaces for interactions with the non-polar residues from the pro peptide. Perhaps not coincidently, HNP1 carries, on one side of the molecule, three net cationic charges positioned at 14, 15 and 24 (HNP1 numbering) along the second and third β-strands. (The only other cationic residue, Arg^5, forms a conserved salt bridge with Glu^{13} important for defensin stability[20].) Although highly speculative, Arg^{14}, Arg^{15} and Arg^{24} on HNP1 can conceivably interact with the three functionally important anionic residues from the pro peptide, i.e., Glu^{15}, Asp^{20} and Glu^{23}, providing additional specificity for the recognition of HNP1 by its pro peptide (Figure 9). Structural studies are needed to unveil at the molecular level residues involved in the defensin-pro peptide interactions.

Experimental procedures

Synthesis of HNP1 and pro peptide analogs

Peptide synthesis was carried out on an Applied Biosystems 433A synthesizer using an in-house Boc chemistry[35; 36], tailored from the HBTU activation and DIEA *in situ* neutralization protocol previously developed by Kent and coworkers[37]. Crude peptides, after chain assembly and hydrogen fluoride cleavage/deprotection, were purified by C18 reversed phase high performance liquid chromatography (RP-HPLC). Reduced HNP1 was folded using the oxidative folding protocol described previously[35], followed by RP-HPLC purification. Purified peptides were quantified spectroscopically at 280 nm using molar extinction coefficients calculated according to a published algorithm[38], and their molecular masses were verified by electrospray ionization mass spectrometry (ESI-MS).

Synthesis of proHNP1 analogs using native chemical ligation

Synthesis of proHNP1 and various proHNP1 analogs using native chemical ligation[39; 40] was essentially as described previously[16]. Briefly, the N-terminal thioester peptides were synthesized on Trityl-SCH_2CH_2COO-Leu-OCH_2-phenylacetamidomethyl resin, to which the C-terminal defensin domain starting with an N-terminal Cys residue was ligated, resulting in a full-length wild type proHNP1 and four proHNP1 analogs. Oxidative folding of proHNP1 and its analogs was carried out as previously described[16; 20]. To verify correct pro defensin folding, mature HNP1 was cleaved off the folded products by CNBr using a previously detailed protocol[16], and analyzed by analytical RP-HPLC and ESI-MS.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) – based kinetic measurements were performed on a Biacore 3000 (Biacore AB, Uppsala, Sweden). The experiments were conducted at 25 °C in HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4). HNP1 equivalent to 180 response units (RUs), prepared in 10 mM acetate buffer, pH 5.5, was immobilized to a CM4 sensor chip using the coupling procedures recommended by the

manufacture. A reference surface was prepared by a similar procedure without injection of HNP1. The pro peptide and its analogs were injected at 30 μl min^{-1} for 4 min, followed by a 5-min dissociation. Salt-dependent binding affinity of the wild type pro peptide was determined at different NaCl concentrations of 25, 50, 100 and 150 mM on a similarly prepared high-density (CM4) chip with HNP1 immobilized at 1036 RUs.

A parallel competition assay was also carried out for the synthetic pro peptides. Briefly, 800 RUs of the wild type pro peptide were immobilized (in 10mM acetate buffer, pH 4.0) to a CM4 sensor chip using the amine coupling chemistry. Kinetic analysis of the binding to the wild type pro peptide by 100 nM HNP1, either alone or in the presence of a varying concentration of each pro peptide analog, was carried out at 25 °C in HBS-EP buffer. Defensin HNP1 of 100 nM was incubated at room temperature for 15 min with different concentrations of pro peptide analogs, and the incubation mixture injected at a flow rate of 20 μl/min for 2 min, followed by a 3-min dissociation. The concentration of free HNP1 in solution (not complexed with pro peptide analogs) was deduced, based on the initial rate (slope) of association, from a standardized calibration curve established by kinetic measurements of HNP1 injected alone at different concentrations. Non-linear regression analysis was performed using GraphPad Prism 4 to give rise to IC_{50} values, concentrations of pro peptide at which 50% of HNP1 was sequestered in pro peptide – HNP1 complexes. Salt-dependent binding affinity of the wild type pro peptide for HNP1 was also determined at varying NaCl concentrations of 25, 50, 100, and 150 mM.

Antibacterial activity assays

To evaluate functional inhibition of HNP1 by its pro peptide and pro peptide analogs, antimicrobial assays against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were conducted using a previously detailed 96-well turbidimetric method dubbed virtual colony counting[41]. A twofold dilution series of pro peptide, ranging from 0.4–100 μM, was mixed with 10 μM HNP1 for 30 min in 10 mM sodium phosphate, pH 7.4, and subsequently incubated at 37 °C for 2h with *E. coli* or *S. aureus* (1×10^6 CFU/ml), followed by addition of twice-concentrated Mueller-Hinton broth (2 × MHB) and 12-h kinetic measurements of bacterial growth at 650 nm. In addition, direct antimicrobial activities of wild type proHNP1 and four proHNP1 analogs were determined using the virtual colony counting protocol[41].

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Figure 1.

Sequence alignment of 34 unique pro peptides of known mammalian alpha-defensins (human, mouse, rat, rhesus macaque – MACMU, rabbit – RABIT, chimpanzee – PANTR, and guinea pig – CAVPO). The acidic residues in the proHNP1 pro peptide are in bold typeface and underlined. The central hydrophobic regions are shaded in grey (residues 10–19) and black (residues 24–28). Also shaded in black are hydrophobic motifs conserved in the pro peptides of most mammalian alpha-defensins.

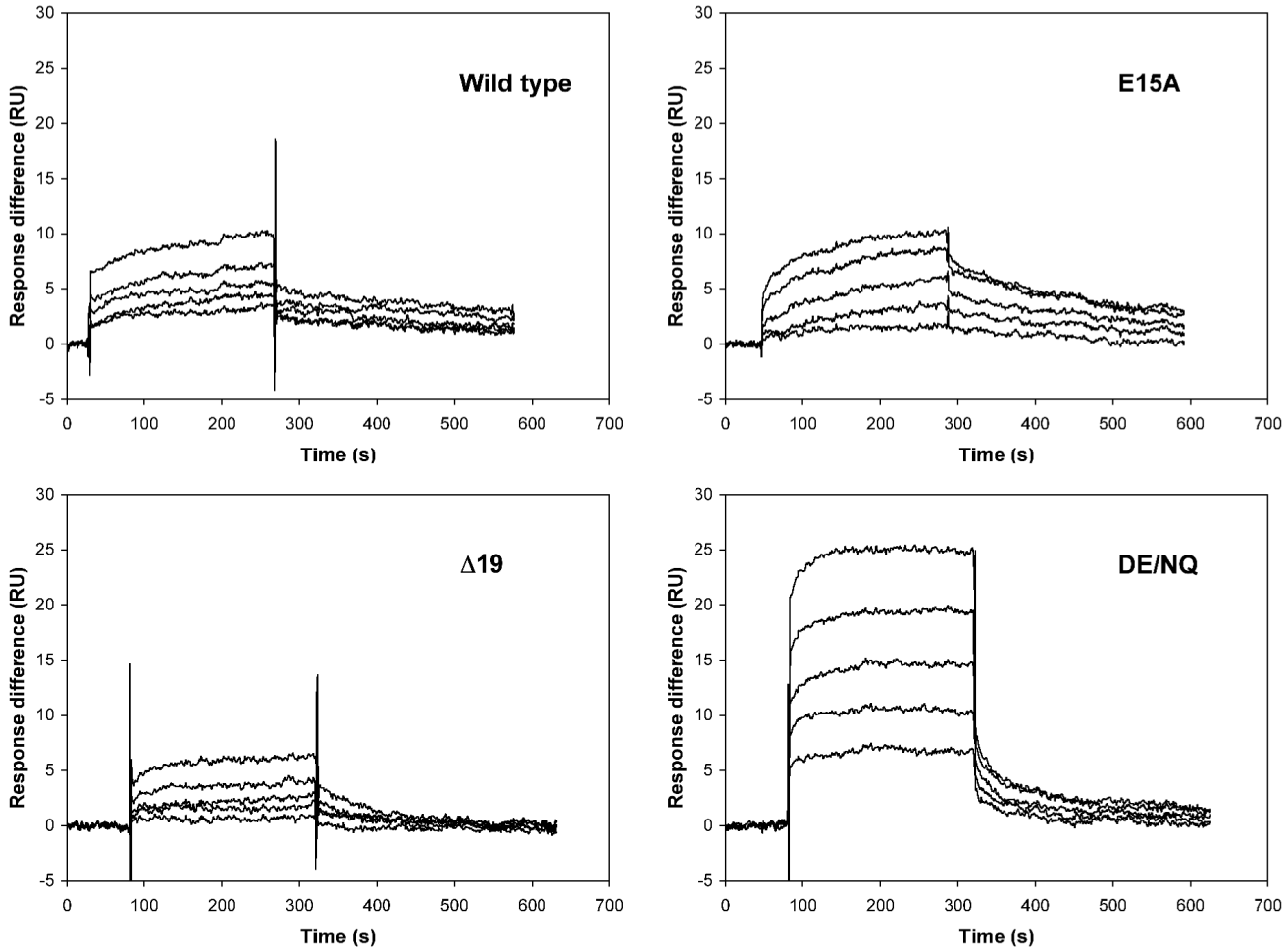

Figure 2.

Representative direct binding of wild type, E15A, Δ19, and DE/NQ pro peptides to the wild type HNP1, monitored by surface plasmon resonance (Biacore). Measurements were conducted on a CM4 chip with HNP1 immobilized. Concentrations ranging from 2 to 0.125 μM were used for wild type and E15A pro peptides; higher concentrations ranging from 20 to 1.25 μM were used for analogs Δ19 and DE/NQ.

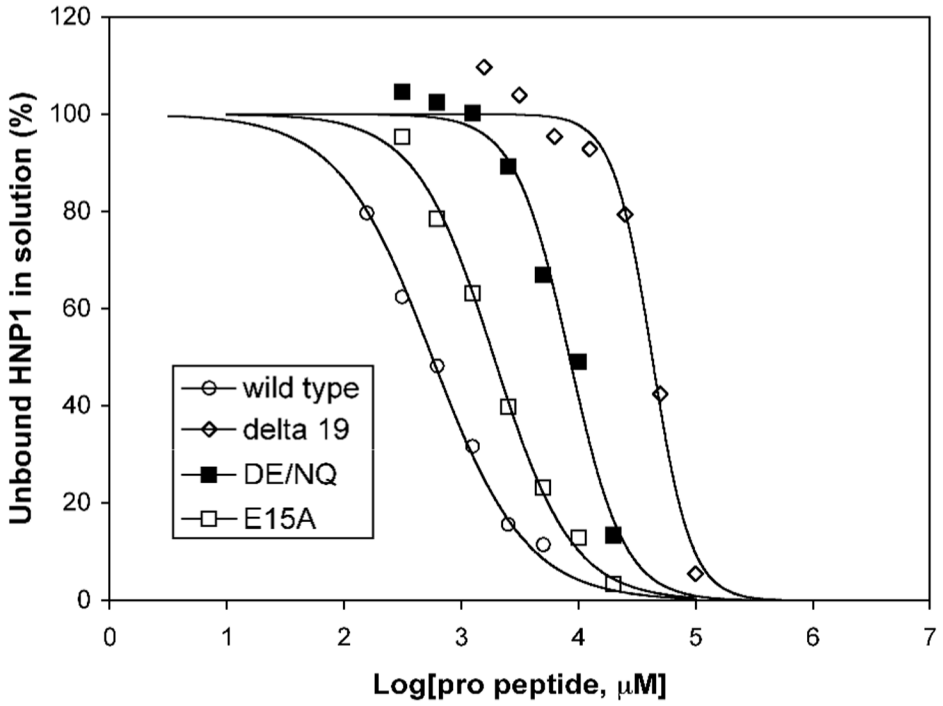

Figure 3.

Representative competitive binding of HNP1 to the wild type pro peptide in the presence of wild type (circle), E15A (empty square), Δ19 (diamond), and DE/NQ (filled square) pro peptides, monitored by surface plasmon resonance (Biacore). Measurements were conducted on a CM4 chip with the wild type pro peptide immobilized. HNP1 of 100 nM was incubated with varying concentrations of pro peptide, followed by an injection of the incubation mixture. Free HNP1 in the solution was deducted, based on the initial rate (slope) of association, from a standardized calibration curve established by kinetic measurements of HNP1 injected alone at different concentrations.

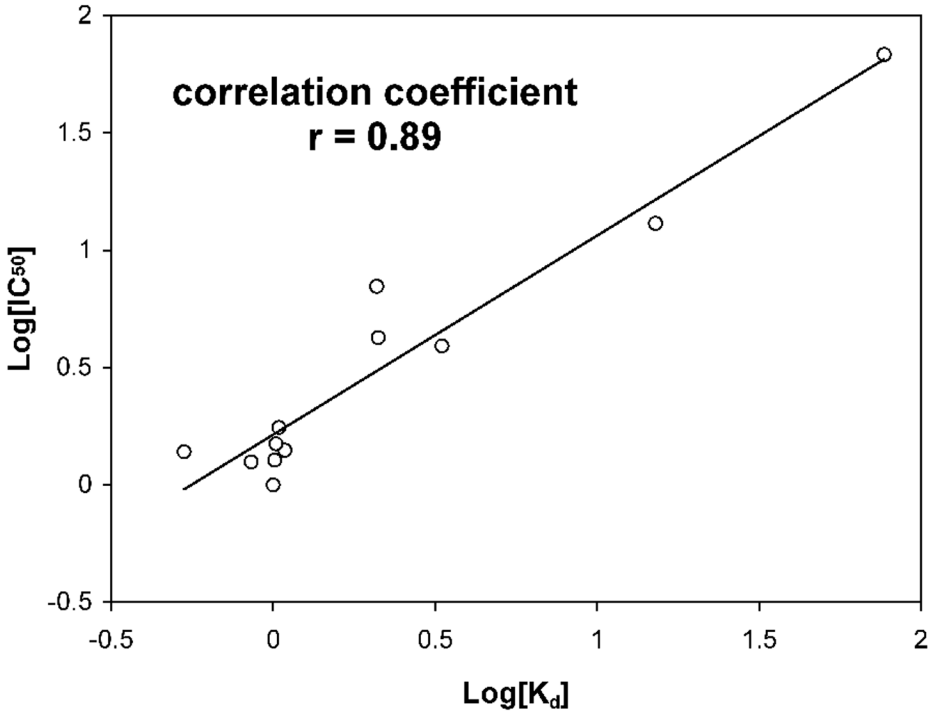

Figure 4.
Correlation between the binding affinities of HNP1 for selected pro peptides (W30A and VVVL/4A excluded), measured by SPR-based direct binding assays and competitive binding assays.

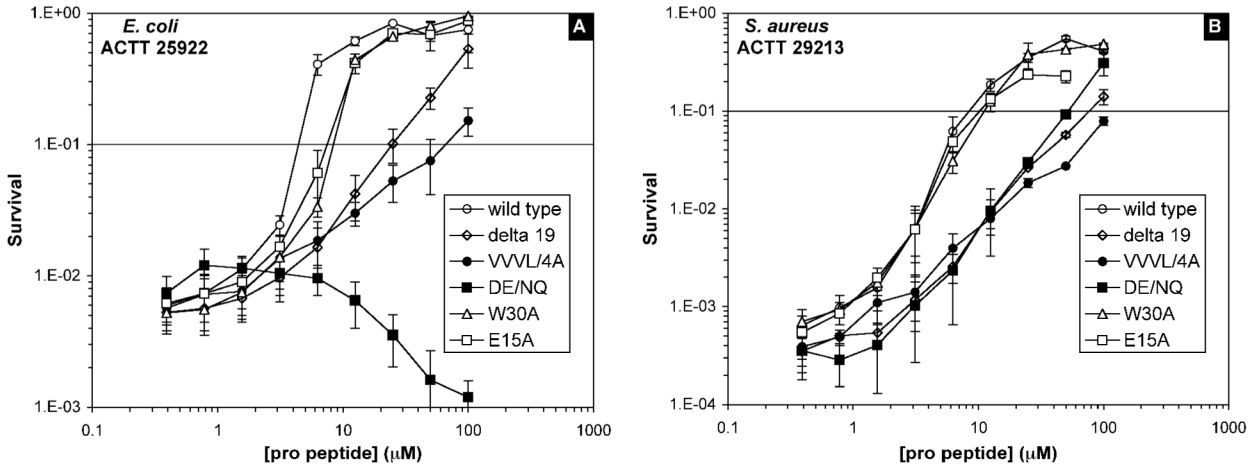

Figure 5.

Inhibition of the antibacterial activity of HNP1 by pro peptides at 37 °C. The data are from two independent vCC assays for *E. coli* (panel A) and *S. aureus* (panel B) with 10 μM HNP1, titrated by the wild type pro peptide (empty circle), Δ19 (diamond), VVVL/4A (filled circle), DE/NQ (filled square), E15A (empty square), or W30A (triangle).

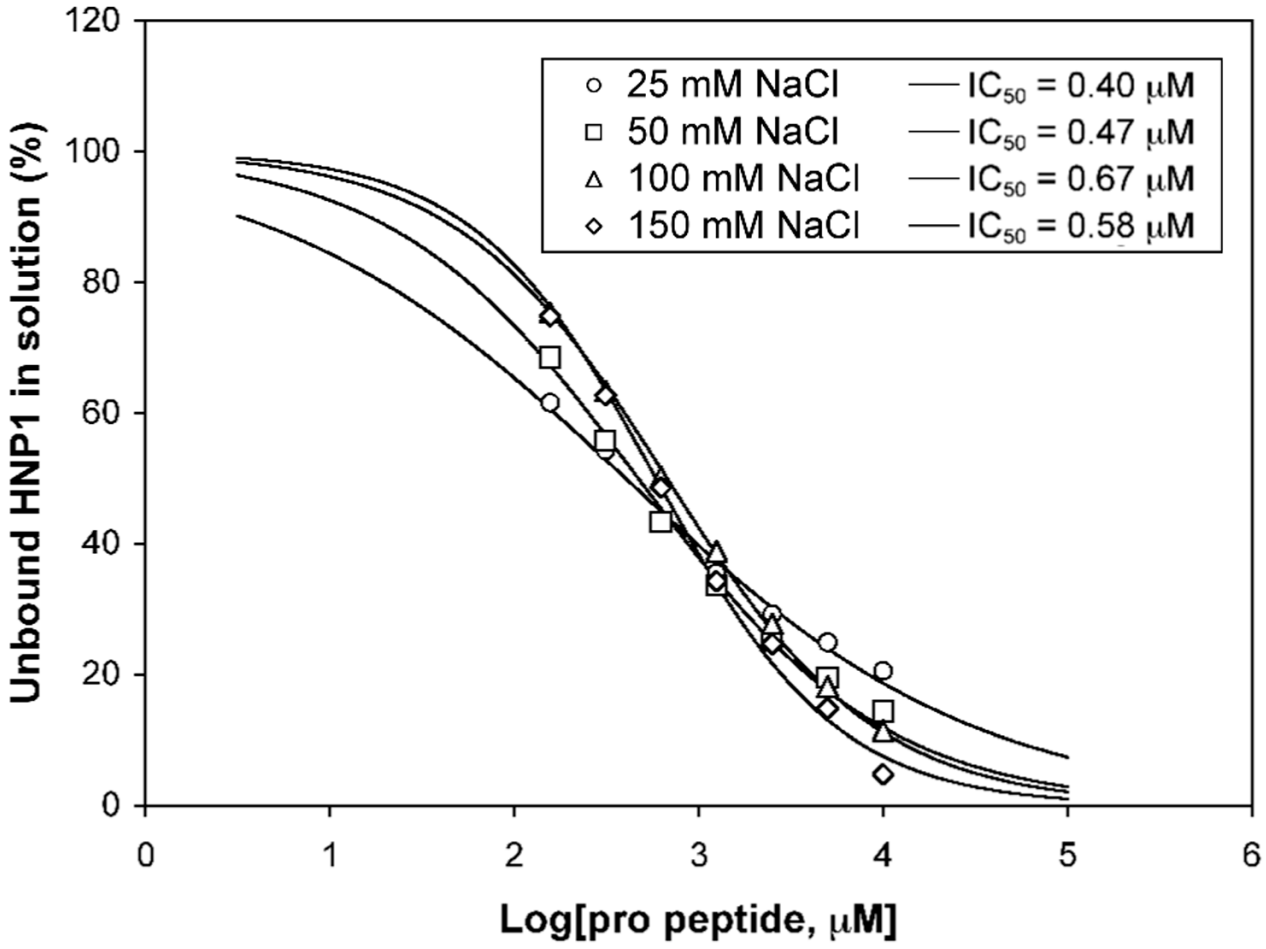

Figure 6.
Competitive binding of 100 nM HNP1, at different salt concentrations, to immobilized wild type pro peptide in the presence of varying concentrations of the same pro peptide, monitored by surface plasmon resonance (Biacore). Free HNP1 in the solution was deducted from the slopes of sensor grams as described in the legend of Figure 3.

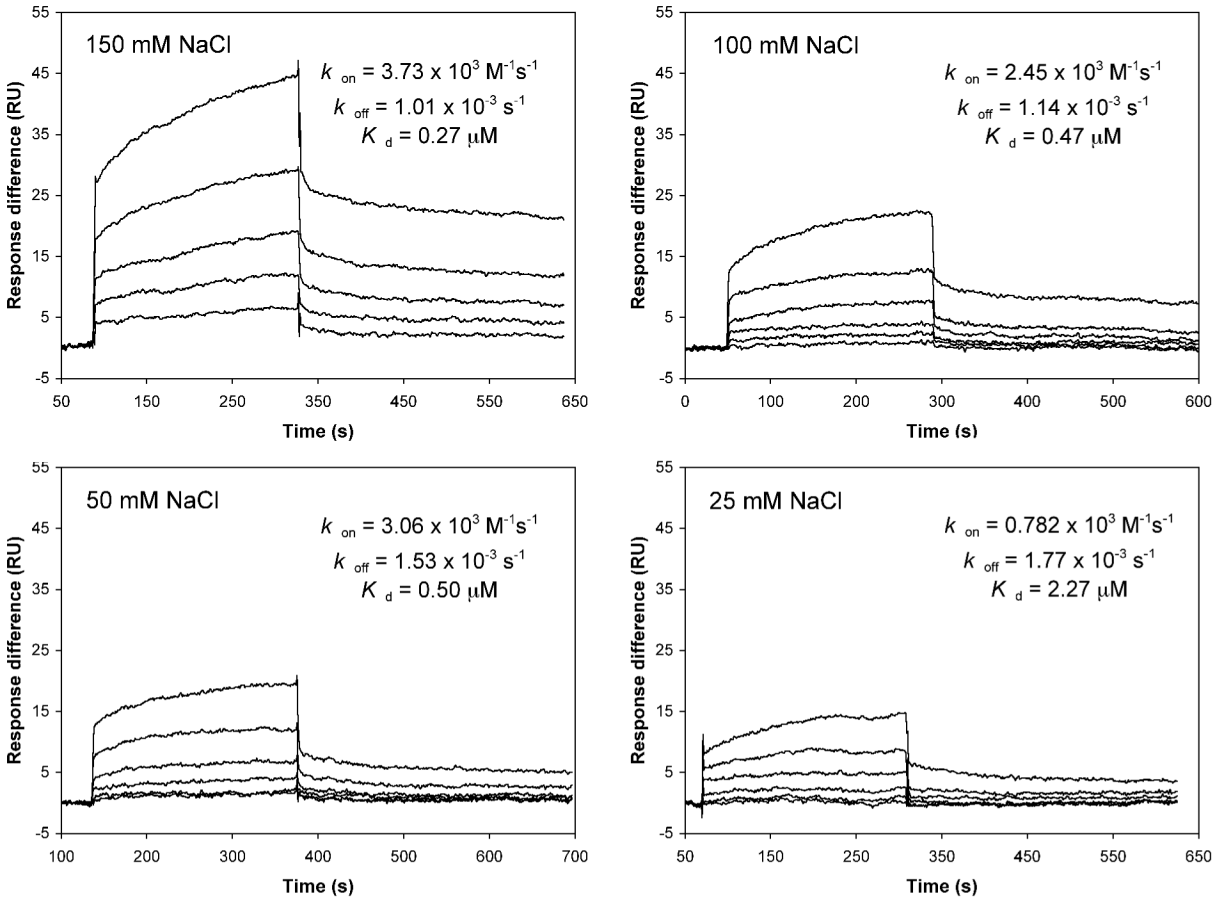

Figure 7.

Direct binding of varying concentrations of wild type pro peptide to immobilized HNP1 in the presence of different concentrations of salt, monitored by surface plasmon resonance (Biacore).

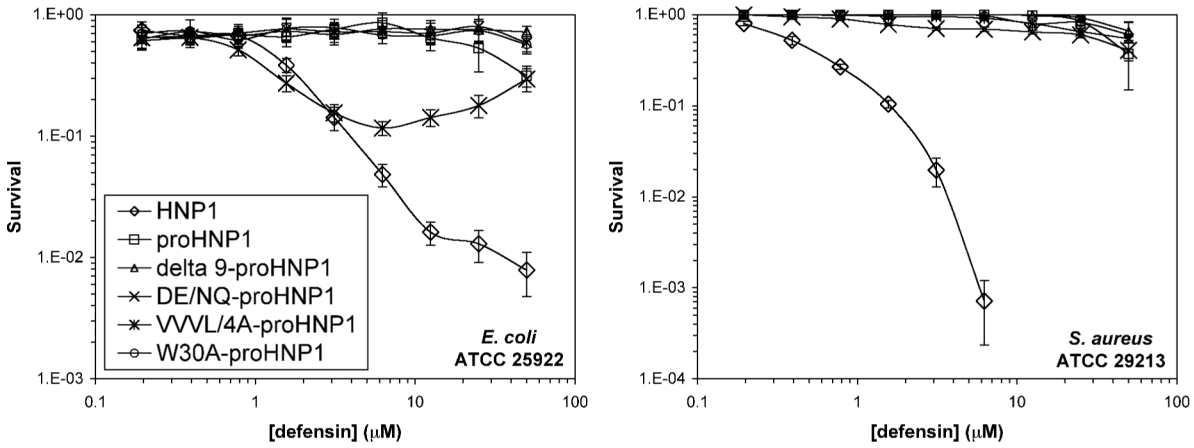

Figure 8.
Survival curves of *E. coli* and *S. aureus* exposed to full-length wild type proHNP1 and its analogs. Each curve is the mean of two independent experiments.

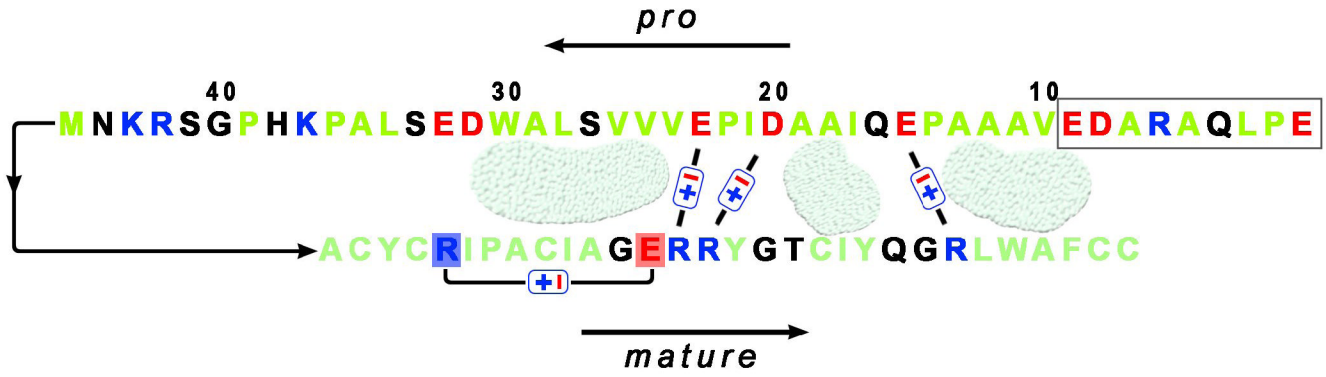

Figure 9.

Schematic representation of stabilizing interactions between the pro peptide (the top sequence) and mature HNP1 (the bottom sequence). The basic, acidic, and hydrophobic residues are colored in blue, red, and light-green, respectively. The first nine residues of the pro peptide, marked with a box, were shown in this study as dispensable for HNP1 binding and inhibition. Three acidic residues, Glu^{15}, Asp^{20}, and Glu^{23}, that stabilize an interaction with HNP1, may participate in the electrostatic interactions (i.e. salt bridges) with three accessible arginines of mature defensin. Hypothetical hydrophobic contacts between the pro peptide and HNP1 are indicated by grainy light-green shapes. For clarity, every tenth residue of the pro peptide is numbered, and two charged residues of HNP1 (Arg^5 and Glu^{13}) forming an intra-molecular salt bridge, thus inaccessible for intermolecular interactions, are also indicated.

Table 1

Binding parameters for HNP1 interacting with its pro peptide and various pro-peptide analogs, determined by SPR-based direct binding assays (K_d) and competition binding assays (IC_{50}).

	K_d (μM)	$K_{d,mut}/K_{d,wt}$	IC_{50} (μM)	$IC_{50,mut}/IC_{50,wt}$
Wild type Mutants	0.200		0.574 ± 0.010	
E1A	0.106	0.53	0.794 ± 0.070	1.38
D8A	0.217	1.08	0.808 ± 0.196	1.40
E9A	0.202	1.01	0.732 ± 0.026	1.27
E15A	0.422	2.11	2.44 ± 0.54	4.25
D20A	0.417	2.08	4.03 ± 0.12	7.02
E23A	0.835	4.17	2.25 ± 0.24	3.91
D31A	0.204	1.02	0.861 ± 0.101	1.50
E32A	0.208	1.04	1.01 ± 0.07	1.76
W30A	n.r.[a]	n.r.	2.47 ± 0.33	4.30
Δ9	0.171	0.855	0.72 ± 0.16	1.26
Δ19	15.4	77.0	39.3 ± 3.3	68.4
DE/NQ	3.03	15.2	7.48 ± 0.89	13.0
VVVL/4A	n.b.[b]		n.b.	

[a] n.r., not reported due to erratic curve fitting

[b] n.b., no binding detected.

Table 2

Effective concentrations (EC_{90}, μM) of pro peptide at which 90% of input cells were killed by 10 μM HNP1, determined by vCC assays. The values in the top half of the table were the means of two separate experiments; the entries in the bottom half of the table were averages of three independent experiments performed at a later date.

	S. aureus		*E. coli*	
	EC_{90}	**ratio**	**EC_{90}**	**ratio**
WT	7.8 ± 1.1	1.0	3.6 ± 0.1	1.0
E15A	9.6 ± 0.5	1.2	6.8 ± 0.5	1.9
W30A	10.9 ± 1.8	1.4	7.0 ± 0.0	1.9
Δ19	74.5 ± 7.8	9.6	25.6 ± 4.2	7.1
DE/NQ	51.8 ± 1.6	6.6	n.a	
VVVL/4A	137 ± 20	18	66.4 ± 20.2	18
WT	4.1 ± 0.3	1.0	2.3 ± 0.3	1.0
E1A	5.8 ± 0.8	1.4	3.3 ± 0.3	1.4
D8A	5.0 ± 0.6	1.2	2.8 ± 0.2	1.2
E9A	4.8 ± 0.5	1.2	3.0 ± 0.7	1.3
E15A	5.7 ± 0.7	1.4	2.9 ± 0.5	1.3
D20A	8.9 ± 0.5	2.2	3.3 ± 0.3	1.4
E23A	5.4 ± 1.0	1.3	3.6 ± 0.3	1.6
D31A	4.7 ± 0.7	1.1	4.3 ± 1.4	1.9
E32A	4.4 ± 0.5	1.1	3.2 ± 0.6	1.4
Δ9	4.9 ± 0.6	1.2	3.2 ± 0.2	1.4
Δ19	55.6 ± 22.0	14	12.6 ± 1.0	5.5