The pro Region of Human Neutrophil Defensin Contains a Motif That Is Essential for Normal Subcellular Sorting

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Human defensins (human neutrophil peptides) HNP 1-3 are 29-30 amino acid antibiotic and cytotoxic peptides highly abundant in the cytoplasmic granules of polymorphonuclear leukocytes. The peptides are produced from 94 amino acid (aa) prepropeptides by proteolytic cleavage of the signal sequence and stepwise removal of the 44-45 aa anionic propeptide. To study the role of the propeptide, we constructed five in-frame deletions in preproHNP-1 cDNA between the signal peptidease site and the amino-terminus of the mature defensin region (aa 21-64). The wild type HNP-1 cDNA and the deletion mutants were ligated into the pBabe-Neo retroviral vector, expressed in GP + E86 packaging derivative of NIH 3T3 cells, then transfected into the 32D cl3 granulocytic cell line. For each construction and both cell lines, we measured the accumulation of the various defensin forms in cells and media by 24-hour labeling or pulse-chase with 35S-cysteine and immunoprecipitation/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Deletions in the amino-terminal two-fifths of the propeptide, Δ21-28 and Δ21-38, had only minor effects on defensin biosynthesis in both cell lines and did not interfere with the accumulation of mature defensin in the granules of 32D cl3 cells. Deletions in the carboxyterminal three-fifths of the propeptide (Δ21-51 and Δ21-64) diminished net defensin synthesis, blocked constitutive secretion of prodefensin in both cell lines, and interfered with defensin accumulation in cytoplasmic granules of 32D cl3 cells. These effects were reproduced by the smaller deletion Δ40-51, which contains highly conserved secondary structure. The propeptide segment 40-51 appears to be essential for the subcellular trafficking and sorting of HNP-1 defensin.

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EACH NEUTROPHIL contains several thousand cytoplasmic granules that function as storage compartments for macromolecules destined for secretion (specific granules) or for fusion with phagosomes (azurophil granules). The granules proteins, synthesized during the promyelocyte and myelocyte stages, undergo posttranslational modifications commonly including glycosylation and one or more proteolytic cleavage steps. From the Golgi complex, granule proteins are selectively transported to nascent granules. The keys that permit the sorting of each protein to its appropriate subcellular transport system must be encoded in its primary amino acid sequence, but the addressing codes and mechanisms of sorting to the azurophil or specific granules are not known.

Human defensins (human neutrophil peptides) HNP 1-3 are cationic antimicrobial and cytotoxic peptides that are among the principal constituents of the microbicidal granules of polymorphonuclear leukocytes (neutrophils). The peptides are produced only in neutrophil progenitor cells by posttranslational processing of 94 amino acid (aa) preprodefensins. Sequential proteolytic processing from the amino-terminus first cleaves the 19 aa endoplastic reticulum targeting signal sequence to generate a 75 aa prodefensin and then in two or more steps removes the 44-45 amino acid anionic propeptide, leaving the 29 or 30 aa cationic defensin peptides found in mature neutrophils.

Experiments on cell lines transduced with human defensin cDNA suggested that the machinery for efficient processing of prodefensins to their mature form is restricted to cells of myeloid lineage. Nonmyeloid cells secreted most defensin as 75 aa prodefensin by either the constitutive pathway (NIH 3T3 embryonic fibroblasts) or by the regulated exocytic pathway (AtT-20 pituitary adenoma cells), wherein secretory granules are released in response to secretagogues. Among the myeloid cells examined, the granulocytic cell line 32D cl3 transfected with HNP-1 cDNA processed preproHNP-1 to mature 29 aa HNP-2 and accumulated this product in cytoplasmic vacuoles. We refer to these vacuoles as granule-like, based on (1) the stability of mature defensin in these vacuoles and (2) the fusion of the vacuoles to phagosomes.

As documented by pulse-chase studies, transgenic defensin was processed and then rapidly degraded in macrophage-like myeloid cell lines J774.1 or RAW 264.7, but persisted for at least 24 hours in 32D cl3 cells. Moreover, immunolocalization of HNP-1 in 32D cl3 cells by both light microscopy and transmission electron microscopy showed that the defensin-bearing vacuoles fuse to phagosomes containing opsonized zymosan (L. Liu and T. Ganz, unpublished data, November 1993).

The synthesis of prodefensin with a large propeptide that must be removed by myeloid-specific processing enzymes appears to be metabolically wasteful, unless the propeptide has a biologic function either in defensin biosynthesis or independently. In this report, we describe the consequences of deletions in the propeptide of proHNP-1 on posttranslational processing, intracellular trafficking, and constitutive secretion in the myeloid cell line 32D cl3 and the nonmyeloid NIH 3T3-derived GP + E86 cells.

MATERIALS AND METHODS

Cells and media. The cell line GP + E86 (an NIH 3T3-derived packaging cell line provided by Dr A. Bank, Columbia University, New York, NY) was grown in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (GIBCO-BRL, Grand Island, NY) with 10% FCS and 1% antibiotics.

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fetal bovine serum (FBS) and 50 μg/mL gentamicin. The myelolast cell line 32D cl3 (provided by Dr Giovanni Rovera, Wistar Institute, Philadelphia, PA) was grown in Iscove’s modified Dulbecco’s medium (IMDM; Gibco-BRL) with 10% FBS, 50 μg/mL gentamicin, and the required growth factor interleukin-3 (IL-3) provided as 10% WEHI-3 conditioned medium.

Preparation of deletions and retroviral constructs. To generate the deletions in human defensin cDNA, nine primers were used: (1) 5’ TTAGGAGTCGTCGACCCAG, (2) 5’ TCAAGCAGGGCTGATCCGGGGC, (3) 5’ TCTGAGCCCAGGCTGAGGGACATTC CGAGCCACCAGGCTGAGGCCTGCAGAAGTGGTT, (4) 5’ TCTGAGCCCAGGCTGAGGGTTGCT TCAAAAAGCAT, (5) 5’ TCTGAGCCCAGGCTGAGGGACATTC CGAGCCACCAGGCTGAGGCCTGCCTGCAGAAGTGGTT, (6) 5’ TCTGAGCCCAGGCTGAGGGTTGCT TCAAAAAGCAT, (7) 5’ TCTGAGCCCAGGCTGAGGGTTGCT TCAAAAAGCAT, (8) 5’ TCTGAGCCCAGGCTGAGGGTTGCT TCAAAAAGCAT, and (9) 5’ TCTGAGCCCAGGCTGAGGGTTGCT TCAAAAAGCAT. Polymerase chain reactions (PCRs) were performed using Taq (Perkin-Elmer/Cetus, Norwalk, CT) or Pfu (Stratagene, La Jolla, CA) DNA polymerase as specified by the manufacturer. The PCR-generated DNA fragments from each mutation with the highest defensin release into media in the presence of polybrene E86) by the lipofectin (provided by Dr H. Land, ICRF, London, UK), and transformed into retroviral vector pBabe-Neo” (TransfectAce; GIBCO-BRL). Media conditioned by the geneticin-resistant clones were assayed for defensins by enzyme-linked immunosorbent assay (ELISA), and transformed into Escherichia coli XL-1 Blue (Promega, Madison, WI). The nucleotide sequence and correct orientation of the insert were confirmed by dideoxynucleotide sequencing on both strands.

Packaging cell line and gene transfer. The pBabe-Neo vector containing the modified HNP-1 cDNA was introduced into the ecotropic packaging cell line 3T3 (GP + E86) by the lipofectin method, performed according to the manufacturer’s instructions. PCR products from overlap extension reaction were subjected to BamHI digestion, purified, ligated into the retroviral vector pBabe-Neo16 (provided by Dr H. Land, ICRF, London, UK), and transformed into Escherichia coli XL-1 Blue. The cell extracts or the culture supernatants were mixed with 30 μL of antidesfensin rabbit serum.29 After incubation on ice for 1 hour, 40 μL of 1:1 slurry with RIPA buffer protein A-agarose (Boehringer Mannheim Corp, Indianapolis, IN) was added, and the mixture was incubated at 4°C for 1 hour with constant rotation. The immunoprecipitate was washed three times in RIPA buffer and four times in PBS. The pellet was resuspended in 40 μL electrophoresis sample buffer (0.17 mol/L Tris-HCl, pH 8.8; 21% [vol/vol] glycerol; 6% [wt/vol] SDS; 120 mmol/L dithiothreitol) and incubated overnight at 4°C. Samples were boiled for 10 minutes and briefly centrifuged, and the supernatant was analyzed by SDS-tricine PAGE.

RNA extraction and Northern blot analysis. Total cellular RNA was extracted using the guanidinium method.22 The RNA was frac-
tionated on denaturing formaldehyde/agarose gel electrophoresis and transferred to hybridization membrane (Gene Screen Plus, NEN-DuPont, Wilmington, DE) by capillary blotting. DNA-RNA hybridizations were performed overnight at 42°C in 50% (vol/vol) formamide, 5 × SSPE, 5 × Denhardt’s, 0.1% SDS, 100 μg/mL denatured salmon sperm DNA and HNP-1 cDNA probe (labeled with 32P). The filters were washed to a final stringency of 0.1 × standard sodium citrate (SSC), 0.1% SDS at room temperature and exposed to Kodak-XAR film at -80°C.

RESULTS

We previously established that a typical endoplasmic reticulum-targeting signal sequence (aa 1-19) of the nascent preproHNP-1 is rapidly removed in HL-60 cells10 that naturally produce HNP-1, as well as in NIH 3T3 (GP + E86) and 32D c13 cells expressing exogenous HNP-1 cDNA.13 To preserve Glu20, which is highly conserved among known mammalian preprodefensins and may be a part of the signal peptidase recognition site, we constructed four nested deletions in the propiece starting with residue 21 (Fig 1). We suspected that the repetitive arrangement of negatively charged amino acids (Glu, Asp) was functionally important for the interaction of the anionic propiece with the cationic mature peptide, and designed the deletions to bring Glu20 into a peptide environment where Glu or Asp residues would be replaced. For example, the first construct, Δ21-28, had an eight amino acid deletion from 21 to 28 inclusive, so that residue 20 (Glu) was joined to residue 29 (Val), recreating the native GluVal sequence. The phenotypes of the four deletion mutants focussed our attention on the region 40 to 51, which was then selectively deleted in the fifth construct (Fig 1).

The wild type (WT HNP-1) and the deleted HNP-1 cDNAs were inserted into the pBabe-Neo retroviral vector and transfected into the packaging cell line 3T3 (GP + E86). 32D c13 cells were cocultured with the retroviral producer cells, and G418-resistant clones were selected. All the clones of both cell types transfected with mutated HNP-1 cDNAs expressed the modified peptides, as confirmed by immunoprecipitation and enzyme immunoassay. No defensin was detected in supernatants of the cells not treated with retroviral constructs or of the cells that contained the mutated HNP-1 cDNA construct in the antisense orientation.

Northern blots of total cellular RNA from transduced 3T3 (GP + E86) and 32D c13 cells were hybridized with radiolabeled HNP-1 cDNA (Fig 2). In transduced 3T3 (GP + E86) cells, the defensin mRNA levels in cells with the deleted constructions were several-fold lower than with WT HNP-1. In transduced 32D c13 cells, Δ21-28, Δ21-51, and Δ21-64 generated somewhat lower levels of defensin mRNA than Δ21-38 and HNP-1. When comparing the various constructs, there was no relationship between the levels of mRNA and immunoprecipitable defensin (see below), suggesting that the observed differences in net defensin synthesis were determined primarily during or after translation. The major differences in the amounts of peptide detected were not caused by inefficient immunoprecipitation of the shorter forms, as these forms were readily immunoprecipitated when generated as processing intermediates or products in WT HNP-1 cells (Fig 3).

To characterize the effect of deletions on defensin synthesis and posttranslational processing, transfected 3T3 (GP + E86) and transduced 32D c13 cells were grown for 24 hours in the presence of 35S-cysteine to label newly synthesized and processed protein. The cell lysates and media were immunoprecipitated with a conformation-specific antidefensin antibody and analyzed by SDS-tricine PAGE and autoradiography. In both cell lines, WT HNP-1, Δ21-28, and Δ21-38 differed little in the amounts of immunoprecipitable defensin, but Δ21-51 and Δ21-64 generated much less defensin, even when proportionally corrected for the somewhat different levels of defensin mRNA (Fig 3).

The cell lysates of 3T3 (GP + E86) fibroblast cells transfected with WT HNP-1, Δ21-28, and Δ21-38 contained predominantly an intact or deleted prodefensin with only minor amounts of peptide migrating with mature defensin (Fig 3A). The small amount of processing to mature defensin seen with other constructs was nearly undetectable in Δ21-51, suggesting that this deletion interfered with access to the protease involved, or modified the peptide conformation recognized by it. As expected, the construct Δ21-64 generated mature defensin only, indicating that most or all of the signal peptide was proteolytically removed. The 3T3 (GP + E86) cells constitutively secreted the intact or deleted prodefensin into the media, but the mature form was not detected there. Secretion was impaired in Δ21-38 and nearly absent in Δ21-51 and Δ21-64 (Fig 3A). These data suggest that the carboxyterminal region of the propiece was necessary for the efficient constitutive secretion of prodefensin in 3T3 (GP + E86) cells.

In myeloid 32D c13 cells (Fig 3B), Δ21-28 and Δ21-38 showed a processing pattern similar to WT HNP-1. Apparently, the 32D c13 cells use a different intermediate proteo-
lytic site than do HL-60 cells, as the respective intermediate forms differed in size. However, in Δ21-51, the intermediate form disappeared completely, suggesting interference with access to the processing proteases, or the sites that they recognize on the peptide.

The relative amounts of the defensin proforms and mature form were measured by scanning densitometry of the autoradiograms shown in Fig 3B. In 32D cl3 cells transduced with the wild type defensin, about 51% of the total labeled defensin was mature defensin; in the Δ21-28 mutant, the proportion was about 27%; in the Δ21-38 mutant, about 25% was mature defensin; and in the mutant Δ21-51, only 20%. Like 3T3 (GP + E86) cells, the transduced 32D cl3 cells constitutively secreted the propeptides. The shorter deletions, Δ21-28 and Δ21-38, secreted amounts similar to WT HNP-1, but Δ21-51 secreted very little. The construct Δ21-64 generated small amounts of an intracellular defensin form with the electrophoretic mobility of mature peptide, and no secretion was detected.

Deletion Δ21-51 impaired constitutive secretion and intracellular proteolytic processing in both myeloid and fibroblast cell lines. To determine whether these defects were due to the cumulative effect of this large deletion or to the deletion of a small segment critical for the function of the propiece, we constructed a fifth deletion, Δ40-51, that spanned most of the segment present in the mildly affected Δ21-38 but not in the severely affected Δ21-51. In both 3T3 (GP + E86) and 32D cl3 cells, constitutive secretion of the Δ40-51 construct was impaired dramatically compared with WT HNP-1. In myeloid 32D cl3 cells, deletion Δ40-51 ablated posttranslational proteolytic processing of proHNP-1 despite similar amounts of intracellular proHNP-1 in WT HNP-1 and Δ40-51 (Fig 4).

Our previous studies found that HNP-1 expressed in the transduced macrophage cell line RAW 264.7 cells was not stable but was degraded within a few hours after synthesis. To determine whether the decreased amount of intracellular defensin in cells transduced with Δ21-51 and Δ21-64 was due to the instability of deleted proHNP-1, we performed pulse-chase experiments to examine the fate of the modified peptides. The modified peptides present at 1 hour remained stable for 24 hours in both cell lines, 3T3 (GP + E86) and 32D cl3 (Fig 5). Similarly, the Δ40-51 construct in 32D cl3 cells also produced a stable peptide (data not shown). During 24 hours’ chase, there was little accumulation of secreted peptides in the medium of 3T3 (GP + E86) cells (Fig 5A), and none in the medium of 32D cl3 cells (Fig 5B), confirming that the propiece deletions were interfering with the constitutive secretion of the peptides.

We previously demonstrated that in 32D cl3 cells transgenic human defensin accumulated in cytoplasmic vacuoles that resembled the nascent granules in granulocyte precursors. To locate the site of accumulation of defensins generated from deleted constructs, each transduced 32D cl3 cell line was immunostained with anti-HNP antibody (Fig 6). In Δ21-28 and Δ21-38 HNP-1 mutants (Fig 6A and B), the brown immunoperoxidase product localized in vacuoles in a manner similar to wild type HNP-1 cDNA constructs (Fig 6F). In Δ21-51, Δ21-64, and Δ40-51 (Fig 6C, D, and E), the immunoperoxidase product was not concentrated in vacuoles. 32D cl3 cells that were not transduced, or were transduced with mutated HNP-1 cDNAs in antisense orienta-
Fig 4. Autoradiogram of SDS-tricine-PAGE of $^{35}$S-cysteine–labeled HNP-1 and mutant Δ40-51. The transduced 32D cl3 or 3T3 (GP + E861) cells were incubated for 24 hours with $^{35}$S-cysteine to metabolically label their proteins. The various defensin forms contained in cells (C) and released into media (M) were immunoprecipitated with anti-HNP serum, and the immunoprecipitate was analyzed by SDS-tricine-PAGE/autoradiography.

Fig 5. Pulse-chase studies of defensin synthesis and processing in 3T3 (GP + E861) cells (A) and 32D cl3 cells (B) transduced with deleted HNP-1 cDNA (Δ21-51 and Δ21-64). The cells were labeled with $^{35}$S-cysteine for 1 hour and then transferred into nonradioactive media. At the times indicated above each lane, the cells were lysed, and the lysate was immunoprecipitated with anti-HNP serum. The immunoprecipitate was analyzed by SDS-tricine-PAGE/autoradiography.
Fig 6. Photomicrograph of 32D cl3 cells expressing various defensin constructs. The cells were stained for human defensins by the immunoperoxidase method (brown product) and counterstained with hematoxylin. Parental 32D cl3 cells were processed in parallel and did not stain (not shown). Experiment 1: A, Δ21-28; B, Δ21-38; C, Δ21-51; and D, Δ21-64. Experiment 2: E, Δ40-51; and F, WT HNP-1. Variations in cell size and vacuolar morphology were related to the duration of culture.

cating that targeting information may also reside in the mature peptide. Propiece deletions often severely reduce net peptide synthesis by an unknown mechanism. Although only two short motifs in the nerve growth factor (NGF) propiece were strictly required for the expression of active NGF in COS-7 cells, extensive propiece deletions outside these segments greatly diminished net NGF synthesis. As NGF is constitutively secreted by COS-7 cells, the effects of deletions on granule targeting could not be addressed. In the aggregate, these observations suggest that the propieces may have multiple functions in the biosynthesis and subcellular sorting of secretory granule proteins, and that the functions of the propiece may differ from one peptide to another.

Defensins are synthesized as prepropeptides that undergo posttranslational proteolytic processing and subcellular sorting that resemble the proteolytic cleavage of neuroendo-
The local secondary structure in plots of preprodefensins. The PC-GENE NOVOTNY program (Intelligenetics, Inc. Mountain View, CA) was used to display the local secondary structure indices (turn, β-sheet, α-helix, charge, and hydrophobicity) of prepropeptides HNP-1, HNP-4, NP-1, NP-3A, NP-4, NP-5, GPDEF, CRYPTDIN, and HD-5. The horizontal axis displays the amino acid position number. The prepropeptides are aligned at their amino- and carboxy-termini. Dark line, HNP-1; lighter lines, other preprodefensins. The arrows point to an area of conserved secondary structure in the propiece.

To begin our exploration of the mechanisms of defensin processing and subcellular sorting in neutrophil granulocytes, we studied the effects of propiece deletions on net defensin synthesis, posttranslational processing, constitutive secretion, and targeting to cytoplasmic vacuoles. We compared the effects of these deletions in granulocyte-like 32D c13 cells that process defensins and accumulate them in cytoplasmic vacuoles and in 3T3 (GP + E86) cells that constitutively secrete prodefensin. We found that deletions involving the region aa 40-51 generally diminished net defensin synthesis and conversion of prodefensin to defensin, interfered with constitutive secretion of prodefensin, and blocked accumulation of defensin in the vacuoles of 32D c13 cells.

Net defensin synthesis, measured as the amount of accumulated immunoprecipitable defensin after 24-hour labeling, depends on the rate of mRNA translation, proportion of correctly folded propeptide, and the stability of the propeptide and its processed forms. The pulse-chase studies indicate that immunoprecipitable defensin forms generated by Δ21-51, Δ40-51, and Δ21-64 are not appreciably degraded during 24 hours of chase. Further studies will be required to ascertain whether the low observed net defensin synthesis in Δ21-51 and Δ21-64 is a result of interference with ribosomal function and translation, production of a peptide that misfolds and is not recognized by our conformation-specific antibodies, and/or cotranslational proteolysis.

The fraction of total defensin converted to mature peptide within 24 hours was decreased by each deletion in the propiece: slightly in Δ21-28 and Δ21-38, markedly in Δ21-51, and almost completely in Δ40-51. Although the diminished rate of processing could also be due to altered interaction with the processing enzymes, the associated defects in secretion and vacuolar accumulation suggest a block in intracellular transport into compartments where processing takes place.

Constitutive secretion in both 3T3 (GP + E86) cells and 32D c13 cells was impaired in the propiece deletion mutants Δ21-51, Δ40-51, and Δ21-64 as compared with wild type or Δ21-28 and Δ21-38. There is much evidence that the constitutive secretion pathway is the default pathway that requires no additional signals besides the endoplasmic reticulum-targeting hydrophobic signal sequence. As the construct joining the signal sequence directly to mature peptide is not secreted, we suspect that structural features of the...
mature peptides that would interfere with secretion are normally masked by the propiece. The selective accumulation of defensin in the cytoplasmic vacuoles of transduced 32D c13 cells was not appreciably affected by the less extensive deletions (Δ21-28 and Δ21-38), but further deletion (Δ21-31 and Δ21-64) or the specific deletion Δ40-51 blocked defensin accumulation in granule-like vacuoles (Fig 6). Thus, the propiece, especially in the amino acid 40-51 region, contains sequences important for transport of defensin to granule-like vacuoles either directly or by negating the effects of mature peptide sequences that interfere with transport or sorting to vacuoles.

The interaction of the propiece with its mature peptide could influence prodefensin exit from the endoplasmic reticulum and Golgi in two ways. If the propiece is required for the correct folding of mature defensin, deletions in the propiece could result in misfolding of the mature peptide, exposure of a normally inaccessible peptide retention signal, and the resulting retention of the misfolded propeptide in the endoplasmic reticulum. However, the antibody used in these experiments is directed against mature 30 aa HNP-1 and is conformationally specific as indicated by its lack of interaction with reduced and denatured HNP-1 in Western blots (unpublished data, September 1986). We consider it unlikely that this 30 aa peptide with six disulfide-bonded cysteines would misfold in a manner that would preserve a normally inaccessible peptide retention signal. Instead, we propose that the correctly folded mature defensin HNP-1 contains motifs that would cause retention in a subcellular compartment (endoplasmic reticulum and Golgi) proximal to the point where constitutive secretion diverges from sorting to cytoplasmic vacuoles and propeptide proteolytic processing. These motifs would normally be covered by the propiece, directly or via a chaperone, and the aa 40-51 region is apparently required for this interaction. An analysis of the known prodefensins did not identify any conserved primary structure in the propiece, but a hydrophobic segment with β-sheet-forming composition was conserved in all prodefensin molecules and was centered on the aa 40-51 region (Fig 7). During defensin synthesis and subcellular transport, this segment could interact with a complementary hydrophobic β-sheet aspect of the mature defensin peptide or with a chaperone protein, forming a structure that facilitates transit through the requisite subcellular compartments.

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The pro region of human neutrophil defensin contains a motif that is essential for normal subcellular sorting

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