Proteolysis of Macrophage Inflammatory Protein-1α Isoforms LD78β and LD78α by Neutrophil-derived Serine Proteases*

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Macroproteolytic activity at sites of infection is important, since excess accumulation of leukocytes may contribute to localized tissue damage. Neutrophil-derived serine proteases modulate the bioactivity of chemokine and cytokine networks through proteolytic cleavage. Because MIP-1α is temporally expressed with neutrophils at sites of infection, we examined proteolysis of MIP-1α in vitro by the neutrophil-derived serine proteases: cathepsin G, elastase, and proteinase 3. Recombinant human MIP-1α isoforms LD78β and LD78α were expressed and purified, and the protease cleavage sites were analyzed by mass spectrometry and peptide sequencing. Chemotactic activities of parent and cleavage molecules were also compared. Both LD78β and LD78α were cleaved by neutrophil lysates at Thr16-Ser17, Phe24-Ile25, Tyr28-Phe29, and Thr31-Ser25. This degradation was inhibited by serine protease inhibitors phenylmethylsulfonyl fluoride and 4-(2-aminoethyl)-benzenesulfonyl fluoride. Incubation of the substrates with individual proteases revealed that cathepsin G preferentially cleaved at Phe31-Ile32 and Tyr28-Phe29, whereas elastase and proteinase 3 cleaved at Thr16-Ser17 and Thr31-Ser25. Proteolysis of LD78β resulted in loss of chemotactic activity. The role of these proteases in LD78β and LD78α degradation was confirmed by incubation with neutrophil lysates from Papillon-Lefèvre syndrome patients, demonstrating that the cell lysates containing inactivated serine proteases could not degrade LD78β and LD78α. These findings suggest that severe periodontal tissue destruction in Papillon-Lefèvre syndrome may be related to excess accumulation of LD78β and LD78α and dysregulation of the microbial-induced inflammatory response in the periodontium.

A major process in the pathogenesis of many inflammatory diseases, such as periodontitis, rheumatoid arthritis, and asthma, is the recruitment and activation of leukocytes by chemokines. During inflammation, leukocytes transgress the vascular endothelium and migrate into peripheral tissues in response to a gradient of chemotactic factors such as chemokines. Chemokine production is triggered by proinflammatory cytokines (e.g. IL-1α and tumor necrosis factor-α), bacterial products like lipopolysaccharides, and immune complexes (1–4). The functional activity of cytokines and chemokines is controlled in part through proteolytic cleavage by neutrophil-derived serine proteases (NSPs) (5). For example, N-terminal truncation of ENA-78, IL-8, and IL-1β produced more potent cytokines (6–8). Cleavage of active cytokines, such as tumor necrosis factor-α, IL-6, and IL-8, results in the loss of their biological activity (9–12).

Dysregulation of NSPs may be important for some human diseases characterized by microbial infection and localized tissue destruction. Such a functional role for NSPs is supported by the study of Chediak-Higashi syndrome patients with neutrophil granule deficiency (13). Another example may be seen in Papillon-Lefèvre syndrome (PLS), which is characterized by early onset, severe periodontal destruction. As a consequence of cathepsin C gene mutations, this autosomal recessive condition results in a complete loss of cathepsin C enzyme activity and subsequent failure to activate serine proteases such as cathepsin G, elastase, and proteinase 3 (14). In periodontitis, the inflammatory response to microbial infection in the gingival sulcus results in destruction of connective tissues and resorption of alveolar bone. Chemokine-positive cells and chemokine receptor-positive cells are present in inflamed periodontal tissues, and macrophage inflammatory protein-1α (MIP-1α)-positive cells are more abundant than other chemokine-positive cells (15, 16).

MIP-1α is a C-C chemokine produced at inflammatory sites (17–20) by many cell types, such as neutrophils, epithelial cells, cells of the monocoy/macrophage lineage, bone-derived cells, dentritic cells, and endothelial cells (21–24). Human MIP-1α has two different isoforms, LD78β and LD78α, which are expressed by two highly related nonallelic genes on chromosome 17q21.1–3 (25). Both genes are relatively small (∼3.0 kb), consist of three exons and two introns, and are transcribed upon inflammatory cytokine stimulation. The LD78β and LD78α genes encode preproteins of 93 and 92 amino acid residues, respectively.

The abbreviations used are: IL, interleukin; NSP, neutrophil-derived serine protease; PLS, Papillon-Lefèvre syndrome; MIP-1α, macrophage inflammatory protein-1α; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; TLCK, N-tosyl-lys chloromethyl ketone; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone; PBS, phosphate-buffered saline; HBS, Hanks’ balanced salt solution; MES, 4-morpholineethanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; TOP, time-of-flight; EST, (25, 3S)-trans-epoxysuccinyl-l-leucylamido-3-methylbutane ethyl ester.

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respectively. After signal peptide cleavage, mature LD78β and LD78α both contain 70 amino acids with theoretical molecular masses of 7798.7 and 7788.7 Da, respectively. There are no apparent glycosylation sites on LD78β and LD78α, and they share 94% sequence similarity, differing by only three amino acids. The biological activity of the two different MIP-1α isoforms has been studied extensively in HIV-1 infection. LD78β is the more potent HIV-1-inhibiting chemokine, acting as a CCR5 agonist. Its binding affinity to CCR5 is 6-fold higher than that of LD78α (26, 27). Truncated forms of MIP-1α are found in vivo. LD78α, when isolated from natural sources, has only 66 amino acids, LD78α-(5–70). Three different LD78β forms, LD78β-(1–70), LD78β-(3–70), and LD78β-(5–70), have been identified in conditioned media from stimulated mononuclear cells. Conversion of LD78β-(1–70) to LD78β-(3–70) results from processing of the precursor by a membrane-associated serine protease, dipetidyl peptidase IV. Truncated LD78β-(3–70) has increased chemotactic activity and enhanced anti-HIV-1 activity due to an increased receptor binding affinity compared with intact LD78β (28).

At present, the fate of MIP-1α at sites of infection is not well characterized. We investigated the proteolysis of the MIP-1α isoforms, LD78β and LD78α, by NSPs in order to identify the specific cleavage sites and to assess the chemotactic activity of the parent molecules and their cleavage products. We hypothesized that in PLS, the failure to activate NSPs due to cathepsin C inactivity prevents proteolytic degradation and inactivation of MIP-1α. Dysregulation of MIP-1α in inflamed periodontal tissues provides an environment favoring localized tissue destruction. Here, we report that LD78β and LD78α are cleaved by NSPs and that the cleavage products exhibit loss of chemotactic activity. We also report that NSPs from PLS patients fail to degrade LD78β and LD78α, supporting the hypothesis that this underlies the inflammation-associated destruction of the periodontium seen in this condition.

**EXPERIMENTAL PROCEDURES**

**Materials**—Histoplaque-1119-1 and Histoplaque-1077-1 were purchased from Sigma. Protease inhibitors phenylmethylsulfonyl fluoride and EDTA were obtained from Sigma, and AEBSF, E-64, EST, leupeptin, TLCK, and TPCK were purchased from Calbiochem. Human neutrophil cathepsin G and elastase were purchased from Calbiochem, and human neutrophil proteinase 3 was purchased from Athens Research & Technology (Athens, GA).

**Construction of Recombinant Human LD78α and LD78α Expression System**—Human LD78β and LD78α cDNAs were amplified by PCR using a macrofage-derived cDNA as a template and LD78β- and LD78α-specific primer sets (LD78α sense, 5'-ACA TTC CAT CAG CTG CTC CC-3'; LD78α sense, 5'-ACA TTC CGT CAC CAG CTC AG-3'; LD78α antisense, 5'-AAG CGT CAT GAC CCC TCA GG-3'). The PCR was performed as follows: 94°C for 1 min, 60°C for 30 s, 72°C for 1 min, for 34 cycles. Amplified LD78β and LD78α PCR products were cloned into the TA vector (Invitrogen), and the cDNAs were confirmed by sequence analysis. Using these two cDNAs, NdeI and BamHI restriction sites were introduced, and LD78β and LD78α cDNAs were generated by PCR (LD78β NdeI, 5'-ATA CAT ATG GCA CCA CCT GCT GCT GAC AGC-3' (NdeI site underlined); LD78β NdeI, 5'-ATA CAT ATG GCA CCA CCT GCT GCT GAC AGC-3' (NdeI site underlined); LD78α BamHI, 5'-CGG GAA TTC TCT GAC GCT CAG TCT GAG-3' (BamHI site underlined)). Amplified PCR products were digested with NdeI and BamHI and subcloned into the pET11a vector (Novagen, Madison, WI) digested with NdeI and BamHI. The clones were verified by sequence analysis and transformed into BL21(DE3) (Novagen) for LD78β and LD78α protein expression.

**Expression of Recombinant Human LD78β and LD78α from Escherichia coli**—A single colony was inoculated into 10 ml of NZCYM media supplemented with 50 μg/ml ampicillin in a 50-ml disposable centrifuge tube and grown overnight with shaking at 37°C. This bacterial culture was seeded into a liter flask containing 500 ml of NZCYM-ampicillin media and grown to an OD (at 550 nm) of 0.6. Isopropyl-β-thiogalactopyranoside was then added to a final concentration of 0.5 mM, the flasks were shaken for 2–3 h, and the cells were pelleted by centrifugation. The cells were resuspended in 20 ml of phosphate-buffered saline (PBS), sonicated, and centrifuged at 15,000 rpm for 10 min. After the pellets were resuspended in 10 ml of 6 M urea and centrifuged, the supernatant was ultrafiltered using YM-30 and YM-3 membranes.

The protein solution obtained following ultrafiltration was injected into HPLC with a reverse phase column ODP-50 (Supelco, St. Louis, MO). The proteins were eluted by 0–80% acetonitrile in 50 mM ammonia water for 45 min at a flow rate of 1 ml/min. The purified proteins were stored at −20°C after freeze-drying.

**Preparation of Cell Lysates**—Human neutrophils and mononuclear cells were isolated from peripheral blood of healthy donors using Histoplaque-1119-1 and Histoplaque-1077-1 as described in Sigma procedure 1119 (leukocyte separation). The neutrophils were treated with cold NH4Cl buffer to lyse contaminating erythrocytes and washed with PBS. Neutrophils and mononuclear cells were assessed by light microscopy in Giemsa stained, air-dried membrane. Cells were suspended at a concentration of 1 × 106 cells/ml in Hank's balanced salt solution (HBSS) and sonicated for 30 s. After centrifugation at 4°C for 5 min at 13,000 rpm, the supernatants were used as the cell lysate. For neutrophil preparation from PLS patients, blood was obtained from three Turkish patients, who were previously shown to have cathepsin C mutations and to lack cathepsin C activity (29). All patients had complete loss of cathepsin C activity (less than 5 μmol/min/mg protein; control range, 685–1200 μmol/min/mg protein). The genotypes of the three patients were p.G139R/p.G139R, p.R272P/p.R272P, and p.W429X/p.W429X. As controls, blood was obtained from five population- and age-matched healthy individuals.

**Proteolysis of LD78β and LD78α**—Substrate solutions, 100 μM LD78β and LD78α, were prepared with Hank's balanced salt solution containing 1 mM Tris-HCl buffer (pH 7.4) and 10 mM CaCl2. Lysates of neutrophils or mononuclear cells were separately mixed with the substrate solutions at a ratio of 1:1 (v/v). The reaction mixtures were incubated for 4–24 h at 37°C and analyzed using SDS-PAGE. In order to determine the effect of inhibitors, the cell lysates were pretreated with protease inhibitors by incubating them at room temperature for 1 h, prior to adding the cell lysates. Test concentrations of the inhibitors used were: 1 mM phenylmethylsulfonylfuoride, 1 mM AEBSF, 5 mM EDTA, 10 μM E-64, 100 μM EST, 100 μM leupeptin, 100 μM TLCK, 100 μM TPCK.

For the reaction with human NSPs, cathepsin G, elastase, and proteinase 3 were dissolved according to the manufacturer's instructions and diluted with 50 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and mixed with substrate solution at a molar ratio of 1:10 (29). All patients had complete loss of cathepsin C activity (less than 5 μmol/min/mg protein) with a mean of 0.6 μmol/min/mg protein. The pH of the reaction mixtures was adjusted (pH 5.5) with 50 mM sodium acetate buffer containing 150 mM NaCl. Reaction mixtures were incubated for 4–24 h at 37°C.

**Protein Gel Electrophoresis and Coomassie Staining**—For protein gel electrophoresis, each sample was mixed with 4× NuPAGE LDS sample loading buffer (Invitrogen) and boiled for 5 min, and run in a 12% NuPAGE Bis-Tris polyacrylamide gel with MES-SDS running buffer (Invitrogen). Proteins were visualized by Coomassie staining using SimplyBlue SafeStain (Invitrogen).

**Determination of the N Terminus Amino Acid Sequence and Mass Values**—The electrophoresed proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) using Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The membranes were washed with distilled water for 3 × 5 min, stained with 0.25% Coomassie Brilliant Blue R-250 in 40% methanol, and washed with 50% methanol until the background became light blue. The protein bands were excised from the dried membrane and used for N terminus amino acid sequencing. Edman sequencing was performed by the FDA protein core laboratory, which uses a gas/liquid phase protein sequenator (Applied Biosystems model 477A) with an on-line HPLC determination of derived phenylthiohydantoin-derivatives.

Mass fingerprint analyses of intact LD78β and LD78α proteins and their proteolytic peptides were made by MALDI-TOF mass spectrometry, whereas peptide sequencing was obtained through nanospray tandem mass spectrometry. For mass analysis of intact proteins, samples were prepared using Bio-Rad matrix (MS/MS) mass spectrometer (a MALDI-TOF) in linear positive ion mode. Sineapinic acid matrix (5 mg/ml) was prepared in 50% acetonitrile containing 0.1% trifluoroacetic acid. The dried droplet method was employed for sample preparation. In brief, 1 μl of the sample and matrix mixture was dried on MALDI target plates at room temperature. Two protein standards, bovine insulin (average M + H = 5734.51 Da) and equine cytochrome c (average M + H = 12,361.96 Da), were used as the calibrants. For peptide mass
fingerprints, mass values of purified LD78β and LD78α, were characterized by N-terminal amino acid sequence and mass value (Table I). The N-terminal amino acid sequence of LD78α started with ASLAADTP, excluding the first methionine, with a molecular mass of 7790.8 Da. LD78β had two different forms, MAPLAADTP- (minor, 10%) and PLAADTPMA- (major, 90%, lacking the first two amino acids), with a molecular mass of 7930.4 and 7728.0 Da, respectively.

**Proteolysis of LD78β and LD78α by Cell Lysates—**To examine proteolysis of LD78β and LD78α, the substrates were incubated for 8 h at 37 °C with human peripheral blood mononuclear cell lysates or neutrophil lysates. Fig. 2A shows the protein profiles of cell lysates and substrates. As shown in Fig. 2B, neutrophil lysates cleaved both substrates, whereas mononuclear cell lysates did not detectably degrade LD78β or LD78α. Neutrophil lysates degraded the parent substrates (7.8 kDa) to cleavage products with molecular masses of <6.5 kDa.

**Effect of Protease Inhibitors on Degradation of LD78β and LD78α by Neutrophil Lysates—**Neutrophil lysates were preincubated with various protease inhibitors at 4°C for 1 h, and the lysates were reacted with LD78β or LD78α at 37 °C for 4 and 24 h. When incubated without inhibitors for 4 h, the lysates cleaved the parent 7.8-kDa LD78β to products with apparent molecular masses of ~6.5 and 5 kDa. These cleavage products were further degraded to smaller peptides following a 24-h incubation (Fig. 3A). Fig. 3B showed that full length LD78α was initially cleaved to 4.5–6.5-kDa cleavage products and further degraded to peptides of <4.0 kDa following a 24-h incubation. Degradation of LD78β and LD78α was almost totally blocked in the presence of serine protease inhibitors, phenylmethylsulfonyl fluoride and AEBSF, but not inhibited by EDTA, E-64, EST, leupeptin, TLCK, and TPCK (Fig. 3, A and B), suggesting that the proteolysis of LD78β and LD78α may be by serine proteases in neutrophils. A 7.5-kDa band appeared during incubation of both LD78β and LD78α in the presence of AEBSF. Edman sequencing revealed that the 7.5-kDa band had the same N-terminal amino acid sequence as the parent molecules, suggesting a portion of the C terminus part may be removed by specific protease activity (data not shown).

**Processing of LD78β and LD78α by Cathepsin G, Elastase, and Protease 3—**Each NSP, cathepsin G, elastase, and protease 3, was tested to determine its capacity to degrade LD78β and LD78α. Different cleavage patterns were produced by each serine protease, with cathepsin G and protease 3 producing major cleavage products for LD78β of 6.5 and 5.5 kDa, whereas the major elastase cleavage products were ~5 kDa (Fig. 4A). The cleavage pattern of LD78α generated by each serine protease was quite similar to that of LD78β (Fig. 4B and Table II).

The serine protease cleavage patterns were different from those produced by neutrophil lysates, suggesting that degradation of LD78β and LD78α may occur by the action of two or more serine proteases.
and LD78β by neutrophil lysates, cathepsin G, elastase, or proteinase 3 were analyzed by liquid chromatography-mass spectrometry to determine the mass values and peptide sequencing of their cleavage products. Peptide sequence-matched mass values were assigned to the cleavage products, as shown in Table II. For LD78β, neutrophil lysates cleaved Thr16-Ser17, Phe24-Ile25, Tyr28-Phe29, Thr31-Ser32, Asp53-Pro54, and Tyr62-Val63 preferentially. Whereas cathepsin G cleaved Gln22-Asn23, Phe13-Ser14, Tyr15-Thr16, Phe24-Ile25, Tyr28-Phe29, Thr31-Ser32. These results confirmed that the cleavage sites by neutrophil lysates were combinations of those by cathepsin G and elastase or proteinase 3.

No Degradation of LD78β with Neutrophil Lysates Derived from PLS Patients—Neutrophil lysates obtained from three PLS patients and five healthy individuals were incubated with LD78β at 37 °C for 24 h, and the reaction mixtures were separated on polyacrylamide gels, and the gels were stained with Coomassie solution. M, marker.

FIG. 2. Proteolysis of LD78β and LD78α by neutrophil lysates. Neutrophil and mononuclear cell lysates were prepared by suspending 1 × 10^6 cells in 1 ml of HBSS followed by sonication. Substrate solutions, 100 nM LD78β and LD78α, were prepared in HBSS containing 20 mM Tris-HCl buffer (pH 7.4) and 10 mM CaCl2. A, cell lysates or substrate alone were loaded as controls. B, the substrate solutions were mixed with lysates of mononuclear cells (MN) or neutrophils (Neu) at a volume ratio of 1:1. The reaction mixtures were incubated at 37 °C for 8 h and run on 12% polyacrylamide gels, and the gels were stained with Coomassie solution. β and α, LD78β and LD78α, respectively.

FIG. 3. Degradation of LD78β (A) and LD78α (B) by neutrophil lysates in the absence/presence of inhibitors. Neutrophil lysates were prepared by suspending 1 × 10^6 cells in 1 ml of HBSS followed by sonication. The cell lysates were pretreated with protease inhibitors by incubating at room temperature for 1 h and mixed with 100 nM LD78β or LD78α at a ratio of 1:1. The concentrations of inhibitors used were as follows: 1 mM phenylmethylsulfonyl fluoride, 1 mM AEBSF, 1 mM TPCK. The reaction mixtures were incubated at 37 °C for 4 or 24 h and run on 12% polyacrylamide gels, and the gels were stained with Coomassie solution. As controls, neutrophil lysate and substrate alone were loaded. M, marker.

FIG. 4. Proteolysis of LD78β (A) and LD78α (B) by the neutrophil-derived serine proteases, cathepsin G, elastase, or proteinase 3. Human neutrophil cathepsin G, elastase, and proteinase 3 were dissolved in 50 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and separately mixed with substrate solution at a molar ratio of 1:10–100 (1 millimolar of protease for 6 μg of substrate). The mixtures were incubated for 4 h at 37 °C, products were separated on 12% polyacrylamide gels, and the gels were stained with Coomassie solution. As controls, neutrophil lysate and substrate alone were loaded. M, marker.

PLS patients and five healthy individuals were incubated with LD78β at 37 °C for 24 h, and the reaction mixtures were separated on polyacrylamide gels. As shown in Fig. 5, LD78β was cleaved by neutrophil lysates from healthy controls. In contrast, cell lysates from PLS patients did not detectably degrade LD78β. Incubation of LD78α also demonstrated the absence of cleavage by neutrophil lysates donated from PLS patients (data not shown).

Chemotactic Activity of Intact LD78β, LD78α, and Their Cleavage Products—After LD78β was digested with three NSPs (cathepsin G, elastase, and proteinase 3) for 24 h, its chemotactic activity was compared with the parent molecules LD78β and LD78α. The LD78β cleavage products had molecular masses of <4.5 kDa after cleavage by the three serine proteases (data not shown). After incubation of mononuclear cells with chemottractant, the migrated cells were measured by fluorescence labeling of the cellular nucleic acids. The results showed that LD78β and LD78α had similar chemottractant potency, and LD78β cleavage products lost the biological activity (Fig. 6).

DISCUSSION

In addition to their role in intralysosomal degradation of engulfed cell debris and microorganisms, the function of extracellularly released NSPs at sites of inflammation has received increasing attention (5). NSPs impact the bioactivity of cytokine networks through proteolytic release of active cytokines from precursor molecules, cleavage inactivation of active cytokines, cytokine receptor shedding, and proteolysis of cytokine-binding proteins. For example, certain cytokines (e.g. IL-1β, tumor necrosis factor-α, and IL-18) are activated by NSPs, whereas the activity of other cytokines and chemokines (e.g. IL-8, NAP-2, and ENA-78) is amplified by N terminus processing by cathepsin G and proteinase 3 (6–8). In contrast, cytokines such as tumor necrosis factor-α, IL-6, and IL-8 are inactivated by cathepsin G, elastase, and proteinase 3 (9–12). The proteolytic cleavage of tumor necrosis factor-α into at least two fragments results in the loss of its cytotoxic activity. Likewise, IL-6 is inactivated by cleavage at Phe28→Asn29. Proteolysis has emerged as an important regulatory mechanism within the cytokine network, and NSPs function as essential proteases in the physiological repertoire of cytokine bioactivity control mechanisms, especially in neutrophil-dominated inflammatory processes.

MIP-1α is a chemotactic factor that induces transendothelial...
### Table II

**Determination of mass values and peptide sequences of LD78β (A) and LD78α (B) cleavage products by neutrophil lysates and serine proteases**

LD78β or LD78α were mixed with each neutrophil lysate, cathepsin G, elastase, and proteinase 3 at a molar ratio of 1:10–100, and the mixtures were incubated for 4–24 h at 37 °C. Reaction was stopped by adding trifluoroacetic acid to a final concentration of 0.1%. The proteolytic mixtures were analyzed by liquid chromatography-mass spectrometry, and the mass/charge (m/z) ratios of peptides and their fragmented ions were determined. The raw data files were searched against the human database, and the peptide sequence-matched mass values were obtained.

*Measured mass values are in parentheses.*

| (A) LD78β | APLAADTPTACCPSYTSRQIPQNFADYFETTSSQSCKPSVIIFLTGRQVCADPSEBWQKYVSDELLSA |
| --- | --- |
| + neutrophil lysate | IPQNFADY (1081.2)* |
|  | SRQIPQNF (990.1) |
|  | SRQIFQNFADY (1452.6) |
|  | SRQIPFQNFADYET (1829.9) |
|  | PLAADTPTACCPSYTSRQIPQNF (2550.9) |
|  | APLAADTPTACCPSYTSRQIPNF (2603.9) |
|  | PLAADTPTACCPSYTSRQIPNFADY (2993.1) |
|  | FETSSQSCKPSVIIFLTGRQVCADPSEBWQKYVSDELLSA (4748.6) |
|  | (5211.1) IADYFETSSQSCKPSVIIFLTGRQVCADPSEBWQKYVSDELLSA (1352.4) ADPSEBWQKY (1166.3) PSEBWQKY |
| + cathepsin G | PLAADTPTACCPSYTSRQIPQNF (2271.6) |
|  | PLAADTPTACCPSYTSRQIPNF (2552.8) |
|  | APLAADTPTACCPSYTSRQIPNF (2603.9) |
|  | APLAADTPTACCPSYTSRQIPNFADY (2995.3) |
| + elastase | NFIADYFET (1120.2) |
|  | RQIPQNFADYFET (1742.6) |
|  | SRQIFQNFADY (1174.3) |
|  | SRQIFQNFADYET (1829.9) |
|  | PSYRQIPQNFADYET (2328.5) |
|  | ACCPSYRQIPQNFADYET (2605.9) |
|  | SSQCSKPSVIIFLTGRQV (3137.6) |
| + proteinase 3 | RQIPQNFADYFET (1742.9) |
|  | SRQIPQNFADYET (1829.9) |
|  | SRQIPQNFADYETFS (1917.1) |
|  | YTSRQIPQNFADYET (2094.3) |
|  | PSYRQIPQNFADYET (2328.5) |
|  | ADTPACCSYTSRQIPQNFADYET (3091.4) |
|  | SSQCSKPSVIIFLTGRQV (3137.6) |
|  | (1265.4) PSEBWQKY (1253.4) QKYSDELLSA |

| (B) LD78α | ASLAAADPACCPSYTSRQIPQNFADYFETTSSQSCKPSVIIFLTGRQVCADPSEBWQKYVSDELLSA |
| --- | --- |
| + neutrophil lysate | ASLAAADPACCPSYTSRQIPQNF (2593.9)* |
|  | ASLAAADPACCPSYTSRQIPQNFADYET (3054.7) |
|  | SYTSRQIPQNFADYET (3433.8) |
|  | TSRQIPQNFADYET (2181.4) |
|  | SRQIFQNFADYET (1829.9) |
|  | SRQIPQNFADYET (1829.9) |
| + cathepsin G | ASLAAADPACCPSYTSRQIPQNF (2593.9) |
|  | ASLAAADPACCPSYTSRQIPQNFADYET (3056.4) |
|  | SYTSRQIPQNF (1341.5) |
|  | SYTSRQIPQNFADY (1803.9) |
|  | TSRQIPQNFADYET (1553.7) |
|  | (1167.3) PSEBWQKY (382.1) BEBWQKY |
| + elastase | SRQIPQNFIA (1174.3) |
|  | SRQIFQNFADYET (1829.9) |
|  | RQIPQNFADYET (1742.9) |
|  | RQIPQNFADYETSSQCSKPGV (2616.9) |
|  | NFIADYFET (1120.2) |
| + proteinase 3 | ASLAAADTPACCPSYT (1622.8) |
|  | TSRQIPQNFADYET (1553.7) |
|  | SRQIPQNFADYET (1829.9) |
|  | RQIPQNF (1616.2) |
|  | RQIPQNFADYET (1742.9) |
|  | SSQCSKPGVIFLTGRQVCADPSEBWQKY (2410.4) |
|  | (3655.9) SSQCSKPGVIFLTGRQVCADPSEBWQKY (1265.4) PSEBWQKY (734.8) SDLLESA |
migration, protease release, and Ca^{2+} release by leukocytes (17, 23, 31). It binds to the chemokine receptors CCR1, CCR4, and CCR5 and inhibits HIV-1 infection and murine hematopoietic stem cell proliferation (32). MIP-1α expression in many cell types is triggered by microbial pathogens and proinflammatory cytokines (19–22). Since excess accumulation of leukocytes may contribute to localized tissue damage, controlling the activity of MIP-1α at sites of infection is important. Little is known about the fate of MIP-1α secreted at inflammatory sites, and inactivation of MIP-1α is not well characterized. The lysosomal cysteine protease, cathepsin D, is reported to degrade MIP-1α, but the study was limited to a tumor invasion model, and the cleavage sites for MIP-1α substrates were not determined. Additionally, the most efficient cleavage by cathepsin D was observed at pH 4, which is rarely found in the extracellular environment (33). Because MIP-1α expression is temporally related to neutrophil infiltration at inflammatory sites, we evaluated proteolysis of the MIP-1α isoforms LD78β and LD78α by the three NSPs: neutrophil elastase, proteinase 3, and cathepsin G. Our findings indicate that cleavage sites of LD78β or LD78α generated by the NSPs are different for each protease. Cathepsin G preferentially cleaved aromatic amino acids in the P_1 position. Elastase and proteinase-3 preferred aliphatic side chain amino acids, threonine, valine, serine, and alanine, at the P_1’ position. The major cleavage sites for all three proteases were the same for both LD78β and LD78α.

Because the amino acid sequences of chemokines are highly homologous, their secondary and tertiary structures are similar. NMR studies indicate that chemokines share the same basic features, including a relatively disordered N terminus followed by an extended loop that leads into three antiparallel β-pleated sheets and a C-terminal α-helix (34). Human MIP-1β shares 68% amino acid sequence homology with LD78β and LD78α and has a triple-stranded β sheet (residues 26–31, 39–44, and 48–52) arranged on top of an α-helix (residues 57–68). MIP-1β tightly binds the CCR5 receptor as part of its natural function in the immune response and in doing so also blocks the ability of other ligands to bind CCR5. The single most important MIP-1β residue known to contribute to its CCR5 receptor interaction is Phe^{13} (35). Fig. 7 shows the predicted two-dimensional structure of LD78β and LD78α and the major cleavage sites generated by each protease. Our data demonstrate four cleavage sites within four amino acids of Phe^{13} in MIP-1α. MIP-1α cleavage products generated by all three serine proteases lose their chemotactic activity for mononuclear cells, compared with the parent molecules. Although we did not evaluate anti-HIV activity, we would predict that these cleavage products will lose the biological activity to inhibit HIV infection.

The LD78β and LD78α isoforms studied were generated from E. coli. Expressed protein, present as an inclusion body, was purified in the presence of organic solvent. Both LD78β and LD78α contain four cysteine residues, raising the possibility of intermolecular disulfide bond formation. To check this, we electrophoresed the isolated recombinant proteins under native conditions by mixing 0.25 mg/ml of solution with native sample loading buffer (1:2 ratio by volume). A single band was

![Fig. 5. Comparison of LD78β proteolysis by neutrophil lysates from PLS patients and healthy individuals.](image)

![Fig. 6. Comparison of the chemotactic activity of LD78β, LD78α, and LD78β cleavage products.](image)

![Fig. 7. Two-dimensional structure and major cleavage sites of LD78β and LD78α by cathepsin G, elastase, or proteinase 3.](image)
visualized at 7.8 kDa, corresponding to a monomeric form (data not shown), demonstrating the lack of intermolecular disulfide bond formation. It is known that high concentrations of MIP-1α induce high molecular mass aggregates by self-assembly, and serial dilution breaks the aggregate to a monomer form by a reversible process. Both aggregated and disaggregated forms have equivalent G-protein-coupled receptor-mediated biological activity (36). Therefore, we postulated that the recombinant LD78β and LD78α used in our experiment is properly folded. Even if enzymatic forms were formed at high concentrations, this would not influence the results of this study.

Cytokines are constitutively expressed only at low levels in healthy periodontal tissues (15, 16). Microbial lipopolysaccharides and proinflammatory cytokines rapidly induce MIP-1α expression, and chemokine-positive cells, particularly MIP-1α-positive cells, are more abundant than other chemokine-positive cells in inflamed periodontal tissues. This suggests that MIP-1α expression increases with increasing levels of inflammation in the periodontium, and MIP-1α activity may be important in periodontitis-associated inflammatory tissue destruction. Whereas the common, chronic, adult onset forms of periodontitis are etiologically complex and probably heterogeneous conditions, several Mendelian forms of severe periodontitis are known. PLS is characterized by early onset aggressive periodontitis with connective tissue destruction and alveolar bone loss. PLS results from mutation of the gene encoding cathepsin C, a dipeptidyl peptidase. The mutated alleles associated with PLS have complete loss of protease activity (29). Therefore, we postulated that the recombinant LD78α and LD78β, the downstream effect of loss-of-function mutations in cathepsin C prevents activation of cathepsin G, elastase, and proteinase 3. Our current findings indicate these NSPs are able to proteolytically degrade the MIP-1α isoforms LD78β and LD78α. Incubation of MIP-1α with neutrophil lysates from three patients with PLS failed to cleave the LD78β and LD78α parent molecules. We believe this failure of MIP-1α degradation is etiologically important in PLS. Choi et al. (37) reported MIP-1α also functions as an osteoclast stimulating factor, resulting in bone resorption. In the pathophysiology of PLS periodontitis, we suspect that leukocyte recruitment is effectively prolonged because MIP-1α is not inactivated, resulting in an exaggerated immune response and the subsequent destruction of connective tissue and alveolar bone.

In summary, the present findings demonstrate that NSPs can inactivate LD78β and LD78α, extending the role of NSPs in regulating chemokine bioactivity. These findings offer a mechanism for MIP-1α function in localized periodontal tissue destruction in PLS.

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