Posttranslational Processing of Defensins in Immature Human Myeloid Cells

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Human neutrophil promyelocytes synthesize, process, and package several microbialidal proteins, including the highly abundant defensins, into their azurophil granules. As deduced from their cDNA sequences, defensins are initially synthesized as 94 amino acid (aa) precursors that must undergo extensive processing. We performed metabolic labeling studies of defensin synthesis in the human promyelocytic cell line HL-60 and in chronic myeloid leukemia cells, and showed that preprodefensins are processed to mature 29 to 30 aa defensins over 4 to 24 hours via two major intermediates: a 75 aa prodefensin generated by the cleavage of the signal sequence, and a 56 aa prodefensin that results from a subsequent preaspartate proteolytic cleavage. Almost all of the 75 aa form was found in the cytoplasmic/microsomal fraction, whereas the 56 aa prodefensin and mature defensins predominated in the granule-enriched fraction. The 75 aa prodefensin was also selectively released into the culture supernatant. Treatment of HL-60 cells with monensin, chloroquine, or ammonium chloride, substances that neutralize acidic subcellular compartments, partially blocked conversion of the 75 aa prodefensin into 56 aa prodefensin, but did not increase the extracellular release of the 75 aa form. Further studies will be required to determine the role of this processing pathway in subcellular targeting to azurophil granules and avoidance of autocytotoxicity.

DEFENSINS ARE 29 to 34 amino acid (aa) cysteine- and arginine-rich peptides that have a broad spectrum of antimicrobial and cytotoxic properties in vitro.1 In humans, the three major defensins, human neutrophil peptides HNP-1 through -3, constitute more than 5% of the total cellular protein in mature neutrophils, and at least 30% of the protein of their azurophil granules.2 Because HNP-2 (29 aa), the smallest human defensin, differs from HNP-1 and HNP-3 only by the absence of an amino-terminal alanine or aspartate respectively, the known human defensin cDNAs for HNP-1 and HNP-3 may in fact encode all three major defensin peptides.3 The 94 aa putative defensin precursors, deduced from the cDNAs, contain a typical 19 aa signal sequence and a 45 aa propeptide. The 30 aa mature defensin peptide comprises the entire carboxyl terminus of the preprodefensin.3

The processing of myeloid granule proteins myeloperoxidase, elastase, cathepsin G, and lactoferrin has been characterized in myeloid cell lines and bone marrow.4-11 Despite the common belief that azurophil granules are homologous to lysosomes of nonmyeloid cells, there is increasing evidence that azurophil granule proteins are processed by pathways distinct from those responsible for the processing of lysosomal enzymes in nonmyeloid cells.12 Unlike other myeloid granule proteins, defensins and their deduced precursors lack any consensus site for attachment of N-linked oligosaccharide side chains that can interact with the receptor system responsible for the targeting of lysosomal enzymes. Defensins thus present a compelling model for studying the processing and targeting of myeloid granule proteins.

MATERIALS AND METHODS

Materials. Chloroquin and monensin were from Sigma Chemical Company (St Louis, MO) and ammonium chloride was from Fisher Scientific (Tustin, CA). Prestained electrophoresis molecular weight standards were from Bethesda Research Laboratories (Bethesda, MD). Dog pancreas microsomes13 were a generous gift from Dr David Meyer (University of California, Los Angeles).

Labeling of cells. HL-60 cells (passage 4 to 8 from stock CCL 240; American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, and 50 mg/L gentamicin at 37°C in humidified 5% CO2. Before metabolic labeling, 2 × 106 cells/mL were incubated in cysteine-free RPMI 1640 (Selectamine; GIBCO-BRL) with 5% FCS for 60 minutes to allow depletion of the intracellulare cysteine pool. Cells were labeled by incubation with 50 μCi/mL 35S-cysteine (1,000 Ci/mmol; NEN-Dupont, Boston, MA). In “chase” experiments, the cells were washed after 1 hour of labeling, suspended in RPMI 1640 with 10% FCS at a density of 105 cells/mL, and then incubated for the indicated chase times. When used, the lysosomal acidification inhibitors were added 30 minutes before addition of 35S-cysteine.

Chronic myelogenous leukemia (CML) cells were purified from blood samples obtained from consenting patients. The cells were sedimented in 3% dextran/normal saline, and depleted of residual red blood cells by brief hypotonic lysis. Metabolic labeling of CML cells with 35S-cysteine was performed for 24 hours as described for HL-60 cells. Labeling with 3H-leucine (156 Ci/mmol; NEN-DuPont) was performed after 1 hour of leucine starvation, by incubation with 10 μCi/mL of [3H]leucine in leucine-free RPMI 1640/5% FCS (Selectamine; BRL-GIBCO) for 24 hours.

In vitro translation of CML messenger RNA (mRNA). Total and poly-A-selected mRNA from CML cells was purified as previously described.14 Either 10 μg of total RNA or 5 μg poly-A-selected mRNA was mixed with wheat germ extract (Promega, Madison, WI) and 25 μCi/35S-cysteine in 50 μL total volume. For cotranslational processing experiments, 2 μL dog pancreas microsomes (60 OD260/mL) were also included in the reaction. The translation mix was incubated at 25°C for 1 hour and then subjected to immunoprecipitation as described below.

Subcellular fractionation. Labeled CML cells (2.5 × 106) were suspended in 1 mL 0.34 mol/L sucrose (pH 7.4), and disrupted by
two 10-second bursts with a probe sonicator. Cell lysis was monitored by light microscopy. Nuclei were collected by centrifugation at 200g for 10 minutes. The granule-rich fraction was pelleted from the supernatant by centrifugation at 16,000g for 30 minutes at 4°C, leaving a supernatant enriched in microsomes and cytosol.

**Immunoprecipitation of radioactive defensin peptides.** Labeled cells were extracted with radioimmunoprecipitation assay (RIPA) buffer (100 µL/106 cells) containing 0.15 mol/L NaCl, 30 mmol/L HEPES pH 7.3, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma; added just before use). Extracts were incubated on ice for 1 hour, centrifuged at 16,000g for 30 minutes at 4°C, and the supernatant was stored frozen until used for immunoprecipitation. Subcellular fractions were lysed by the addition of 1/3 volume of a threefold concentrated solution of RIPA buffer. Samples were incubated on ice for 30 minutes and then stored at −20°C until used for immunoprecipitation.

Cell extracts (300 µL) and culture supernatants (3 mL) were mixed with 30 µL of antidefensin rabbit serum. After incubation on ice for 1 hour, 60 µL of protein A-agarose (Biorad, Richmond, CA; 1:1 slurry with RIPA buffer) was added and the mixture rotated at 4°C for 1 hour. The immunoprecipitate was washed five times in RIPA buffer and three times in phosphate-buffered saline (PBS). The pellet was resuspended in 30 to 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). Samples were boiled for 10 minutes, briefly centrifuged, and the supernatant was analyzed by electrophoresis.

**SDS-Tricine polyacrylamide gel electrophoresis (PAGE) and fluorography.** SDS-Tricine PAGE was performed on 0.75-mm thick minigels containing 16.5% acrylamide in the running layer and 4% polyacrylamide in the stacking layer (Hoefer Scientific Instruments, San Francisco, CA). Electrophoresis was conducted at 30 mA/gel without cooling. Gels were stained with Coomassie blue, destained in 25% methanol/0.4% formaldehyde, washed in water for 10 minutes, and treated with 1 mol/L sodium salicylate/2% glycerol for 20 minutes. After drying, the gels were exposed to Kodak XAR 5 X-ray film (Eastman Kodak, Rochester, NY) at −80°C.

For Edman degradation analysis, samples were subjected to electrophoresis as above and then electroblotted to polyvinylidene difluoride (PVDF; Biorad) membranes. Transfer time (Trans blot; Biorad) was 2 hours in 0.05 mol/L sodium borate, pH 9, 20% methanol, 0.05% SDS at 20 V (0.2 A). The membranes were exposed to Kodak XAR 5 film to identify the appropriate bands for radioactive amino acid sequencing.

**Amino acid sequence alignment.** To characterize the defensin precursors, 35S-cysteine- or 3H-leucine–labeled proteins bound to PVDF membranes were subjected to Edman degradation on an ABI 475 (Applied Biosystems, Foster City, CA) automated amino acid sequencer. Fractions were collected directly from the cartridge and subjected to liquid scintillation counting. The radioactivity (cpm) in each cycle versus the cycle number were plotted, and the peaks were aligned with the known cysteines or leucines in the preprodefensin amino acid sequence.

**RESULTS**

**Biosynthesis.** Although mature polymorphonuclear leukocytes (PMN) contain about 5 µg of defensins HNP-1 through -3 per 10⁶ cells, they do not actively synthesize these proteins. Accordingly, biosynthetic studies were performed in HL-60 cells or CML cells. In acid-urea polyacrylamide gels or their Western blots, the defensins produced by HL-60 cells comigrated with those found in human PMN (data not shown). As judged by microbicidal assays and analysis in acid-urea PAGE or SDS-PAGE, mature defensins prepared from leukophoresed CML cells were identical to those prepared from mature PMN (unpublished data). Because cysteines represent 6 of the 29 or 30 aa residues in human defensins, 35S-cysteine was used to metabolically label defensin precursors. The 94 aa preprodefensins deduced from the cDNA sequence do not contain additional cysteines outside the mature defensin region. Consequently, the intensity of labeling of each defensin precursor should be proportional to its relative abundance in the extract, and independent of the size of the precursor.

An autoradiogram of a representative pulse chase experiment in HL-60 cells is shown in Fig 1. The upper and lower panels show labeled defensin proteins immunoprecipitated from cell lysates and the culture medium, respectively. In cells labeled for 24 hours (lane EQ), three major protein species could be identified, with apparent molecular weights (MW) of 7.1 Kd, 4.9 Kd, and 3.1 Kd. The earliest form (7.1 Kd, form 1) was seen after 15 to 30 minutes of chase. In some experiments, this band appeared to be a closely spaced doublet at 15 and 30 minutes. Between 1 and 4 hours, form 1 was gradually converted to a 4.9-Kd protein (form 2). From 4 to 24 hours, most of the form 2 precursor was cleaved to the 3.1-Kd species (form 3) that comigrated with mature defensins HNP-1 through -3. As shown in the lower panel, a protein with the same MW as form 1 accumulated in the medium. A second extracellular protein with an approximate MW of 15 Kd could also be seen in some experiments (see 4h and EQ lanes), but was not further investigated in these studies.

When we compared labeled forms 1 through 3 biosynthetized by myeloid cells to the immunoprecipitated products of in vitro translation of CML mRNA, the translated product migrated with an apparent MW somewhat higher than form 1 (data not shown). If dog pancreas microsomes were present during in vitro translation, a second band appeared that comigrated with biosynthetically labeled form 1. These observations are consistent with the initial synthesis of defensins as 94 aa preproproteins that are rapidly processed to a lower MW precursor form 1. Further proteolytic events occur over a 24-hour period to give form 2 (4.9 Kd) and finally the 3.1-Kd mature defensin peptide (form 3).

**Defensin precursor identification.** In preliminary experiments in CML cells, we found that they contained the same pattern of defensin precursor forms as HL-60 cells (data not shown) but labeled much more intensely. To determine the exact cleavage pattern that generates defensin precursors, CML cells were allowed to incorporate 3H-leucine for 24 hours. Preprodefensins contain 9 leucine residues: 5 in the putative signal sequence, 3 in the propiece, and 1 in the mature defensin sequence. Alternatively, the cells were labeled with 35S-cysteine for 24 hours as before. The immunoprecipitated proteins were blotted to PVDF membranes and subjected to 30 cycles of N-terminal Edman degradation. Each fraction was subjected to scintillation
counting and the radioactive peaks were aligned with the leucines or cysteines in the known preprodefensin amino acid sequence (Fig 2). Forms 2 and 3 could be aligned unequivocally (Fig 2C through E) because at least two distinct peaks with characteristic spacing were detected in each case. Only one large leucine peak was detected in form S (Fig 2A), and the proposed alignment is based on the assumption that any additional leucine before the tenth N-terminal residue would be detectable if it were present. With an estimated repetitive Edman degradation yield of 0.9, this is a realistic assumption. Form 1, which comigrated with form S in SDS-PAGE, yielded the same peak as form S but also contained two additional small peaks, presumably due to contamination with small amounts of form 2. Based on these observations, we conclude that the form 1 precursor is a 75 aa proprotein that is formed after cleavage of the signal sequence. The radiosequencing confirmed that the same form 1 precursor was also released into the culture medium as the predominant defensin species. Form 2 is a 56 aa product resulting from cleavage between an alanine and an aspartic acid residue (residues 38 and 39). The mature defensin peptides HNP-1, -2, and -3 correspond to form 3. Given the approximate nature of protein MW derived from electrophoretic mobility in SDS-PAGE, there is reasonable agreement between the MW calculated from the proposed amino acid sequences of the three forms and the MW derived from their electrophoretic mobilities (form 1: 8.2 Kd calculated, 7.1 Kd electrophoretic; form 2: 6.3 Kd calculated, 4.9 Kd electrophoretic; form 3: 3.4 Kd calculated, 3.1 Kd electrophoretic).

Subcellular fractionation. After labeling CML cells for 24 hours with 35S-cysteine, crude nuclear, cytoplasmic/microsomal, and granule fractions were isolated as described in Materials and Methods, and were subjected to immunoprecipitation. As seen in Fig 3, the nuclear fraction was devoid of defensin precursors. Form 1 was found only in the conditioned medium and the cytoplasmic/microsomal fraction. Forms 2 and 3 were detected predominantly in the granule-enriched fraction but were present also in the cytoplasmic/microsomal fraction. Although the results suggest that the sequential proteolytic cleavages may take place in distinct compartments, more detailed studies will be required to ascertain the subcellular location of these processing steps precisely.

Effects of monensin and weak bases on processing. To define the role of acid compartments in the processing of defensin precursors, pulse chase experiments in HL-60 cells were performed in the presence of monensin and the weak bases chloroquin and ammonium chloride. The ionophore monensin exchanges protons for Na+ ions leading to an increase in the pH of acidic organelles. Figure 4 shows the effects of 0.5 μmol/L and 1 μmol/L monensin on defensin processing. Cells were labeled for 1 hour and chased for 1, 7, and 24 hours. Another set of cells was labeled for 24 hours with no chase (lane E). After 7 hours of chase, there was accumulation of form 1 in monensin-treated cells compared with the control cells. Form 2 was virtually absent from the monensin-treated 24-hour labeled cells. These data suggest that monensin inhibits the conversion of form 1 to form 2, but does not interfere with the conversion of form 2 to the mature defensin peptide. Chloroquin and ammonium chloride, weak bases that act by a different mechanism to increase the pH of acid compartments, had a similar effect to that of monensin (Fig 5). Cells cultured in the presence of 100 μmol/L chloroquin or 50 mmol/L ammonium chloride also accumulated form 1 at all chase times. A similar effect was seen with cells cultured in 10 mmol/L ammonium chloride (data not shown). However,
depletion of form 2 in 24-hour labeled cells was much more prominent with ammonium chloride than with chloroquin. These three inhibitors had no detectable effect on the release of the 75 aa prodefensin into the culture medium (data not shown). The pH of the culture media was not significantly affected by the addition of ammonium chloride or chloroquin. Taken together, these data suggest that processing of form 1 to form 2 involves a pH-sensitive step, whereas the conversion of form 2 to form 3 is resistant to neutralization of acid compartments.

**DISCUSSION**

The azurophil granules of PMN encompass a variety of proteolytic, membrane-active, and cytotoxic proteins that participate in the destruction of microbes in the phagolysosome and in tissue injury during inflammation. These
proteins, synthesized during the promyelocyte stage of
neutrophil maturation in the bone marrow, must undergo
posttranslational processing and transport to azurophil
granules without incurring autocytoxicity. Although similar
constraints also govern the synthesis and intracellular
transport of lysosomal enzymes in nonmyeloid cells, there is
increasing evidence that the processing and intracellular
targeting of lysosomal enzymes versus azurophil granule
proteins involves separate and distinct mechanisms. Lysoso-
mal enzymes undergo N-linked glycosylation in the endo-
plasmic reticulum (ER), followed by modification of the
oligosaccharide side chains in the ER and Golgi. The
resulting mannose-6-phosphate-containing oligosaccha-
rides are recognized by specific receptors that mediate the
intracellular transport and targeting of lysosomal enzymes.
Upon reaching the acid pH of (pre)lysosomal compart-
ments, the receptor–enzyme complexes dissociate and the
receptors are recycled. Interventions that interfere with
glycosylation, oligosaccharide processing, or the mainte-
nance of acid pH in these compartments result in incom-
plete processing and intracellular accumulation or extracel-
ular release of proenzymes. Like lysosomal enzymes,
myeloperoxidase (MPO) is N-glycosylated and normally
contains mannose-6-phosphate residues that can function
to mediate the endocytosis of the protein by an M6P-
receptor–dependent pathway. Unlike the processing of
lysosomal enzymes in fibroblasts, MPO processing is only
modestly affected by agents that increase the pH of acid
compartments, and not affected by substances that inhibit
the sequential trimming of oligosaccharides to structures
recognized by the M6P receptors. Based on these observa-
tions, it appears that the M6P receptor is not required for
MPO processing or targeting. The case for M6P-indepen-
dent processing of myeloid granule proteins is made even
more clearly by defensins because their precursors lack the
consensus sequence for N-glycosylation and are thus un-
likely to form the oligosaccharide side chains required for
recognition by the M6P receptor.

We have shown that the processing of human defensins
involves the sequential cleavage of the 94 aa preprodefensin
to 75 aa prodefensin form 1, then 56 aa prodefensin form 2,
and subsequently to the 29 to 30 aa mature defensin (Fig 6).
However, the pulse-chase method preferentially detects the
major longer-lived intermediates, and the existence of
additional short-lived intermediates cannot be excluded.
Some of these potential intermediates are described by
Harwig et al.

Of the three major sites of preprodefensin cleavage, the
signal sequence cleavage site that generates form 1 closely
follows the consensus for similar sequences. The cleavage
that generates form 2 takes place on the amino side of an
aspartate residue, reminiscent of the preaspartate endopro-

![Fig 3. Subcellular fractionation of CML cells labeled for 24 hours with 35S-cysteine. The cells were disrupted by sonication in 0.34 mol/L sucrose, pH 7.4, and subjected to differential centrifugation to collect nuclei (fraction N, 200g sediment), granule-enriched fraction (fraction G, 16,000g sediment), and cytoplasmic/microsomal fraction (fraction C, 16,000g supernatant). For comparison, an equivalent amount of the CML culture medium (M) was also analyzed. Each fraction (5 x 10⁶ cell equivalents) was subjected to lysis, immunoprecipitation, and analyzed by Tricine SDS-PAGE. An autoradiogram of the gel is shown, with the fraction indicated above each lane. Forms 1, 2, and 3 correspond to those shown in Fig 1.](image)

![Fig 4. The effect of monensin on defensin processing in HL-60 cells. The autoradiogram of a Tricine SDS-PAGE is shown. Each group of four lanes (monensin 0.5 µM/L, monensin 1.0 µmol/L, and control) represents the immunoprecipitates of cell lysates from a single pulse-chase study. The cells were preincubated for 30 minutes with the concentration of monensin indicated before labeling for 1 hour with 35S-cysteine. In control, the monensin was omitted and only its solvent added. Each aliquot of cells was then subjected to cold chase for the times shown above each lane (1, 7, or 24 hours). Lanes E were labeled for 24 hours without cold chase.](image)
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Fig 5. The effects of chloroquin and ammonium chloride on defensin processing in HL-60 cells. The autoradiogram of a Tricine SDS-PAGE is shown. Each group of four lanes (chloroquin 100 μmol/L, ammonium chloride 50 mmol/L, and control) represents the immunoprecipitates of cell lysates from a single pulse-chase study. The cells were preincubated for 30 minutes with the concentration of chloroquin or ammonium chloride indicated before labeling for 1 hour with 35S-cysteine. In control, the chloroquin and ammonium chloride were omitted. Each aliquot of cells was then subjected to cold chase for the times shown above each lane (1, 7, or 24 hours). Lanes E were labeled for 24 hours without cold chase.

tease activity detected in media that had been conditioned by phytohemagglutinin-stimulated peripheral blood mononuclear cells. Although we found form 1 (75 aa prodefensin) as the predominant prodefensin in fresh HL-60 conditioned media, we also noted that form 2 became detectable after storage of conditioned media at -20°C for several weeks (data not shown). The recovery of a prodefensin identical to our form 2 (56 aa prodefensin) from media conditioned by HL-60 cells has been previously reported, and may be explained perhaps by the presence of proaspartate endoprotease activity released by HL-60 cells into the media. The final maturation cleavage that generates form 3 (mature defensin) takes place in the vicinity of a dibasic sequence with a nearby upstream monobasic residue, as has been described for prohormone processing. The possibility that several sequential proteolytic steps are involved in the conversion of form 2 to form 3 is discussed in the accompanying manuscript.

The effects of inhibitors of acidification on defensin processing were similar to their effects on MPO processing. Incubation with these inhibitors resulted in a partial block of conversion of prodefensin form 1 to prodefensin form 2, suggesting that the activity of intracellular transporters or enzymes involved in this step takes place in an acid compartment and is somewhat affected by pH. However, the influence of these inhibitors on defensin processing in HL-60 cells was modest compared with their effects on the sorting of lysosomal enzymes in fibroblasts. Because the ability of monensin, ammonium chloride, and chloroquin to neutralize vacuolar pH in HL-60 cells is well documented, these observations suggest that defensin processing and subcellular transport are not critically dependent on an acid-dissociable carrier analogous to the mannose-6-phosphate receptor.

Why are preprodefensins processed in this relatively complex manner? During defensin synthesis, processing, and intracellular transport, the prepropiece and its fragments could subserve several functions that are no longer required once the protein is packaged into the azurophil granule. In its intact initial form the prepropiece may assist in the folding of the defensin molecule into its correct shape, probably by electrostatic interactions of the arginines of the mature defensin with the negatively charged glutamate and aspartate residues in the propiece. After removal of the signal sequence that functions to anchor the nascent peptide in the membrane of the endoplasmic reticulum, the anionicity of the propiece balances the cationicity of the mature protein, and may act to neutralize the latter's cytotoxicity or facilitate its transit through organellar membranes. Alternatively, the propiece may contain addressing information that sequentially directs the nascent defensin into the appropriate subcellular compartments. Finally, the endoproteolytic fragments generated during defensin processing may exert as yet unknown intracellular or extracellular functions. In the future, the structure of preprodefensin can be subjected to selective mutagenesis to test these conjectures.

In summary, the processing of defensins from the 94 aa preprodefensins to mature 29 to 30 aa defensins takes place over 4 to 24 hours and involves at least three endoproteolytic cleavages. In the process, two major transient intermediates, a 75 and a 56 aa prodefensin, are generated.
and the larger intermediate is released extracellularly. Additional studies will be required to determine the subcellular location of the processing steps, the nature of the enzymes involved, and the targeting mechanism responsible for the selective delivery of defensins to azurophil granules.

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