Amino-Terminal Processing of Chemokine ENA-78 Regulates Biological Activity†

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ABSTRACT: Epithelial cell-derived neutrophil-activating protein-78 (ENA-78) is a potent stimulator of neutrophils, inducing a variety of biological responses such as chemotaxis, enzyme release, up-regulation of surface receptors, and intracellular calcium mobilization. Proteolysis of ENA-78 with cathepsin G and chymotrypsin yielded a time-dependent increase in elastase-releasing activity, predicting the formation of truncation products with higher potency than native ENA-78. To investigate the biological implications of progressive truncation of ENA-78, the N-terminal variants ENA(5−78), ENA(9−78), and ENA(10−78) were cloned and expressed in *E. coli*. When tested in the neutrophil elastase release assay, the variants ENA(5−78) and ENA(9−78) had a 2−3-fold higher potency than full-length ENA-78, while ENA(10−78) was 3-fold less potent. In the chemotaxis assay, the variant ENA(5−78) exhibited an 8-fold and ENA(9−78) a 2-fold higher potency than native ENA-78. ENA(10−78), conversely, was 10-fold less potent, but reached a comparable efficacy to ENA-78 at 10^{-7} M concentration. In summary, the rank order in potency with respect to elastase release was ENA(9−78) > ENA(5−78) > ENA-78 > ENA-(10−78), while for chemotaxis it was ENA(5−78) > ENA(9−78) > ENA-78 > ENA(10−78). Variant ENA(5−78) had a higher overall potency and efficiency for chemotaxis than interleukin-8 (IL-8), while ENA(9−78) exhibited a higher efficiency at concentrations of 1−100 nM. The fact that neutrophil cathepsin G produces the stable ENA(9-78) variant in vitro strongly suggests a role for this N-terminal proteolysis during inflammatory processes in vivo.

Epithelial cell-derived neutrophil-activating protein-78 (ENA-78)[1] is a member of the CXC subfamily of chemokines, originally isolated from the stimulated human type-II alveolar epithelial cell line A549 (*1*). ENA-78 displays the four conserved cysteine residues and the Glu-Leu-Arg (ELR)-motif preceding the first cysteine residue. This structural element has shown to be essential for neutrophil-stimulating activity (*2*, *3*). In contrast to interleukin-8 (IL-8) which interacts with both IL-8 receptors, CXCR1 and CXCR2, ENA-78 only binds to CXCR2 with high affinity (*4*, *5*). ENA-78 is a potent activator of neutrophils in vitro, up-regulating cell surface expression of the integrin Mac-1 (*5*), inducing chemotaxis, enzyme release, and a rise of intracellular calcium (*1*). ENA-78 has been detected as an abundant chemotaxin present in certain disease states such as rheumathoid arthritis, chronic pancreatitis, and inflammatory bowel diseases (*6*−*9*), and may thus play an important role in leukocyte recruitment during these inflammatory conditions in vivo. The major form of ENA-78 released by human epithelial and endothelial cells is a 78 amino acid protein (*1*, *8*). Supernatants of bovine alveolar macrophages and blood monocytes yielded a homologue of human ENA-78 that is N-terminally truncated (*10*). As demonstrated earlier, proteolytic processing is a fundamental process in the formation of neutrophil-activating protein-2 (NAP-2). NAP-2 is formed from inactive precursors stored in α-granules of blood platelets by the leukocyte protease cathepsin G to yield a highly potent neutrophil chemotaxin and activator (*11*−*13*). Modulation of biological activity associated with N-terminal truncation has also been observed with IL-8. When isolated from tissue cells, IL-8 consists of 77 amino acids, while when isolated from supernatants of human monocytes IL-8 is primarily obtained as an N-terminal truncated 72 amino acid protein (for review, see ref *14*). The progressive cleavage of the N-terminus of IL-8 yielded an increase in potency; however, truncation within the ELR-motif preceding the first cysteine residue caused a dramatic loss of biological activity (*2*).

In the present study, we have used proteolysis of ENA-78 to study the formation of active variants. Using recombinant technologies, we have produced full-length ENA-78 and three N-terminal truncated ENA-78 variants which were characterized for their properties to activate human neutrophils. Our results demonstrate that leukocyte proteases produce ENA-78 variants with strongly elevated potencies, and that progressive cleavage has differential effects on specific neutrophil functions.

MATERIALS AND METHODS

PCR Amplification of the Expression Cassettes. cDNA segments encoding full-length ENA-78 and the three amino-terminal variants were amplified from a cDNA prepared

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[1] Abbreviations: ELR, Glu-Leu-Arg; ENA-78, epithelial cell-derived neutrophil-activating protein-78; IL-8, interleukin-8; NAP-2, neutrophil-activating protein-2; BSA, bovine serum albumin; CXCR, CXC chemokine receptor; rp-HPLC, reversed-phase high-performance liquid chromatography.

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previously (*15*) using the oligonucleotide sequences as indicated in Table 1. PCR reactions were performed in 60 μL mixtures containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 333 μM dNTP, 1 μM of each primer, 0.001% gelatin, 1 μL of the ENA-78 cDNA template, and 2.5 units of *Taq* DNA polymerase. The samples were subjected to 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C. PCR products were then separated on 1.2% agarose gels, and bands of the anticipated sizes were electroeluted (Model UEA, Kodak, Lausanne, Switzerland).

Transformation with the Expression Constructs. Amplicons containing ENA-78 and variants were digested with *Bam*HI and *Hin*dIII and ligated into the pQE-8 vector (Qiagen AG, Basel, Switzerland) according to standard procedures (*16*). Ligation products were then transformed into *E. coli* strain XL1-Blue by electroporation (*17*), and ampicillin-resistant colonies were picked and analyzed for their proper insert by a PCR screening method. Briefly, single colonies were resuspended in 50 μL of water, 10 μL of the suspension was incubated at 95 °C for 10 min, and after centrifugation at 13 000 rpm for 15 min, supernatants were used for PCR reactions with the oligonucleotides QE8For and AS/36C (Table 1). The samples were subjected to 30 cycles of 15 s at 95 °C, 30 s at 45 °C, and 45 s at 72 °C with a final extension at 72 °C for 7 min. The PCR products were fractionated on a 1.4% agarose gel and visualized by ethidium bromide. Clones containing inserts of the correct size were then analyzed for protein expression. Samples of induced (2 mM IPTG) and noninduced cultures were analyzed by 6 M urea/SDS/18% PAGE and protein bands visualized by Coomassie R-250 stain, or after transfer to nitrocellulose membranes (0.45 μm; Schleicher & Schuell, Dassel, Germany) analyzed for ENA-78 immunoreactivity by Western blot analysis using a rabbit polyclonal anti-ENA-78 antibody (*18*). Plasmids from positive expression clones were isolated and ENA-78 genes analyzed by DNA sequencing using oligonucleotides QE8For and QE8Rev and the Sequenase reagent kit (United States Biochemical Corp., Cleveland, OH).

Protein Expression and Purification. For large-scale expression, the plasmid constructs were transformed into the *E. coli* strains BL21 and M15 using methods described (*19*). Both strains contained the pREP4 repressor plasmid encoding the *lac* repressor (Qiagen AG, Basel, Switzerland). Cultures from single colonies were grown and expressed in 1 L volumes of LB containing 100 μg/mL ampicillin and 25 μg/mL kanamycin. After the induction, the bacteria were harvested by centrifugation at 2400*g* for 30 min at 4 °C, and the pellets were stored at −80 °C until further use. The recombinant ENA-78 proteins were then purified from bacterial lysates by Ni^{2+}-nitrilotriacetic acid (NTA) agarose affinity purification according to the manufacturer's application manual (Qiagen AG, Basel, Switzerland). Purified proteins were analyzed by 6 M urea/SDS/18% PAGE as described above. From the two *E. coli* expression strains used, BL21 consistently yielded higher expression for all the constructs than strain M15.

Refolding and Factor Xa Cleaving. Purified proteins were denatured and reduced in 8 M urea, 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 0.1 M NaH_2PO_4, 0.01% CHAPS at a protein concentration of 50 μg/mL. Refolding was then initiated by stepwise addition of 1 volume of dilution buffer (40 mM NaH_2PO_4, pH 8.0, 50 mM NaCl, 5% glycerol, 0.01% CHAPS, 1 mM reduced glutathione, 0.5 mM oxidized glutathione) every 1.5 h. After five such dilutions, the protein solution was left to stir for 48 h at 4 °C. After pH adjustment to pH 7.4, the solution containing the refolded protein was loaded onto a phosphocellulose column as previously described (*1*). Proteins were step-eluted with 2 M NaCl, and then dialyzed against 50 mM Tris-HCl, pH 8.3, 100 mM NaCl using dialysis tubes with a 3500 Da cutoff. The fusion proteins were cleaved with 1% (w/w) bovine coagulation factor Xa (FXa) (Haematology Technologies Inc., Essex Junction, VT) at 37 °C. The unprocessed proteins and the cleaved histidine tags were then removed from the reaction mixture by a 1 h incubation with Ni^{2+}-NTA-resin on a rotary shaker at RT. After centrifugation at 2000*g*, the FXa processed proteins remaining in the supernatant were subjected to purification by rp-HPLC using a semipreparative wide-pore C_{18} column (10 × 250 mm; Baker Research Products, Phillipsburg, NJ). The protein was eluted from the column at 2 mL/min with a linear gradient of 0−75% acetonitrile in 0.1% TFA (1.2%/min). Protein peaks eluting according to the absorbance at 216 nm were collected, lyophilized, and resuspended in water. Confirmation of correct cleavage was obtained by mass spectroscopy (Perkin-Elmer SCIEX API III ion-spray mass spectrophotometer) and amino acid analysis (*20*).

Biological Characterization. Neutrophil chemotaxis was carried out with freshly isolated neutrophils. Venous blood was diluted 10-fold in ACD (135 mM D-glucose, 75 mM sodium citrate dihydrate, 40 mM citric acid monohydrate), and neutrophils were isolated by centrifugation through neutrophil isolation medium (Cardinal Assoc. Inc., Santa Fe, NM). Isolated cells were resuspended in 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4, and incubated for 10 min on ice in order to lyse contaminating erythrocytes. After 3 washes with GEY's buffer containing 0.25% BSA and 20 mM HEPES, pH. 7.5, neutrophils were counted and stained with DADE-Diff-Quik (Merz & Dade, Duedingen, Switzerland) to ensure a purity of greater than 92%. Chemotaxis was performed in 48-well chemotaxis chambers with 5 μm pore diameter poly(vinylpyrrolidone)-free polycarbonate membranes as previously described (*21*). Briefly, neutrophils and chemokines were diluted in 0.25% bovine serum albumin, GEY's buffer, and 20 mM HEPES, pH 7.5. Per well, 50 000 cells were loaded in a volume of 50 μL and incubated for 30 min in humidified air with 5% CO_2 at 37 °C. Membranes were removed, air-dried, and stained with Diff-Quik. Migrated cells were evaluated microscopically by counting 10 randomly chosen 400× fields. Established methods were used to assess cytosolic free calcium changes (*22*, *23*) and elastase release activity from cytochalasin B-pretreated human neutrophils (*24*). In neutrophil chemotaxis and elastase release assays, human IL-8 (72 amino acid form) has been used as control.

RESULTS

Proteolytic Processing of ENA-78. The formation of proteolytic cleavage products with altered biological activity from full-length ENA-78 was investigated (Figure 1a). Highly purified recombinant ENA-78 was subjected to proteolysis by bovine chymotrypsin, human cathepsin G, and trypsin. At various time points during the incubation,

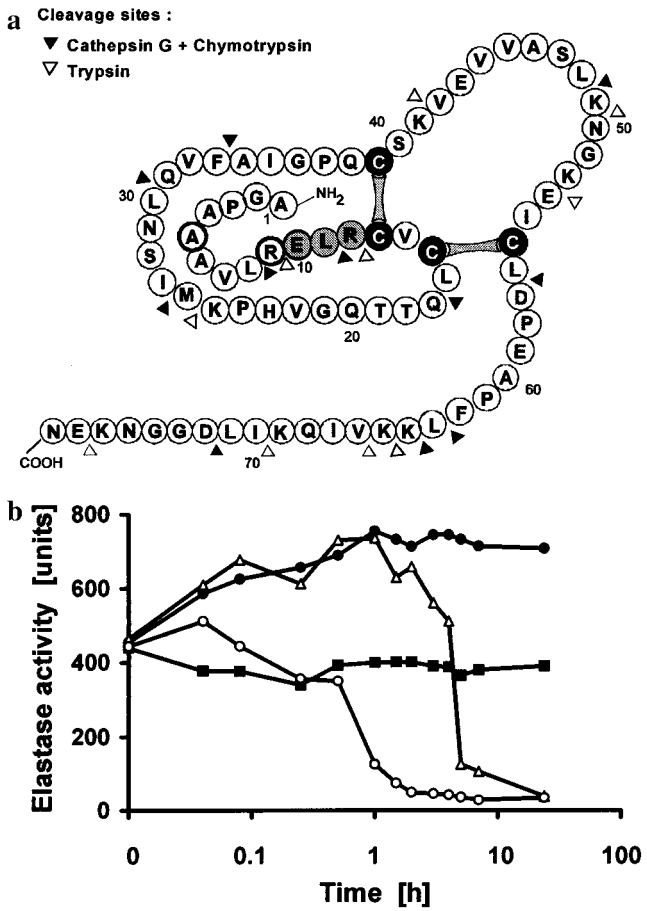

FIGURE 1: (a) Two-dimensional structure of ENA-78 and putative cleavage sites for cathepsin G, chymotrypsin, and trypsin. Conserved cysteine residues are shown in black. Shaded circles indicate the Glu-Leu-Arg (ELR)-motif. Bold circles indicate aminotermini of the cloned variants ENA(5−78), ENA(9−78), and ENA(10−78). (b) Proteolysis of recombinant human ENA-78. Thirty micrograms of ENA-78 was incubated at 37 °C in 0.5 mL of PBS with 1 μg of either bovine chymotrypsin (open triangles), human cathepsin G (closed circles), or trypsin (open circles); untreated control (squares). At the time points indicated, 45 μL samples were taken, and 100 μg of bovine serum albumin was added and immediately quick-frozen. Activity was determined by elastase release from cytochalasin B-treated human neutrophils. A representative of four independent experiments is shown.

neutrophil-stimulating activity was determined by measuring elastase release activity (Figure 1b). Both cathepsin G and chymotrypsin yielded a significant, time-dependent increase in activity. While the increase in degranulation activity observed with cathepsin G remained stable for up to 24 h at 37 °C, chymotrypsin only caused a transient effect, reaching maximal elastase release activity at 1 h incubation time, whereafter a sudden decrease in activity was observed. Trypsin reduced biological activity to zero levels within 1−2 h without a significant formation of intermediates with elevated activity (Figure 1b). The active cleavage product of cathepsin G-treated ENA-78 was isolated by reversed-phase HPLC and analyzed by amino-terminal sequencing. The sequence determined was RELRCVC, predicting the existence of a highly active ENA(9−78) variant (Figure 1a). Similarly, ENA(9−78) was also identified as the major active cleavage product upon chymotrypsin digestion of full-length ENA-78 (data not shown).

Cloning and Expression of ENA-78 and N-Terminal Variants. The coding sequences of ENA-78 and three variants, truncated at the N-terminus by 4, 8, and 9 amino acids, were amplified using PCR technology (Figure 1a, Table 1). To facilitate specific isolation of the expressed proteins by Ni^{2+}-nitrilotriacetic acid (NTA) agarose affinity chromatography, all expression plasmids coding for the ENA-78 isoforms contained an N-terminal polyhistidine sequence followed by a factor Xa sensitive cleavage site (IEGR) for later removal of the His-tag (MRGSHHHHH-HGSIEGR) (Table 1). Expression constructs were under the control of T5 phage promoter and two adjacent *lac* operator sequences that allowed rapid induction of protein expression by the addition of IPTG. Positive clones were identified by SDS−urea−PAGE analysis and Western immunoblots using an anti-ENA-78 antibody.

Purification and Refolding of the Fusion Proteins. One liter batches of recombinant *E. coli* cultures yielded on average 8 g of wet weight bacterial pellet. The fusion proteins were purified from bacterial lysates by nickel affinity chromatography in the presence of 8 M urea and 2.5 mM β-2-mercaptoethanol to prevent protein−protein interactions and the formation of ENA-78 dimers via intermolecular disulfide bonds. Despite these conditions, elution of the nickel column with low-pH buffer (pH 3.5) often yielded a minor fraction containing dimerized ENA-78 molecules (data not shown). Per 8 g of bacterial pellet, about 25 mg of recombinant protein was obtained for ENA-78, and about 12 mg for the truncated variants, except for ENA(9−78) which consistently yielded only about 6 mg of protein. No sequence anomalies have been detected within the promoter region of this construct, suggesting that the lower expression is probably not due to impaired transcription. The purified fusion proteins were diluted in 8 M urea to a concentration

Table 1: Oligonucleotide Probes Used for PCR Amplification and Sequencing[a]

oligonucleotide	sequence (5′→3′)
5′ FXENA-78	AGGGATCC*ATCGAGGGTAGA***GCTGGTCCTGCCGCTGCT**
5′ FXENA(5−78)	AGGGATCC*ATCGAGGGTAGA***GCTGCTGTGTTGAGAGAG**
5′ FXENA(9−78)	AGGGATCC*ATCGAGGGTAGA***AGAGAGCTGCGTTGCGTT**
5′ FXENA(10−78)	AGGGATCC*ATCGAGGGTAGA***GAGCTGCGTTGCGTTTGT**
3′ ENA-78	TTC**AAGCTT**A**TCAGTTTTCCTTGTTTCCACC**
AS/36C	**CCTCTAGAAGCTTATGGCGAACACTTGCAGATTACT**
QE8For	CGGATAACAATTTCACACAG
QE8Rev	GTTCTGAGGTCATTACTGG

[a] The primer sequences written in boldface correspond to base pairs coding for human ENA-78. Sequences written in italic indicate base pairs coding for the IEGR (Ile-Glu-Gly-Arg) cleavage motif recognized by the factor Xa protease. The *Bam*HI restriction site in the 5′-end primers is underlined by a straight line. The boldface underlined sequence corresponds to the *Hin*dIII restriction site in the 3′-end primer. Oligonucleotides QE8For and QE8Rev were used for DNA sequencing.

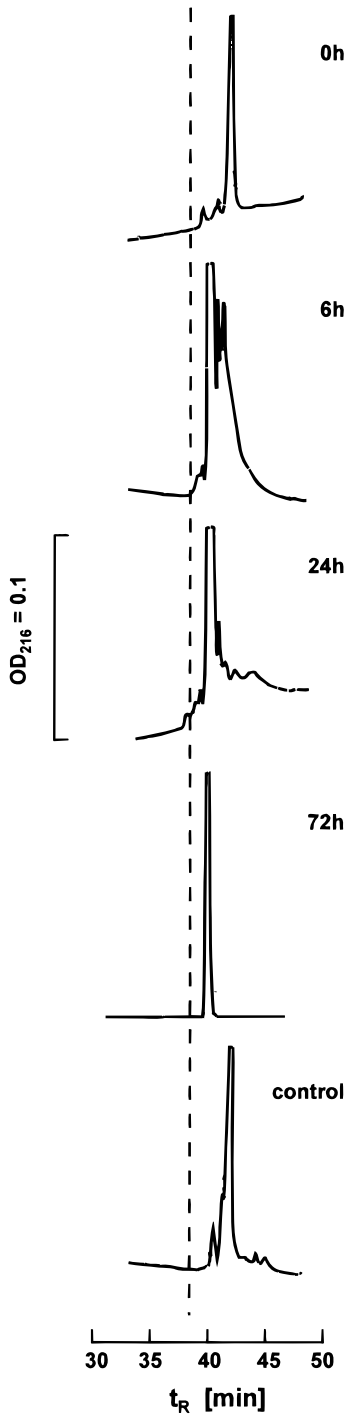

FIGURE 2: Reversed-phase HPLC analysis of protein refolding. Protein samples (4 μg) were taken at the time points indicated from the refolding and oxidation reaction and analyzed on an Aquapore butyl column (7 μm, 2.1 × 100 mm; Applied Biosystems, San Jose, CA). The protein was eluted from the column at 0.3 mL/min with a linear gradient of 0−75% acetonitrile in 0.1% TFA at an increment of 1.25% per minute. The analysis of the fusion protein PHTFx:ENA(5−78) is shown as a representative example. The retention times (t_R) of the reduced and oxidized forms were 42.79 and 40.6 min, respectively. A reduced sample of the 72 h fraction is shown (control). The dashed line is a reference marker.

of 50 μg/mL, and after addition of 0.5 mM DTT, detergent-assisted refolding was initiated by stepwise dilution in a buffer containing a zwitterionic detergent (0.01% CHAPS) and a glutathione redox pair (*25*). Protein refolding was monitored by rp-HPLC (Figure 2). Under the running

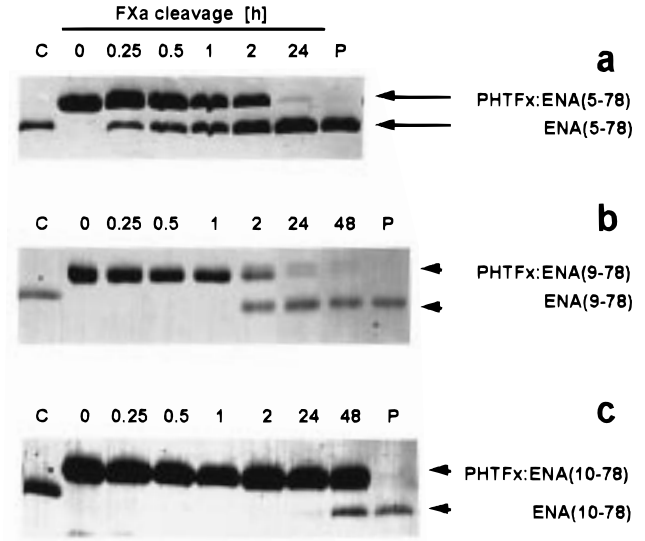

FIGURE 3: Cleavage of the fusion proteins by factor Xa (FXa). Coomassie-stained PAGE of the fusion proteins incubated at 37 °C with 1% (w/w) FXa in 50 mM Tris-HCl, pH 8.3, 100 mM NaCl. Aliquots were taken at the times indicated. Lanes marked with C contained 500 ng of ENA-78; lanes marked with P contained the respective purified proteins after cleavage.

conditions used, the difference of the retention times for the denatured and refolded protein was about 2 min. Renaturation was usually complete after 72 h. After refolding, the proteins were concentrated by ion exchange chromatography on phosphocellulose.

Enzymatic Cleavage and Final Purification. Cleavage with factor Xa was carried out at the optimal pH of 8.3, except for ENA(10−78) where a pH of 8.0 was selected in order to prevent protein precipitation (pI = 8.3). At several time points during the incubation, samples were taken and analyzed by SDS−urea−PAGE and Coomassie staining. Cleavage of the ENA-78 and ENA(5−78) fusion proteins was visible after 15 min incubation at 37 °C, and the reaction was complete after 24 h incubation time (Figure 3a). Under similar conditions, generation of ENA (9−78) was less efficient, but the reaction was complete after 48 h (Figure 3b). The shortest variant, ENA(10−78), was cleaved very inefficiently, and only a partial cleavage was obtained at 48 h (Figure 3c). Proteins ENA(9−78) and ENA(10−78) exhibited a much lower staining intensity with Coomassie than their corresponding His-tagged versions. Silver staining, on the other hand, was not affected. Cleaved proteins were repurified by nickel affinity chromatography to remove residual uncleaved fusion proteins as well as cleaved His-tags, and then further purified by rp-HPLC to obtain a final purity of greater than 95%. Analysis by mass spectroscopy (MS) and gas-phase hydrolysis revealed the correct calculated masses and the quantitative amino acid compositions for full-length ENA-78 and all ENA variants (data not shown).

Elastase Release Activity of the ENA-78 Variants. All of the tested ENA variants induced a concentration-dependent elastase release from human neutrophils between 1 and 100 nM (Figure 4a). ENA(5−78) and ENA(9−78) exhibited a 2−3-fold higher potency (ED_{50}) than ENA-78, while variant ENA(10−78) was 3-fold less potent (Table 2). The overall rank order among the ENA variants for induction of elastase release was ENA(9−78) > ENA(5−78) > ENA-78 > ENA-

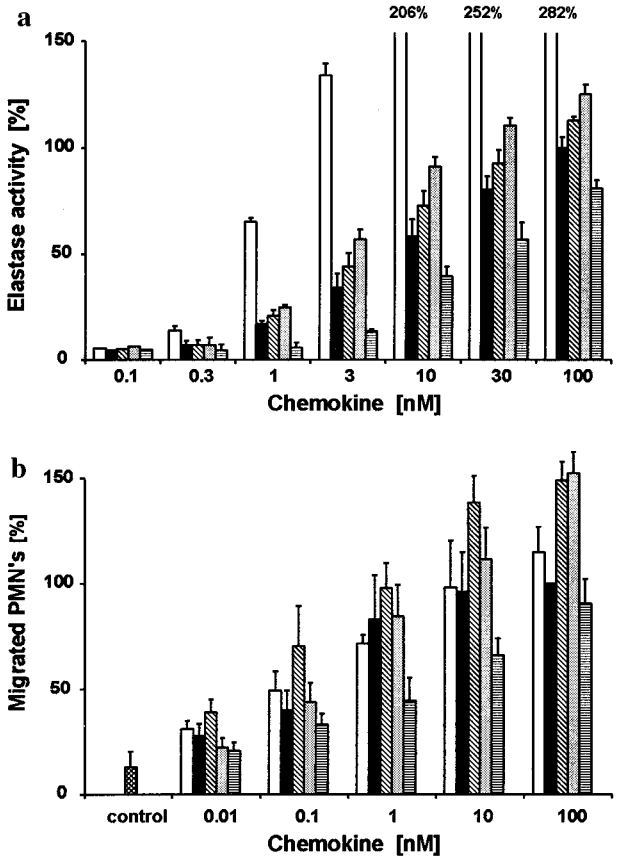

FIGURE 4: Biological characterization of recombinant ENA-78 (black), ENA(5−78) (hatched), ENA(9−78) (dotted), and ENA-(10−78) (horizontal stripes), and IL-8 (white). (a) Elastase release from cytochalasin B-treated human neutrophils. Data were standardized to the activity obtained by 100 nM ENA-78 (100%), and represent the mean ± SEM of four independent experiments in duplicate. (b) Migration of human neutrophils. Chemotaxis was carried out in 48-well microchambers. Data were standardized to the cell count obtained with 100 nM ENA-78 (100%) and represent the mean ± SEM of four independent experiments.

Table 2: Summary of the Relative Potencies of ENA-78 and Variants

	ED_{50} (nM)[a]	
variant	elastase release	chemotaxis
ENA-78	7.6	0.31
ENA(5−78)	4.4	0.04
ENA(9−78)	2.6	0.23
ENA(10−78)	22.3	3.25
IL-8 (1−72)	0.8	0.13

[a] The ED_{50} values of the indicated variants and IL-8 were estimated from data shown in Figure 4 by determining the protein concentration (nM) at 50% of the maximal response obtained with 100 nM ENA-78.

(10−78). IL-8 was significantly more potent than all ENA-78 variants in inducing elastase release (Figure 4a).

Chemotactic Activity of the ENA-78 Variants. Significant chemotactic migration of human neutrophils was elicited by ENA-78 and the variants at concentrations between 0.1 and 100 nM (Figure 4b). Variants ENA(5−78) and ENA(9−78) exhibited an 8- and 2-fold higher potency (ED_{50}) than full-length ENA-78, respectively (Table 2). In contrast, ENA-(10−78) was about 10 times less potent than ENA-78. The rank order of ENA variants in their potency to induce neutrophil chemotaxis was ENA(5−78) > ENA(9−78) > ENA-78 > ENA(10−78). While ENA-78 and IL-8 were equipotent in exhibiting neutrophil chemotaxis, variant ENA-(5−78) had a significantly higher potency and efficacy than IL-8. On the other hand, ENA(9−78) was less potent but more efficient than IL-8 in neutrophil chemotaxis.

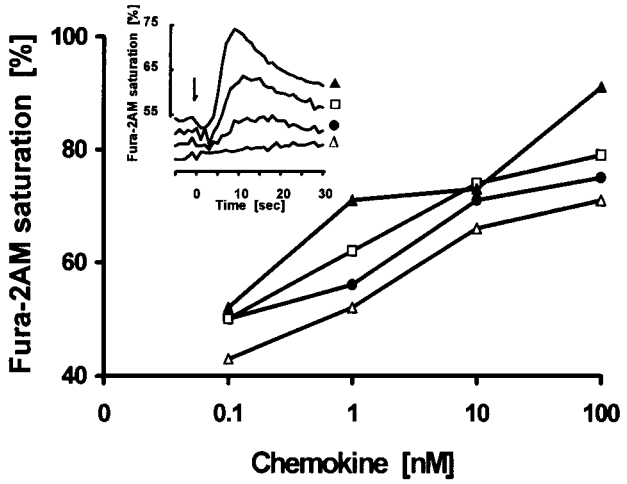

FIGURE 5: Calcium rise in human neutrophils induced by ENA-78 variants at different concentrations. Maximal calcium rise is shown for full-length ENA (closed circles), ENA(5−78) (squares), ENA-(9−78) (closed triangles), and ENA(10−78) (open triangles). Real time measurements of cytosolic calcium changes induced with 1 nM of the variants are shown in the inset. The results are representative of three experiments using different blood donors.

Induction of Cytosolic Free Calcium Changes. A rapid and transient rise in neutrophil cytosolic free $[Ca^{2+}]_i$ was observed after stimulation with all of the ENA variants. As shown in Figure 5, the maximal calcium rise for a given variant was dose-dependent, and the maximal responses were reached at the highest concentrations used (100 nM). ENA-78 variants applied at 1 nM concentration demonstrated a clear difference in the maximal extent and initial rate of free $[Ca^{2+}]_i$ rise (Figure 5, inset). The potency rank order for calcium mobilization was ENA(9−78) > ENA(5−78) > ENA-78 > ENA(10−78).

DISCUSSION

In this study, we have investigated the biological implications of N-terminal truncations on the CXC chemokine ENA-78. As released from pulmonary epithelial cells, ENA-78 has a stretch of 12 amino acids preceding the first cysteine residue. In analogy to the related three-dimensional structure of IL-8 (*26*), we assumed that the N-terminus of ENA-78 is exposed and conformationally disordered, and thus prone to proteolytic truncation. In fact, proteolysis of native ENA-78 with human cathepsin G and bovine chymotrypsin demonstrated an increase in neutrophil-degranulation activity, thus predicting the presence of ENA-78 variants with higher potency than native ENA-78. The predominant active constituent of ENA-78 proteolysis with cathepsin G was identified as ENA(9−78).

To study the effects of N-terminal processing on the potency to stimulate neutrophil responses, such as exocytosis, chemotaxis, and rise of intracellular calcium, full-length ENA-78 and the three truncated isoforms ENA(5−78), ENA-(9−78), and ENA(10−78) were prepared by recombinant technologies (Figure 1a). The variants ENA(5−78) and ENA(9−78) had significantly greater potency than ENA-78

in all assay systems. The shortest variant, ENA(10−78), ending with the essential ELR-motif was about 10-fold less active than variant ENA(9−78) containing an additional N-terminal arginine residue (Table 2). Increasing activity with progressive cleavage was also observed with N-terminal truncations of IL-8 (*2*). However, in contrast to our results with ENA-78, the shortest variants of IL-8, IL-8(3−72) (KELRC...) and IL-8(4−72) (ELRC...), exhibited the highest potency in neutrophil elastase release and chemotaxis.

The most potent variant in inducing elastase release was ENA(9−78). Despite a 3-fold potency increase over full-length ENA-78, ENA(9−78) was still substantially less active in inducing elastase release than IL-8. Interestingly, the potency rank order for neutrophil chemotaxis was different. ENA(5−78) was the most active variant, being about 8-fold more potent than full-length ENA-78, and having an overall 3-fold higher potency than IL-8 (Table 2). Even variant ENA(9−78), when used at concentrations of 100 nM, was significantly more efficient than IL-8 in inducing neutrophil chemotaxis (Figure 4b). In general, N-terminal truncation of ENA-78 had significantly more influence on neutrophil chemotactic activity than on degranulation. These differences may be due to different signaling mechanisms that have overlapping but distinct requirements for receptor binding and triggering. The rank order obtained for the rise of intracellular calcium was the same as obtained for elastase release. This is not surprising due to the fact that calcium mobilization is a necessary intermediate event in the control of exocytosis and respiratory burst (*27*). In contrast, calcium depletion of neutrophils did not effect shape change, a correlate of chemotaxis (*28*).

The increase in potency of N-terminally truncated variants of ENA-78 clearly demonstrates that ENA-78, as originally isolated from the stimulated alveolar type-II epithelial cell line A549, is not the most active form. In analogy, A549 cells release the less active form of IL-8 (77 amino acids) (*1*), the predominant variant also secreted from endothelial cells and fibroblasts (*29*, *30*). Supernatants of blood leukocytes, on the other hand, preferentially contain the more active IL-8(72) form. Conversion of IL-8(77) to IL-8(72) has been observed with thrombin (*31*) and plasmin (*32*). From the neutrophil proteinases, only proteinase-3 was effective to produce the more potent IL-8(70) in vitro (*33*).

In contrast to IL-8, ENA-78 has the unique property of being converted by cathepsin G to a stable and more potent neutrophil chemotaxin. Cathepsin G is a serine proteinase stored in azurophil granules of neutrophils (*34*), but it is also released from stimulated monocytes, and is expressed at the monocyte surface (*35*, *36*). In addition to its role in degrading various structural proteins, cathepsin G might modulate the degree of the inflammatory response in situations such as rheumatoid arthritis, where both cathepsin G and high levels of active ENA-78 are present (*37*, *7*).

The strong modulation of activity by leukocyte proteases, and its local persistence in an active form, thus qualifies ENA-78 as a puissant mediator of inflammation. Its predominant expression by epithelial cells, monocytes or immigrant macrophages, points to a major role for ENA-78 in the pathogenesis of various inflammatory conditions of the airways, the lungs, or the intestinal tract.

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