# Intramolecular Inhibition of Human Defensin HNP-1 by Its Propiece

Erika V. Valore, Edith Martin, Sylvia S.L. Harwig, and Tomas Ganz

Will Rogers Institute Pulmonary Research Laboratory, Department of Medicine, UCLA School of Medicine, Los Angeles, California 90095-1736

## Abstract

We examined mechanisms that protect host defense cells from their cytotoxic effector molecules. Human neutrophil peptides (HNP) 1-3 are microbicidal and cytotoxic defensins, initially synthesized as 94-amino acid preproHNP<sub>1-94</sub>, cotranslationally proteolyzed to proHNP20-94, then converted by removal of the anionic propiece to mature HNP<sub>65-94</sub> (HNP-1 and -3) and HNP<sub>66-94</sub> (HNP-2). We hypothesized that during synthesis and subcellular sorting the anionic propiece inhibits the cytotoxicity of the cationic defensin. We expressed preproHNP-1 cDNA in recombinant baculovirus-infected insect cells that secreted the normally transient proHNP-1<sub>20-94</sub> into the medium. Cyanogen bromide cleaved proHNP-1<sub>20-94</sub> at the fortuitously located Met<sub>64</sub> to yield mature recombinant HNP-165-94 and unlinked propiece. Recombinant and native HNP-1 purified from PMN were identical as judged by mass spectrometry, retention time in reverse-phase high performance liquid chromatography, migration on acid-urea polyacrylamide gels, and reaction with a conformation-specific antibody. Recombinant and native HNP-1 had comparable microbicidal activity towards Listeria monocytogenes and were similarly potent in permeabilizing K562 leukemia cells, but proHNP-1<sub>20-94</sub> was virtually inactive in both assays. Addition of unlinked propiece (proHNP-120-64 with Met64→homoserine) inhibited the bactericidal and cell-permeabilizing activity of mature HNP-1 in a dose-dependent manner. Linked, and to a lesser extent unlinked, propiece interfered with the binding of HNP-1 to target cells. The propiece thus acts as an efficient intramolecular inhibitor of defensin HNP-1 cytotoxicity. (J. Clin. Invest. 1996. 97:1624–1629.) Key words: neutrophil • antimicrobial peptide • posttranslational processing • cytotoxicity • inflammation

### Introduction

Members of the defensin family are small, variably cationic, cysteine- and arginine-rich mammalian peptides, defined by their conserved disulfide pattern (1). Peptides of similar size, composition, and function have also been detected in insects and even plants (2–4). In vitro, defensins exhibit a wide range

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/96/04/1624/06 \$2.00 Volume 97, Number 7, April 1996, 1624–1629 of antimicrobial activity against gram-positive and gram-negative bacteria, fungi, enveloped viruses, and parasites (1, 5, 6), but are also cytotoxic to various types of mammalian cells (7, 8). Defensins kill bacterial and mammalian cells by permeabilization of the cell membrane, followed by additional events that result in irreversible progression to cell death. During the initial phase of cytotoxicity, cells can be rescued from death by preventing the binding of defensins to cell surface or reversing the defensin-mediated permeabilization of the cell membrane (9, 10).

Mammalian defensins are abundant in the cytoplasmic granules of neutrophils, Paneth cells of the small intestine, and some macrophages. The defensins human neutrophil peptides (HNP)<sup>1</sup> 1–3 together with the less abundant HNP-4 constitute 30% of the protein of the microbicidal azurophil granules of neutrophils, and two other human defensins, HD-5 and HD-6, have been localized recently by in situ hybridization to the intestinal Paneth cells.

During the synthesis of defensins HNP-1-3, the initial translation product is a 94-amino acid (aa) precursor that contains a 19-aa endoplasmic reticulum signal sequence, a 45-aa anionic propiece, and a 29-30-aa cationic mature peptide (11). The signal sequence is cotranslationally removed and over several hours the proprotein is sequentially cleaved from its amino terminus to generate the mature defensin peptide (Fig. 1). Since the metabolic cost of defensin biosynthesis is approximately doubled by the relatively large anionic propiece, we hypothesized that the propiece has an important functional role. In previous work we established a model system of murine myeloid 32D cl3 cells transduced with HNP-1 cDNA in a retroviral vector (12). Deletion of preproHNP-1 residues 21-39, at the amino-terminal end of the propiece, had only minor effects on defensin synthesis, processing, and subcellular sorting, but all of these processes were dramatically impaired by deletions in the carboxy-terminal segment of the propiece (aa 40-64). In addition to the essential role of the propiece in defensin biosynthesis, we earlier proposed that the anionic propiece may neutralize the cytotoxicity of the cationic mature peptide during its biosynthesis, posttranslational processing, and transport through subcellular compartments (13). However, due to the transient nature of prodefensin in myeloid cells, we were unable to isolate sufficient amounts from natural sources to test this conjecture. In this study, we biosynthesized preproHNP-11-94 using a baculovirus/insect cell system wherein the insect cells cleaved the signal (pre) sequence and released the 75-aa proHNP-1<sub>20-94</sub> into the culture medium. Comparison of the biological activities of proHNP-1 and HNP-1 and the analysis of the effect of the propiece on the activity of mature HNP-1 were facilitated by the serendipitous location of the lone methionine (Met<sub>64</sub>) residue at the carboxy-

Address correspondence to Tomas Ganz, Ph.D., M.D., Department of Medicine, CHS 37-055, UCLA School of Medicine, Los Angeles, CA 90095-1736. Phone: 310-825-6112; FAX: 310-206-8766; E-mail: tganz@medicine.medsch.ucla.edu

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<sup>1.</sup> *Abbreviations used in this paper:* aa, amino acid: CNBr, cyanogen bromide; HNP, human neutrophil peptide; RP-HPLC, reverse-phase HPLC; TSB, trypticase soy broth.

terminal end of the proprotein. ProHNP-1 molecule could be cleaved at this residue with cyanogen bromide (indicated by the large arrow in Fig. 1) to yield the carboxy-terminally modified (methionine to homoserine/homoserine lactone) anionic propiece and the mature defensin HNP-1.

#### Methods

Construction of HNP-1-baculovirus. The baculovirus expression system was used to overexpress preprodefensin in insect Spodoptera furgiperda (Sf21) cells as described by the manufacturer (CLONTECH, Palo Alto, CA). Briefly, HNP-1 cDNA (12) fragment containing the ribosomal binding site, the translation start codon ATG, the entire coding region of preproHNP-1, a stop codon, and BamHI restriction sites at the 5' and 3' ends of the insert was cloned into the BamHI restriction site of the pBacPAK1 transfer vector and transformed into XL-1 Blue Escherichia coli (14). Selection of defensin-containing clones in the correct orientation was accomplished by detection of a PCR-amplified product. The BacPAK1-HNP-1 transfer plasmid was purified by ultracentrifugation on cesium chloride gradients and was cotransfected with Bsu361-digested BacPAK6 (Autographa californica nuclear polyhedrosis) viral DNA into Sf21 cells with Lipofectin (GIBCO-BRL, Grand Island, NY). Recombinant baculovirus was harvested from culture media 72 h after cotransfection. Individual viral plaques were isolated from infected monolayers of Sf21 cells using the viral plaque assay. ProHNP-1-expressing viral clones were selected by a sandwich enzyme immunoassay using monoclonal antibodies to mature HNP-1-3 which also detects the prodefensin precursor (15).

Purification of proHNP-1. Sf21 insect cells infected with recombinant baculovirus-HNP-1 (BacHNP-1) released a prodefensin into the culture medium, where it accumulated to a concentration of 3-5 µg/ml after 60 h of infection as estimated by Coomassie blue-stained PAGE. For larger scale protein expression Trichoplusia ni 5B1-4 (High Five) adherent cells (Invitrogen, San Diego, CA) were used since they grow faster and yield more recombinant protein than Sf21 cells. High Five cells cultured in EX-CELL 405 serum-free medium (JRH Biosciences, Lenexa, KS) were infected at exponential growth phase (70-80% confluent) with 10 plaque forming units BacHNP-1 virus per cell. At  $\sim$  60 h after infection, the cells were removed from the culture medium by centrifugation, and the culture medium was concentrated  $\sim$  500-fold and diafiltered with 5% acetic acid using a tangential flow concentration apparatus (Filtron Technology Corp., Northborough, MA). Although the mass of proHNP-1 is only 8 kD, it was found to be aggregated in the culture medium, and retained by a 10-kD cutoff membrane (Omega polysulfone "minisette"; Filtron Technology Corp.). Concentrated proteins were separated using a continuous flow preparative gel electrophoresis apparatus (model

*Figure 1.* Amino acid sequence of preproHNP-1 defensin. Major proteolytic cleavage sites are indicated by asterisks and the CNBr cleavage site is shown by the arrow. The distribution of charged residues and the conserved disulfide-bonding pattern of the mature peptide are emphasized. 491 Prep Cell; Bio-Rad Laboratories, Richmond, CA) in a 15.8% acid-urea polyacrylamide gel (16). Fractions containing proHNP-1 were assayed by Coomassie-stained acid-urea or SDS-tricine (17) PAGE and further purified by reverse-phase (RP)-HPLC on a  $4.6 \times 250 \text{ mm}$  Vydac C18 column (Separations Group, Hesperia, CA) using a 1% acetonitrile increment per minute in 0.1% trifluoroacetic acid.

Amino acid sequencing. Purified proHNP-1 was analyzed by SDS-Tricine PAGE and then electroblotted to polyvinylidenedifluoride membranes (PVDF; Bio-Rad Laboratories). Electrophoretic transfer (Transblot; Bio-Rad Laboratories) was performed in 0.05 M sodium borate, pH 9, 20% methanol, 0.05% SDS at 20 V (0.18 A) for at least 2 h. The PVDF membrane was stained with 0.4% napthol blue black dye, 25% isopropanol, 10% methanol and destained in water. Amino acid sequence of the predominant protein band (8 kD) was determined by the UCLA Peptide Sequencing Facility by Edman degradation on an ABI 475 (Applied Biosystems, Foster City, CA) automated amino acid sequencer.

*Mass spectrometry.* HPLC-purified proHNP-1 and cyanogen bromide (CNBr) cleavage products were analyzed in the UCLA Center for Molecular and Medical Mass Spectrometry by matrix-assisted laser desorption ionization technique on a Voyager RP Instrument (PerSeptive Biosystems, Framingham, MA) and electrospray measurements were recorded on a Sciex API III (Perkin-Elmer Corp., Foster City, CA).

*CNBr cleavage.* Purified proHNP-1 (200  $\mu$ g) was lyophilized and resuspended in 200  $\mu$ l 1 M CNBr freshly dissolved in 6 M guanidine-HCl, 0.2 N HCl as described previously (18). Samples were incubated in the dark for 12–18 h, at room temperature, under N<sub>2</sub> gas. The reaction was stopped by addition of 100  $\mu$ l H<sub>2</sub>O. The mixture was dialyzed against 5% acetic acid, lyophilized, and again dissolved in 5% acetic acid. The cleavage products were purified by RP-HPLC as described for proHNP-1.

Antimicrobial assay: determination of colony-forming units (CFU). Listeria monocytogenes was chosen as the target organism because it is very sensitive to defensin antimicrobial activity. The CFU assay was performed as follows: overnight cultures of L. monocytogenes were subcultured in 3% trypticase soy broth (TSB) and grown for 2.5 h to exponential phase in a 37°C shaking incubator. Bacterial concentration was estimated photometrically (OD<sub>620</sub> of 0.02 is  $\sim 5 \times 10^7$  bacteria/ml). Bacteria were washed and diluted to  $5 \times 10^5$  cells/ml in 0.01 M sodium phosphate, pH 7.4, 1% TSB. Lyophilized peptide was resuspended in 10 µl 0.01% acetic acid and mixed with 90 µl bacteria, incubated at 37°C in a shaking water bath for 3 h, then diluted 100-fold in 0.01 M sodium phosphate, pH 7.4, 1% TSB, and plated in triplicate on TSB-agar plates. Bacterial colonies were spread on the agar plates using a spiral plater (Spiral Systems Inc., Cincinnati, OH) which deposits a defined volume per area. Plates were incubated for 12-16 h at 37°C, the colonies were counted in specific areas on the plates, and the CFU per milliliter was calculated. For neutralization assays, native HNP-1 and recombinant anionic propiece were mixed, lyophilized, and resuspended in 0.01% acetic acid. The mixture was allowed to associate at room temperature for at least 1 h before the performance of the CFU assay.

Agarose diffusion antimicrobial assay. Microbicidal activity of purified peptides was also tested by radial diffusion assay (19). Briefly, *L. monocytogenes* were prepared as described for the CFU assay except they were kept in 0.01 M sodium phosphate, pH 7.4, without the addition of TSB. Bacteria ( $4 \times 10^6$  cells) were mixed with 10 ml 42°C agarose (0.03% TSB in 0.01 M sodium phosphate, pH 7.4, with 1% low EEO agarose [A6013; Sigma Chemical Co., St. Louis, MO]) and immediately poured into 10 × 10-cm<sup>2</sup> culture plates. Evenly spaced wells of 3 mm in diameter were punched into the solidified agar. The test peptides were lyophilized and resuspended in 0.01% acetic acid. 5 µl of peptide solution or diluent control was added to each well and allowed to diffuse into the agarose for 3 h at 37°C. After overlaying with a 10-ml nutrient agarose layer (6% TSB in 0.01 M sodium phosphate, pH 7.4, 1% low EEO agarose, 42°C), the plates were incubated for 16–18 h at 37°C. Clear zones around the



*Figure 2.* Gel overlay antibacterial assay. *A* and *B* were generated with duplicate acid-urea PAGE. (*A*) Coomassie-stained acid-urea PAGE,  $\sim 1 \mu g$  of protein per lane. *Lane 1*: defensins HNP-1–3 (confluent bands: 1 = fastest, 2 = intermediate, 3 = slowest). *Lane 2*: baculovirus-expressed proHNP-1. *Lane 3*: CNBr-cleaved proHNP-1. (*B*) Gel overlay antibacterial assay: near confluent colonies of *L. monocytogenes* appear as a speckled light background with a faint rectangular outline of the location of gel overlay. Dark (clear) zones show areas where protein bands transferred from the gel inhibited bacterial colony formation. Lane contents are the same as in *A*. Clear zone in *lane 1* corresponds to the bactericidal activity of standards, bands of defensins HNP-1–3; the clear zone in *lane 3* reflects the microbicidal activity of HNP-1 band generated by CNBr cleavage of proHNP-1.

wells were measured and expressed in convenient units defined as: [diameter of clearing (mm) – diameter of the well (3 mm)] × 10.

Bacterial gel overlay antimicrobial assay. A bacterial gel overlay assay was performed as described previously (19). Specifically, defensin proteins were separated by acid-urea PAGE and the gel was neutralized by washing for 5 min in 0.01 M sodium phosphate, pH 7.4, with 0.01 N NaOH, then in 0.01 M sodium phosphate, pH 7.4, for 15 min. Agarose containing L. monocytogenes was prepared as described for the gel diffusion assay with the exception that no wells were formed. The polyacrylamide gel was placed on the agarose and incubated at 37°C for 3 h to allow the proteins in the gel to diffuse into the bacterial layer. The gel was then removed and the agarose overlaid with a nutrient layer as described for the radial diffusion assay. After 18 h at 37°C to allow visible bacterial growth, the agarose plate was stained for 10 min with 0.003% acridine orange dye and destained with 10 mM sodium phosphate, pH 7.4. Acridine orange, a nucleic acid stain, will cause the bacteria present in the agar gel to fluoresce under ultraviolet light. Nonfluorescent clear zones were seen where the polyacrylamide gel had contained protein bands with antimicrobial activity, preventing the growth of Listeria.

Cytotoxicity microassay. The K562 human leukemia cells were used as targets for the cytotoxicity assay. The cells were washed and suspended to 10<sup>6</sup> cells/ml in serum free RPMI-1640 medium (GIBCO-BRL) and aliquoted (10  $\mu$ l/well) in a Terasaki plate (Nunc, Roskilde, Denmark). Peptides were lyophilized and resuspended in 0.01% acetic acid. Stocks (10×) were made so that 1  $\mu$ l of peptide was added to each well with a P2 pipetor (Rainin Instrument Co. Inc., Emeryville, CA). Peptide and K562 cells were incubated in triplicate wells for 12–16 h at 37°C in a 5% CO<sub>2</sub> incubator. Samples were transferred to Eppendorf tubes on ice, 1  $\mu$ l of 0.4% trypan blue in phosphate-buffered saline was added, and trypan-excluding (viable) and trypan-staining cells (nonviable) were counted on a hemocytometer.

Defensin binding to K562 cells. The binding of trace-radioiodinated HNP-1 and proHNP-1 to K562 cells was analyzed as described previously (8, 10). Briefly, purified HNP-1 and proHNP-1 were labeled with <sup>125</sup>I using the lodogen method (Pierce, Rockford, IL). Unincorporated Na <sup>125</sup>I was removed by exhaustive dialysis. Tracelabeled <sup>125</sup>I-HNP-1 peptide was preincubated with the indicated amount of unlabeled propiece (proHNP-1<sub>20-64</sub>) for 1 h in 0.01% acetic acid with 10 mM sodium phosphate, pH 7.4, buffer to facilitate maxi-



*Figure 3.* Comparison of antibacterial activity of recombinant peptides and native HNP-1. The graph shows CFU assays after 3 h of incubation of *L. monocytogenes* with proHNP-1 ( $\blacktriangle$ ), anionic propiece ( $\blacktriangledown$ ), rHNP-1 ( $\blacksquare$ ), and native mature HNP-1 ( $\blacklozenge$ ) at 37°C. Controls with peptide diluent substituted for peptide solution were incubated under the same conditions (t = 3), or incubated on ice immediately (t = 0).

mal association. <sup>125</sup>I-HNP-1/propiece or <sup>125</sup>I-proHNP-1 ( $4 \times 10^6$  cpm) was added to  $5 \times 10^5$  K562 cells/ml of serum-free RPMI 1640 at 4°C and incubated for 20 min. To separate unbound from bound peptide, cells (250 µl) were centrifuged at 12,000 g through 200 µl silicon oil (Versilube F50; General Electric Co., Waterford, NY) at room temperature. After the supernatant and oil layers were removed, the tip of the tube containing the cell pellet was cut off with a hot razor blade and its radioactivity was counted. The radioactivity of an aliquot of the input peptide–cell mixture was also determined for each sample.

*Data analysis.* The Sigma Plot (Jandel Scientific, San Rafael, CA) computer program was used to calculate the mean and its standard error for each data point in Figs. 3–5. An average of five experiments contributed to each data point.

#### Results

Initial attempts to recover proHNP-1 from recombinant baculovirus-infected insect cells or media by gel filtration revealed that proHNP-1 formed very large complexes that could be dissociated by preparative electrophoresis in acid-urea gels but not by chaotropic agents alone. Subsequent RP-HPLC yielded a protein which eluted from the C18 column at  $\sim$  41% acetonitrile and was homogeneous by silver-stained SDS-PAGE and by mass spectrometry analysis. The final yield of purified proHNP-1 from culture supernatant was  $\sim 20\%$  or equivalent to 1 µg/ml culture medium. Analysis of purified proHNP-1 (Fig. 2 A, lane 2) on acid-urea PAGE revealed a single protein band that migrated slightly slower than HNP-3. The first 10 amino acids of this protein were found to be identical to the first 10 residues of the 75-aa proHNP-1 sequence (preproHNP- $1_{20-94}$ ) deduced from the cDNA (20). In Western blot analysis, proHNP-1 reacted with a conformation-specific antibody against native HNP-1 purified from human neutrophils (data not shown). CNBr cleavage at the methionine residue of proHNP-1 generated three protein bands on acid-urea polyacrylamide gels (Fig. 2 A, lane 3). Mass spectrometry and amino-terminal peptide sequencing data (not shown) indi-



*Figure 4.* Comparison of cytotoxic activity of recombinant peptides and native HNP-1. The graph shows cytotoxicity assays of proHNP-1 ( $\blacktriangle$ ), mature HNP-1 ( $\bigcirc$ ), and rHNP-1 ( $\blacksquare$ ). Peptide in 0.01% acetic acid was added to K562 cells (10<sup>6</sup> cells/ml), incubated for ~ 16 h, and the percentage of viable cells was determined by trypan blue dye exclusion.

cated that, from the slowest to the fastest migrating band, these proteins corresponded to: the 45-aa anionic propiece with Met<sub>64</sub> modified to homoserine/homoserine lactone (4778 D/4760 D), uncleaved proHNP-1 (8232 D), and mature HNP-1 (3442 D). During RP-HPLC, the recombinant HNP-1 (rHNP-1) eluted from the C18 column at 34% acetonitrile, same as native HNP-1 purified from leukocytes (21). ProHNP-1 and its CNBr cleavage products were compared in an antibacterial gel overlay assay with L. monocytogenes (Fig. 2 B). Recombinant HNP-1 (fastest migrating band, lane 3) was antibacterial but proHNP-1 (lane 2) and the anionic propiece (slowest migrating band, lane 3) were inactive (Fig. 2 B). In a quantitative CFU assay, shown in Fig. 3, rHNP-1 and the native HNP-1 displayed similar antibacterial activity at concentrations ranging from 0.3 to 30 µM. ProHNP-1 was inactive except at the highest concentration where it slightly inhibited bacterial growth. The CNBr-cleaved anionic propiece had no antimicrobial activity over the same concentration range. These results were confirmed in the radial diffusion antimicrobial assay. Over a 0-60 µM peptide concentration range, native HNP-1 and rHNP-1 were equipotent but proHNP-1 had no antimicrobial activity (data not shown).

Cytotoxicity to K562 cells cultured overnight in the presence of peptides was assessed by trypan blue exclusion (Fig. 4). Permeabilization of K562 to trypan blue is a highly sensitive assay that detects obligatory cytotoxic events preceding chromium release (10). Native HNP-1 and rHNP-1 had similar cytotoxic potency but proHNP-1 was inactive. Even at 30  $\mu$ M concentration, where HNP-1 permeabilized 94% of the cells, proHNP-1 was not detectably cytotoxic. Thus in the range of concentrations tested, the proregion not only substantially blocks the antimicrobial activity of HNP-1 against *L. monocytogenes* but also completely inhibits the cytotoxic activity of HNP-1.

To determine whether the propiece can inactivate HNP-1 even when the two peptides are not covalently linked, the purified anionic propiece and native HNP-1 were mixed together



Figure 5. Neutralization of HNP-1 activity by varying concentrations of unlinked defensin propiece. (A) Antibacterial CFU assay. The molar ratio of propiece to HNP-1 is indicated on the horizontal axis, the assay concentrations of HNP-1 were either 3 µM (hollow circle) or 7.5 µM (solid circle). Mixtures of native HNP-1 and defensin propiece were lyophilized, resuspended in 0.01% acetic acid, and allowed to incubate at room temperature for at least 1 h to maximize peptide interaction. Peptide mixtures were then tested for bactericidal activity against L. monocytogenes by CFU assay, as in Fig. 3 and Methods. Controls were incubated on ice (t = 0) or incubated at 37°C (t = 3)with peptide diluent. Intact proHNP-1 (linked propiece) was tested at concentrations of either 3 µM (hollow circle) or 7.5 µM (solid circle). (B) Cytotoxicity assay. The molar ratio of propiece to HNP-1 is indicated on the horizontal axis, the assay concentration of native HNP-1 was 30 µM. Native HNP-1 was lyophilized with propiece at the appropriate molar ratio, resuspended in 0.01% acetic acid, incubated for at least 1 h at room temperature, added to K562 cells, and incubated for  $\sim$  16 h. Cell viability was assessed by trypan blue dye exclusion. The control contained the peptide diluent, and proHNP-1 (linked propiece) was tested at 30 µM concentration.

before antimicrobial and cytotoxicity assays. In the antimicrobial CFU assay (Fig. 5 *A*), propiece was added at concentrations ranging from zero to four times the molar concentration of native HNP-1. Regardless of the initial HNP-1 concentration (3.0 or 7.5  $\mu$ M), maximum inhibition of antimicrobial activity was reached at 0.5 molar ratio of propiece mature HNP-1. The results suggest that under these conditions one molecule of propiece interacts with two molecules of mature HNP-1. However, even at maximal inhibition of HNP-1 by unlinked propiece, the expected bacterial growth during the 3-h incubation time was not observed. The complex of HNP-1 and the propiece apparently retained bacteriostatic activity to *L. monocytogenes*. In contrast, proHNP-1 (with linked pro-



*Figure 6.* Binding of proHNP-1 and HNP-1 to K562 cells. The height of each bar indicates the fraction of peptide bound to K562 cells after 20 min at 4°C. Mean and its standard error are shown (n = 6) with *P* values above the bars comparing each set of binding measurements with HNP-1 binding measurements (Student's *t* test). The binding assay was performed with radioiodinated proHNP-1 (6  $\mu$ M) alone, or with radioiodinated proHNP-1 (6  $\mu$ M) without or with added propiece (*PP*) at the indicated molar ratio to HNP-1.

piece) had only a slight inhibitory effect on the growth of *L.* monocytogenes after 3 h of incubation. In a cytotoxicity assay (Fig. 5 *B*), the addition of unlinked propiece inhibited native HNP-1 activity in a dose-dependent manner and decreased cytotoxicity from 93 to 17% when the anionic propiece was at twice molar excess over HNP-1. As before, proHNP-1 (with linked propiece) was not cytotoxic to K562 cells.

Since the binding of HNP-1 to the cell surface is a critical determinant of its cytotoxicity to K562 cells (8, 10), we inquired whether the presence of the propiece affects the fraction of HNP-1 that associates with the cell surface after 20 min of incubation at 4°C, conditions that prevent peptide internalization. At 6  $\mu$ M input peptide concentration, more than four times as many molecules of HNP-1 than proHNP-1 bound to K562 cells (Fig. 6). The binding of HNP-1 to target cells was also substantially diminished by the addition of unlinked propiece.

#### Discussion

We used the baculovirus protein expression system to synthesize proHNP-1, a transient precursor form of a human defensin. From this propeptide, CNBr cleavage generated mature rHNP-1 that displayed the same microbicidal and cytotoxic activity as neutrophil-derived HNP-1. Moreover, rHNP-1 had the same electrophoretic mobility as native HNP-1 in acid-urea PAGE, reacted with a conformation-specific antibody to HNP-1, and had the same retention time on HPLC. This is evidence that the recombinant peptide is correctly folded and that the specific arrangement of the three disulfide pairs has been attained. Previous attempts (22) to express functional recombinant HNP-1 in *E. coli* bacteria via a fusion protein were unsuccessful because of problems with protein proteolysis, and misfolding. This is the first report of successful biosynthesis of antimicrobial and cytotoxic defensins in a recombinant protein expression system. It is also an illustration of how the differences between the posttranslational processing pathways for recombinant proteins in insect cells and in the cells of origin of the native peptides can be exploited to generate otherwise inaccessible processing intermediates for structural and functional studies.

Like many peptide hormones and proteolytic enzymes, the antimicrobial proteins of the host defense system are synthesized as larger precursors that are proteolytically cleaved to form a mature and active product. When initially synthesized on ribosomes, the precursor peptides are commonly tripartite, with a signal or presequence that directs the protein to the endoplasmic reticulum, a proregion and a mature protein. The common occurrence of relatively large proregions that are degraded during posttranslational processing argues that they fulfill important roles during protein biosynthesis and subcellular transport. Studies of proteolytic enzymes indicate that the proregion may catalyze correct protein folding (23, 24) or act as a chaperone that prevents promiscuous protein-protein interactions. In some hormones the proregion contains a targeting signal for subcellular sorting (25) thus freeing the mature peptide from the constraints of this function. In many proproteases (zymogens) the propiece functions as an intramolecular protease inhibitor during subcellular transport or storage. The differing functions of the proregions are not mutually exclusive, as evidenced by the dual activity of the proregion of bacterial alpha-lytic protease as both a high-affinity protease inhibitor and a folding catalyst for the enzyme (23). The potential importance of charge interactions between the propiece and the mature protein is illustrated by pepsinogen which is activated to pepsin by the proteolytic removal of a cationic propiece that forms inhibitory salt bridges with the critical anionic residues in the enzyme (26).

The toxic activity of defensins is mediated in part by their ability to form pores in biological membranes after defensin dimer/multimer formation (9, 27, 28). The cationic portion of this amphipathic molecule enables the defensin to interact with negatively charged membrane surfaces and the hydrophobic aspect probably interacts with the interior of the lipid bilayer (29). In this study we developed evidence that the anionic proregion interferes with the activity of mature HNP-1 by diminishing the initial binding of HNP-1 to target cells, most likely by neutralization of cationic charges on HNP-1 that are essential for electrostatic attraction to negatively charged cellular surface molecules.

A previous study (30) modeled the interaction between the cytotoxic peptide eosinophil major basic protein and its anionic propiece by adding anionic amino acid polymers to mature major basic protein and documenting the inhibitory effects on its bioactivity. However, we detected subtle but reproducible differences between the activity of unlinked as compared with linked propiece. As shown in Fig. 5A, 1:2 molar ratio of free propiece to mature HNP-1 was sufficient to achieve maximal inhibition of bactericidal activity but bacteriostatic activity persisted even at 4:1 ratio of propiece to mature HNP-1. Linked propiece (1:1 molar ratio) was an effective inhibitor of both bactericidal and bacteriostatic activity. Although the unlinked anionic propiece generated by CNBr cleavage differs from the native form by the conversion of carboxy-terminal methionine to homoserine or homoserine lactone, it is unlikely that this relatively conservative change accounts for the observed difference in inhibitory activity. Instead, we surmise that unlinked and linked propieces interact differently with mature defensin and that the latter process may not be adequately modeled by the addition of polyanions. However, even the less specific interaction between polyanions and cationic defensins may be important during granulogenesis, when granule proteins condense with sulfated proteoglycans of the azurophil granule matrix (31). In dense azurophil granules that contain fully mature and potentially active defensins (32) the sulfated proteoglycans may assume the neutralizing activity of the propiece and prevent the permeabilization of granule membranes by the high concentrations of defensins contained within.

In resting neutrophils, mature defensins are stored in the azurophil granules and are delivered to phagosomes during phagocytosis (33, 34). Extracellular release of mature defensin from phagocytosing neutrophils in vitro is modest (< 3% of cell content) (35). However, a portion of newly synthesized prodefensin ( $\sim 10\%$ ) is continually released in vitro from unstimulated myeloid HL-60 cells which normally synthesize defensins, as well as from myeloid 32D cl3 cells that have been engineered to express defensin (36). This release may be caused by a leaky subcellular sorting pathway or may have evolved specifically to serve an as yet unknown biological function. If similar release of prodefensins occurs in vivo, the inactive propeptides will not injure the surrounding cells.

Previous work has shown that the defensin proregion contains a motif that is necessary for correct subcellular sorting and processing. We have now shown that the proregion can also inhibit the functional activity of the mature defensin peptide. Further studies will be needed to determine whether the proregion of antimicrobial defensins also functions as a folding catalyst or an intramolecular chaperone.

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#### References

1. Lehrer, R.I., A.K. Lichtenstein, and T. Ganz. 1993. Defensis: antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* 11:105– 128.

2. Hultmark, D. 1993. Immune reactions in *Drosophila* and other insects: a model for innate immunity. *Trends Genet*. 9:178–183.

3. Hoffmann, J.A., and C. Hetru. 1992. Insect defensins: inducible antibacterial peptides. *Immunol. Today.* 13:411–415.

4. Cammue, B.P., M.F. De Bolle, F.R. Terras, P. Proost, J. Van Damme, S.B. Rees J. Vanderleyden, and W.F. Broekaert. 1992. Isolation and characterization of a novel class of plant antimicrobial peptides form *Mirabilis jalapa L.* seeds. J. Biol. Chem. 267:2228–2233.

5. Aley, S.B., M. Zimmerman, M. Hetsko, M.E. Selsted, and F.D. Gillin. 1994. Killing of *Giardia lamblia* by cryptdins and cationic neutrophil peptides. *Infect. Immun.* 62:5397–5403.

6. Daher, K.A., M.E. Selsted, and R.I. Lehrer. 1986. Direct inactivation of viruses by human granulocyte defensins. *J. Virol.* 60:1068–1074.

 Okrent, D.G., A.K. Lichtenstein, and T. Ganz. 1990. Direct cytotoxicity of polymorphonuclear leukocyte granule proteins to human lung-derived cells and endothelial cells. *Am. Rev. Respir. Dis.* 141:179–185.

8. Lichtenstein, A., T. Ganz, M.E. Selsted, and R.I. Lehrer. 1986. In vitro tumor cell cytolysis mediated by peptide defensins of human and rabbit granulocytes. *Blood.* 68:1407–1410.

9. Lehrer, R.I., A. Barton, K.A. Daher, S.S. Harwig, T. Ganz, and M.E. Selsted. 1989. Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. *J. Clin. Invest.* 84:553–561.

10. Lichtenstein, A. 1991. Mechanism of mammalian cell lysis mediated by peptide defensins. Evidence for an initial alteration of the plasma membrane. *J. Clin. Invest.* 88:93–100.

11. Valore, E.V., and T. Ganz. 1992. Posttranslational processing of defensins in immature human myeloid cells. *Blood.* 79:1538–1544.

12. Liu, L., and T. Ganz. 1995. The pro region of human neutrophil defensin contains a motif that is essential for normal subcellular sorting. *Blood.* 85:1095–1103.

13. Michaelson, D., J. Rayner, M. Couto, and T. Ganz. 1992. Cationic defensins arise from charge-neutralized propeptides: a mechanism for avoiding leukocyte autocytotoxicity? *J. Leukocyte Biol.* 51:634–639.

14. Dagert, M., and S. D. Ehrlich. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene (Amst.)*. 6: 23–28.

15. Panyutich, A.V., N.N. Voitenok, R.I. Lehrer, and T. Ganz. 1991. An enzyme immunoassay for human defensins. *J. Immunol. Methods*. 141:149–155.

16. Harwig, S.S., N.P. Chen, A.S. Park, and R.I. Lehrer. 1993. Purification of cysteine-rich bioactive peptides from leukocytes by continuous acid-urea-polyacrylamide gel electrophoresis. *Anal. Biochem.* 208:382–386.

17. Schagger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfatepolyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.

18. Villa, S., G. De Fazio, and U. Canosi. 1989. Cyanogen bromide cleavage at methionine residues of polypeptides containing disulfide bonds. *Anal. Biochem.* 177:161–164.

19. Lehrer, R.I., M. Rosenman, S.S. Harwig, R. Jackson, and P. Eisenhauer. 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods*. 137:167–173.

20. Daher, K.A., R.I. Lehrer, T. Ganz, and M. Kronenberg. 1988. Isolation and characterization of human defensin cDNA clones. *Proc. Natl. Acad. Sci. USA*. 85:7327–7331.

21. Ganz, T., M.E. Selsted, D. Szklarek, S.S. Harwig K. Daher, D.F. Bainton, and R.I. Lehrer. 1985. Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* 76:1427–1435.

22. Piers, K.L., M.H. Brown, and R.E. Hancock. 1993. Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria. *Gene (Amst.)*. 134:7–13.

23. Baker, D., J.L. Silen, and D.A. Agard. 1992. Protease pro region required for folding is a potent inhibitor of the mature enzyme. *Proteins*. 12:339– 344.

24. Baker, D., A.K. Shiau, and D.A. Agard. 1993. The role of pro regions in protein folding. *Curr. Opin. Cell Biol.* 5:966–970.

25. Stoller, T.J., and D. Shields. 1989. The propeptide of preprosomatostatin mediates intracellular transport and secretion of alpha-globin from mammalian cells. J. Cell Biol. 108:1647–1655.

26. James, M.N., and A.R. Sielecki. 1986. Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 A resolution. *Nature (Lond.)*. 319:33–38.

27. Lehrer, R.I., A. Barton, and T. Ganz. 1988. Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in *E. coli* by multiple-wavelength spectrophotometry. *J. Immunol. Methods.* 108:153–158.

28. Kagan, B.L., M.E. Selsted, T. Ganz, and R.I. Lehrer. 1990. Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA*. 87:210–214.

29. Wimley, W.C., M.E. Selsted, and S.H. White. 1994. Interactions between human defensins and lipid bilayers: evidence for formation of multimeric pores. *Protein Sci.* 3:1362–1373.

30. Barker, R.L., R.H. Gundel, G.J. Gleich, J.L. Checkel, D.A. Loegering, L.R. Pease, and K.J. Hamann. 1991. Acidic polyamino acids inhibit human eosinophil granule major basic protein toxicity. Evidence of a functional role of proMBP. *J. Clin. Invest.* 88:798–805.

31. Bainton, D.F., J.L. Ullyot, and M.G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J. Exp. Med.* 134:907–934.

32. Rice, W.G., T. Ganz, J.M. Kinkade, M.E. Selsted, R.I. Lehrer, and R.T. Parmley. 1987. Defensin-rich dense granules of human neutrophils. *Blood.* 70: 757–765.

33. Joiner, K.A., T. Ganz, J. Albert, and D. Rotrosen. 1989. The opsonizing ligand on *Salmonella typhimurium* influences incorporation of specific, but not azurophil, granule constituents into neutrophil phagosomes. *J. Cell Biol.* 109: 2771–2782.

34. Leffell, M.S., and J.K. Spitznagel. 1974. Intracellular and extracellular degranulation of human polymorphonuclear azurophil and specific granules induced by immune complexes. *Infect. Immun.* 10:1241–1249.

35. Ganz, T. 1987. Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infect. Immun.* 55:568–571.

36. Ganz, T., L. Liu, E.V. Valore, and A. Oren. 1993. Posttranslational processing and targeting of transgenic human defensin in murine granulocyte, macrophage, fibroblast, and pituitary adenoma cell lines. *Blood.* 82:641–650.