Isolation and Primary Structure of NGAL, a Novel Protein Associated with Human Neutrophil Gelatinase*

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A 25-kDa protein was found to be associated with purified human neutrophil gelatinase. Polyclonal antibodies raised against gelatinase not only recognized gelatinase but also this 25-kDa protein. Specific antibodies against the 25-kDa protein were obtained by affinity purification of the gelatinase antibodies.

Immunoblotting and immunoprecipitation studies demonstrated the 135-kDa form of gelatinase to be a complex of 92-kDa gelatinase and the 25-kDa protein, and the 220-kDa form was demonstrated to be a homodimer of the 92-kDa protein, thus explaining the 220-, 135-, and 92-kDa forms characteristic of neutrophil gelatinase.

The 25-kDa protein was purified to apparent homogeneity from exocytosed material from phorbol myristate acetate-stimulated neutrophils.

The primary structure of the 25-kDa protein was determined as a 178-residue protein. It was susceptible to treatment with N-glycanase, and one N-glycosylation site was identified. The sequence did not match any known human protein, but showed a high degree of similarity with the deduced sequences of rat α2-microglobulin-related protein and the mouse protein 24p3. It is thus a new member of the lipocalin family.

The function of the 25-kDa protein, named neutrophil gelatinase-associated lipocalin (NGAL), remains to be determined.

A number of normal human cells and tumor cells are known to synthesize and secrete proteins that are members of the metalloproteinase family (for review see Refs. 1 and 2). These enzymes, including interstitial collagenase, 72- and 92-kDa type IV collagenase (gelatinase), stromelysin, and PUMP-1, have been shown to be important participants in the degradation and remodeling of the extracellular matrix (1, 2). The enzymes are all secreted as zymogens, and their collagen-degrading activities are initiated by mechanisms not yet fully known.

1 This work was supported by grants from The Danish Cancer Society, The Danish Medical Research Council, The Lundbeck Fund, Amalie Jørgensen’s Fund, Emil C. Hertz’s Fund, The Novo Fund, Brøchner-Mortensen’s Fund, and Anders Hasselbach’s Fund. The abbreviations used are: TIMP-1 and TIMP-2, tissue inhibitors of metalloproteinases; PMA, phorbol 12-myristate 13-acetate; NGAL, neutrophil gelatinase-associated lipocalin; PBS, phosphate-buffered saline; PEG, polyethylene glycol; Pipes, 1,4-piperazinediethanesulfonic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) P80188.

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resulting supernatant was collected and subjected to ion exchange chromatography on a DE-52 column (Whatman) followed by affinity chromatography on a CNBr-Sepharose 4B column (Pharmacia) to which heat-denatured type I collagen (gelatine) had been coupled.

Production of Polyclonal Antibodies toward Gelatinase and the 25-kDa Protein—Immunization was performed as described (13). The antiserum was affinity-purified (13) on CNBr-activated Sepharose-4B to which 8 mg of purified gelatinase had been coupled. The specificity of the antibodies was tested by immuno blotting of a postnuclear supernatant from disrupted human neutrophils. This showed that the antibody reacted with both gelatinase and with a 25-kDa protein. To remove antibody with specificity for the 25-kDa protein, the following procedure was performed: neutrophils (10 × 10^6) at 1 × 10^9/ml in Krebs Ringer phosphate were stimulated with PMA (2 μg/ml) to exocytose granule proteins. After addition of phenylmethanesulfonyl fluoride (1 mM) (Sigma), the exocytosed material was collected and concentrated approximately 15-fold using an Amicon ultrafiltration cell. In order to separate the 25-kDa protein from gelatinase, the concentrated material was subsequently subjected to gel filtration on a Sephadex G-200 column (Pharmacia) to which heat-denatured type 1 collagen (gelatine) had been coupled. The majority of the 25-kDa protein, was aspirated. K2HPO4 was added to the carboxymethylated protein was desalted and repurified by reverse-phase HPLC on a 2.1 × 150-mm 5-μm C8 column (Vydac), which was eluted by a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile. The instrument used was a 1090 HPLC from Hewlett-Packard.

Carboxymethylation—The purified protein was reduced and carboxymethylated with iodoacetamide as described (16). Thereafter, the carboxymethylated protein was desalted and repurified by reverse-phase HPLC on a 2.1 × 150-mm 5-μm C8 column (Vydac), which was eluted by a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile. The instrument used was a 1090 HPLC from Hewlett-Packard.

Amino Acid Analysis—The carboxymethylated protein was hydrolyzed for 20 h in 6 n HCl gas phase at 110 °C under argon in pyrolyzed tapered microvials (100 μl, Hewlett-Packard). The hydrolysate was dried and redissolved in 20 μl of 0.4 μM sodium borate, pH 10.4. Amino acid analysis was performed on 6 μl using a Hewlett-Packard Aminocouant analyzer (17) (precolumn derivatization with o-phthalaldehyde hydroxanthine followed by 9-fluorenylmethylchloroformate, both reagents supplied by Hewlett-Packard).

Cleavage with CNBr—2 nmol of the carboxymethylated protein

![Diagram](image-url)

**FIG. 1.** Purification of gelatinase and generation of specific polyclonal antibodies against gelatinase and the 25-kDa protein (p25). A, 6 μg of the purified gelatinase was subjected to electrophoresis under nonreducing (lane 1) and reducing conditions (lane 2). B, Western blotting of a postnuclear supernatant with antibodies against p25 and gelatinase.

**REFERENCES**

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Cleavage with CNBr—2 nmol of the carboxymethylated protein
with anti-p25-antibodies; performed in 400 µl pellets by centrifugation. The resulting supernatants were aspirated.

The p25 antibodies against p25 was cleaved with 100-fold excess of CNBr in PBS to the initial volume. Supernatants and pellets were applied to SDS-PAGE under nonreducing or reducing conditions. A total of 5 µg of protein from a postnuclear supernatant was electrophoresed and blotted onto nitrocellulose. Primary antibody was anti-p25 antibody diluted 2000-fold.

0.1% trifluoroacetic acid in acetonitrile.

**Sequence Analysis**—The amino acid sequences of the purified peptides were determined using an automatic protein sequenator (475A, Applied Biosystems) equipped with an on-line HPLC system for detection of the amino acid phenylthiohydantoins. The thiodyantoines were separated on a C18-DB column (5-7843; Supelco). All chemicals and solvents were sequence or HPLC grade and delivered by Applied Biosystems.

**Mass Spectrometry**—An aliquot of each of the isolated peptides was analyzed in a Bio-Ion 20 plasma desorption time-of-flight mass spectrometer (Applied Biosystems). The samples were either applied directly as 2 × 5-µl aliquots to aluminized Mylar foil (coated with nitrocellulose) and evaporated, or dried and redissolved in 10 µl of 0.05% trifluoroacetic acid in 50% methanol before application. The spectra were recorded for 1-6 × 10^{6} primary ions. The method has an accuracy of 0.1%.

**Endoglycosidase Treatment**—Samples of the purified 25-kDa protein (8.25 µg) were dried in a Speed-Vac centrifuge (Savant) and redissolved in 0.5% SDS and 1% β-mercaptoethanol, boiled for 6 min, and diluted 5-fold into either 0.2 M NaHPO4/Na2HPO4, pH 8.6, 10 mM 1,10-phenanthroline, 1.25% Nonidet P-40 (Sigma), or 20 mM Tris-maleate, pH 6.0, 10 mM D-galactonolactone, 1.25% Nonidet P-40 and incubated with either 10 units/ml of N-glycanase (Genzyme), respectively, for 16 h at 37 °C (8). Samples were diluted in SDS-PAGE sample buffer and subjected to SDS-PAGE. A proteolytic fragment was also digested with N-glycanase, as described above but without SDS and mercaptoethanol, and purified by HPLC.

**SDS-PAGE**—SDS-PAGE was performed essentially as described by Laemmli (18), employing 5–20% gradient gels with 3% stacking gels.

**Postnuclear Supernatant**—A postnuclear supernatant of neutrophils was obtained using nitrogen cavitation followed by centrifugation to remove nuclei and unbroken cells as described (19).

**Immunoblotting**—Protein was transferred from SDS-PAGE slabs to 0.2-µm nitrocellulose filters (Bio-Rad) essentially as described by Towbin (20) in a Bio-Rad trans-blot vertical system at 60 V, 210 mA for 4 h. Transfer buffer was either 192 mM glycine, 25 mM Tris, pH 8.3, 20% (v/v) methanol, or 10 mM CAPS, pH 11.0, 10% methanol. Additional binding sites were blocked by incubating the nitrocellulose filters for 1 h in either 2% Tween-20 in PBS, or 3% bovine serum albumin and 10% goat serum (Dakopatts) in PBS. After three washes in PBS, 0.05% Tween, the blots were incubated with primary antibody overnight. Primary antibodies were labeled with peroxidase-conjugated swine anti-rabbit antibody (Dakopatts (P217)) diluted 1:1000.

**FIG. 2. Immunoprecipitation of purified gelatinase with antibodies against p25.** Twenty µg of purified gelatinase in PBS (total volume 170 µl) was incubated for 1 h with either buffer, anti-p25 antibodies (30 µl), or preimmune rabbit IgG in equivalent amounts. 100 µl of protein A-Sepharose 4B (200 mg/ml in PBS) was added and rotated end over end for 1 h. The Sepharose particles were pelleted by centrifugation. The resulting supernatants were aspirated. After washing twice in PBS, the Sepharose pellets were resuspended in PBS to the initial volume. Supernatants and pellets were applied to SDS-PAGE under nonreducing (A) or reducing conditions (B). Lane 1, gelatinase (control); lane 2, gelatinase, supernatant after immunoprecipitation with anti-p25 antibodies; lane 3, gelatinase, supernatant after immunoprecipitation with preimmune rabbit IgG; lane 4, Sepharose pellet from gelatinase after immunoprecipitation with anti-p25 antibodies; lane 5, Sepharose pellet from gelatinase after immunoprecipitation with preimmune rabbit IgG.

was cleaved with 100-fold excess of CNBr in 70% HCOOH as described (16).

**Proteolytic Cleavages**—1–3 nmol of the carboxymethylated protein (see above) was digested with three enzymes. All were sequencing grade from Boehringer Mannheim. Cleavage with 1 µg of trypsin was performed in 400 µl of 100 mM Tris (pH 8.5) for 18 h at 37 °C. Cleavage with 1 µg of endoproteinase Glu-C was performed in 400 µl of 25 mM sodium phosphate, pH 4.0, for 18 h at room temperature. Cleavage with 1 µg of endoproteinase Asp-N was performed in 400 µl of 50 mM sodium phosphate, pH 8.0, for 18 h at 37 °C. Finally, a tryptic fragment was digested with 100 µg of pyroGlu aminopeptidase (Boehringer Mannheim) for 30 min at 37 °C in 200 µl buffer (100 mM sodium phosphate, 10 mM EDTA, 5 mM dithiothreitol, 5% glycerol, pH 7.8). The resulting peptides were isolated on a Hewlett-Packard 1090 HPLC on 2.1 × 150-mm 5-µm C6 and C18 columns from Vydac, by eluting with gradients from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile.

**FIG. 3. Association of p25 with gelatinase and other neutrophil proteins.** A, Western blotting of purified gelatinase with anti-gelatinase- or anti-p25-antibodies. Blotting was performed as described under “Experimental Procedures.” Lane 1, 3 µg of gelatinase probed with anti-gelatinase antibodies depleted of reactivity toward p25 (1/1000); lane 2, 9 µg of gelatinase probed with anti-p25 antibodies (1/8000). B, Western blotting of postnuclear supernatant with antibodies against p25 under nonreducing conditions. A total of 5 µg of protein from a postnuclear supernatant was electrophoresed and blotted onto nitrocellulose. Primary antibody was anti-p25 antibody diluted 2000-fold.

0.1% trifluoroacetic acid in acetonitrile.
and PPI contain the majority of the eluted p25. These peaks were 
themselves precipitated with PEG in a final concentration of 18% (w/v). 
Nonprecipitated protein was applied on a MonoS cation exchange column, 
equilibrated in 50 mM sodium acetate, pH 6.0, and eluted with a gradient 
of NaCl (broken straight line). Elution was monitored by absorbance at 280 nM. 
The peaks, P1 and P2, contain the majority of the eluted p25. These peaks were 
concentrated separately and each subjected to gel filtration on Superose 
12. The elution profile of P1 is shown in the middle panel, P2 in the lower panel. 
The major peaks of both chromatograms contain p25 as shown in Fig. 5.

and incubated for 2 h. The filters were then washed three times in PBS, 0.05% Tween and once in 50 mM Tris, pH 7.6, and developed 
in 50 mM Tris, pH 7.6, containing diaminobenzidine tetrahydrochloride 
chromogen (Dakopatts) 0.20 mg/ml and 0.03% H2O2.

**Assays**—Total protein was measured using a commercial kit from Bio-Rad with catalase as standard (21).

**RESULTS**

Human neutrophil gelatinase was purified as described by Hibbs et al. (12) from material exocytosed from PMA-stimu-

lated neutrophils. SDS-PAGE of the purified preparation is shown in Fig. 1A. In accordance with the findings of Hibbs et al. (12), three bands of 220, 135, and 92 kDa were seen under nonreducing conditions, and one major 92-kDa band appeared under reducing conditions. A faint 25-kDa band, not described by Hibbs et al. (12), was observed in addition to the 92-kDa band, but only under reducing conditions, indicating the existence of a covalent complex between this protein (henceforth termed p25) and gelatinase.

The purified gelatinase was used for immunization of rabbits. This resulted in generation of polyclonal antibodies that possessed reactivity toward both gelatinase and p25, as demonstrated by Western blotting of a postnuclear supernatant of disrupted human neutrophils (Fig. 1B). In order to separate gelatinase and p25 for subsequent use in affinity purification of the antibodies, we performed gel filtration of material exocytosed from neutrophils in response to stimulation with PMA. For this separation to be successful, p25 has to be exocytosed not only in association with gelatinase, but also in a "gelatinase-free" form. This is indeed the case, as shown in Fig. 1C, which demonstrates immunoblotting of two different fractions eluted from the Sephadex G-200 column, probed with the antibodies reacting with both gelatinase and p25. One fraction contained gelatinase, whereas the other fraction contained p25, but was devoid of gelatinase. Fractions containing gelatinase-free p25 were coupled to CNBr-activated Sepharose, and the gelatinase antibodies were applied to this column. Antibodies directed against p25 bound to the column, and were subsequently eluted, whereas the antibodies with specificity toward 92-kDa gelatinase did not bind and were collected in the void. By this procedure, specific antibodies against p25 and gelatinase were obtained as evidenced by Western blotting (Fig. 1D).

A very faint 69-kDa band is noticed in SDS-PAGE of purified reduced gelatinase (Fig. 1A, lane 2) and also in Western Blotting using antibodies obtained after immunization of rabbits with the purified gelatinase (Fig. 1, B and C). The nature of this 69-kDa protein is not known.

The p25 antibody reacted selectively with the 135-kDa form of unreduced gelatinase both by immunoprecipitation (Fig. 2, lanes 2 and 4) and by immunoblotting (Fig. 3A), indicating that the 135-kDa gelatinase is a complex of p25 and 92-kDa gelatinase. No reactivity of the 220-kDa gelatinase with anti-p25 antibodies was observed. The 220-kDa gelatinase is therefore either a homodimer of 92-kDa gelatinase or a complex of two 92-kDa gelatinase and one p25 molecule in which p25 is hidden from the antibodies. This latter possibility is less likely since reduction of the 220-kDa band (after immunoprecipitation of the 135-kDa form) did not reveal any p25 (Fig. 2, lane 2, lower panel). Thus, the association of 92-kDa gelatinase with p25 explains the peculiar 135-kDa form of "pure" gelatinase from human neutrophils.

To investigate the association of p25 with other neutrophil proteins, Western blotting of a postnuclear supernatant was performed under nonreducing conditions, using anti-p25 antibodies. This demonstrated four bands of 135, 70, 46, and 25 kDa, respectively (Fig. 3B). The 25- and 46-kDa bands represent p25 in its monomer and dimeric forms, as shown below. The 135-kDa band could be accounted for as a complex between 92-kDa gelatinase and p25. The nature of the 70-kDa band is unknown, but it is possible that p25 even exists in a trimeric form.

The protein was purified from exocytosed material from PMA-stimulated human neutrophils. After precipitation by 18% PEG, the supernatant (including p25) was subjected to cation exchange chromatography on MonoS. p25 eluted in
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**Fig. 5.** SDS-PAGE protein profiles during purification of p25. Lane 1, supernatant from PMA-stimulated neutrophils (62 μg); lane 2, PEG supernatant (75 μg); lane 3, P1 from MonoS (4.5 μg); lane 4, P2 from MonoS (7.5 μg); lane 5, peak fraction from gel filtration of P1 (4.25 μg); lane 6, peak fraction from gel filtration of P2 (4.25 μg); lane 7, p25 incubated with N-glycanase; lane 8, p25 incubated with O-glycanase. Lane 9, peak fraction from gel filtration of P1 (4.25 μg, nonreducing conditions); lane 10, peak fraction from gel filtration of P2 (4.25 μg, nonreducing conditions). Unless otherwise indicated electrophoresis is run under reducing conditions.

The yield of purified p25, combining the mono- and dimeric forms, was 350 μg from a total of 140 mg of protein present in exocytosed material from approximately 15 × 10⁶ PMA-stimulated neutrophils.

Amino acid sequence analysis of the native p25 failed, presumably due to blockage of the NH₂-terminal amino acid. This was even the case after reduction and carboxymethylation of the protein. The native protein was also resistant to digestion with trypsin (not shown). However, after reduction and carboxymethylation, the protein could be cleaved by trypsin as well as by endoprotease Glu-C, endoprotease Asp-N and CNBr. The resulting peptides were isolated by HPLC. After sequence analysis of these fragments, it was possible to determine the total amino acid sequence of p25 (Fig. 7). The NH₂-terminal sequence of p25 was determined by identification of a tryptic fragment (Fig. 7, T-1) resistant to sequence analysis. Following digestion with pyro-glutamate aminopeptidase, sequence analysis of the resulting peptide was possible. This shows the NH₂-terminal residue to be pyroglutamate. This explains the resistance to sequence analysis of the intact carboxymethylated protein (Fig. 7). The COOH-terminus of p25 as shown in Fig. 7 was deduced from the two fragments (T-21 and E-10) both terminating in a Gly, since neither trypsin nor endoprotease Glu-C are known to cleave at the COOH-terminal side of Gly.

The total amino acid composition of the carboxymethylated p25 was determined by amino acid analysis and was found to be in agreement with the composition deduced from the total...
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The sequence was deduced from fragments obtained by cleavage of the reduced and carboxymethylated protein by trypsin (T-1 to T-21), endoprotease Glu-C (E-2 to E-10), endoprotease Asp-N (D-3' to D-7'), and CNBr (C-2 to C-5). The fragments are numbered starting from the NH₂ terminus according to all hypothetical cleavage points. Note that CNBr induced cleavage both after M and W (occasionally leaving out a potential cleavage point). Fragments including a hypothetical cleavage point are named by the first included fragment and marked by a hyphen (0-3', C-3', T-10', and T-17'). Horizontal lines indicate residues identified by sequence analysis. Vertical lines indicate the NH₂ terminus of a fragment as determined by sequence analysis and the COOH terminus according to the measured Mₐ (Table II), respectively. * denotes glycosylation site as determined from 0-7 before and after deglycosylation with N-glycanase (0-T). Every 10 amino acids in the sequence are grouped in order to improve the clarity of the figure.

**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of residues</th>
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<tr>
<td>Asx</td>
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<tr>
<td>Glx*</td>
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<tr>
<td>Ser</td>
<td>10.7 (12)</td>
</tr>
<tr>
<td>His</td>
<td>2.5 (2)</td>
</tr>
<tr>
<td>Gly</td>
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<tr>
<td>Thr</td>
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<tr>
<td>Ala</td>
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<td>Arg</td>
<td>7.2 (7)</td>
</tr>
<tr>
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<td>9.1 (10)</td>
</tr>
<tr>
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<td>12.0 (13)</td>
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<td>Phe</td>
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<tr>
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<td>15.3 (15)</td>
</tr>
<tr>
<td>Lys</td>
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</tr>
<tr>
<td>Pro</td>
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<tr>
<td>Trp*</td>
<td>(2)</td>
</tr>
<tr>
<td>Cys*</td>
<td>(3)</td>
</tr>
</tbody>
</table>

* CM-Cys elutes as two peaks, one which coelutes with Glx causing slight overestimation.
* Not determined, due to destruction, but the presence confirmed from second derivative of the UV spectrum.
* Not determined due to great variability but the presence confirmed by specific peak in the chromatogram.

amino acid sequence of the protein (Table I). Furthermore, the molecular mass of most peptides depicted in Fig. 7 was measured by mass spectrometry. These values were in agreement with those calculated from the sequence (Table II). The protein was subjected to treatment with N- and O-glycanase. O-Glycanase was without effect, whereas N-glycanase resulted in a partial reduction in the molecular mass to approximately 21 kDa (Fig. 5, lanes 7 and 8), indicating posttranslational addition of N-linked sugar residues. The molecular mass of the deglycosylated protein is in agreement with the molecular mass calculated from the 178 amino acid residues of the protein backbone (Mₐ = 20,542). Sequence analysis of all fragments containing residue 65 resulted in blank cycles for this particular residue. This indicated glycosylation of residue 65 in accordance with the consensus sequence for N-glycosylation, Asn-Xaa-Ser/Thr-. To verify this, an aliquot of the fragment D-7 (residues 61–76, Fig. 7) was digested with N-glycanase. The resulting peptide was less hydrophilic than the original peptide as indicated by the following RP-HPLC purification. Subsequent sequence analysis showed residue 65 to be an Asp, confirming this residue to be a glycosylated Asn in the native protein.

**DISCUSSION**

We report here the purification and identification of p25, a 25-kDa human neutrophil protein, that is in part associated with gelatinase from human neutrophils. In search for proteins with similarity to the amino acid sequence of p25 (Fig.
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7) 74,551 protein sequences included in the MIPSX data base at EMBL (release October 1992) were screened. The deduced sequences of the rat \( \alpha_2 \)-microglobulin-related protein (22) and the mouse 24p3 protein (23), which are 80% identical, showed the highest degree of similarity with p25 (63.5 and 62% identity, respectively), indicating that p25 is a human analogue of these proteins. The function of these rodent proteins is not known. Fig. 8 shows alignment of p25 with the deduced rat \( \alpha_2 \)-microglobulin-related protein precursor. The first 20 residues of the hypothetical mouse protein 24p3 (23) fulfill the criteria for a consensus sequence of a signal peptide (24). This indicates that residue 21 of the precursor is the first residue in the mature protein. In contrast, the deduced sequence of the rat protein contains an Arg in position -3 from the potential signal peptide cleavage site. This violates the rule which predicts the residue in this position to be nonpolar (24), indicating a sequencing error.

The rat \( \alpha_2 \)-microglobulin-related protein and the mouse 24p3 protein both belong to a functionally heterogeneous family of proteins named lipocalins (25, 26). Human proteins belonging to the lipocalins include brain prostaglandin D synthase (32.7% identity with p25), \( \alpha_2 \)-microglobulin (24.6% identity with p25), retinol-binding protein, and apolipoprotein D (27–30). The latter two demonstrated only a low degree of similarity with p25. Despite a low degree of similarity regarding overall amino acid sequence between the different members of this family, certain motifs within the structure of the proteins are conserved (25, 29, 31). These motifs are also present in p25, indicating that p25 is a novel lipocalin member. The motifs are situated very close together in the proteins and are responsible for their common tertiary structure, with one \( \alpha \)-helix and a \( \beta \)-barrel surrounding a hydrophobic core, in which the ability to bind small lipophilic ligands resides (28, 29, 31). For many lipocalins the lipophilic ligand is retinol, but for other members including p25, the lipophilic ligand is unknown.

In addition to similarity regarding the primary structure, p25 shows several traits that are characteristic of the lipocalin family of proteins. First, their protein backbones are all of a size around 20 kDa (32). Second, their compact structure makes them resistant to proteases (26). p25 was hardly degraded by trypsin, unless the protein was reduced and carboxymethylated. Furthermore, their common \( \alpha \)-helix is believed to be responsible for their tendency for self-association (28), as also observed with p25. The ability to associate with other proteins has been described for other lipocalins, namely \( \alpha_2 \)-microglobulin (28), which is covalently bound to IgA, and apolipoprotein D which is bound to lecithin:cholesterol acyltransferase (30). In agreement with this, we have demonstrated that p25 is covalently bound to gelatinase. This 25-kDa protein copurifies with neutrophil gelatinase, forming a covalent complex with a molecular mass of 135 kDa. This association explains the unusual molecular mass forms of "pure" unmodified neutrophil gelatinase. We therefore suggest the protein be named neutrophil gelatinase-associated lipocalin (NGAL).

The existence of a homodimer of 92-kDa gelatinase and a complex between 92-kDa gelatinase and an unidentified 25-kDa protein resulting in the 135-kDa form of neutrophil gelatinase has recently been described by others (33, 34). One would have expected this 25-kDa protein to be TIMP-1 or an

\[ \alpha_2 \] MGLGVLCLALVLLGVLRQQADSTQTNLIPAHELLSVLPOGFWTERFGKRQDSTSDLIPAPELLSKVPLQNFQDNQFGKR

\[ p25 \]

\[ \begin{array}{l}
\text{Fragment} \\
T-1 A \\
\text{Residues} \\
1-15 \\
\text{M} \\
1553.1 (1553.4) \\
\end{array} \]

\[ \begin{array}{l}
\text{Table II} \\
\text{Molecular masses of fragments used for identification of p25} \\
\text{The fragments are identified in Fig. 7. The measured } M, \text{values are compared to those calculated from the sequences, given in brackets. No } M, \text{ was obtained for the glycosylated fragments E-4, D-7, and C-3' nor for the deglycosylated D-7* of which too little was left. Furthermore, D-3' evaded mass determination.} \\
\end{array} \]

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\text{Fragment} \\
T-1 A \\
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1-15 \\
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1553.1 (1553.4) \\
\end{array} \]
analogous protein, since association of gelatinase with TIMP-1 has been reported in other cell types like SV-40-transformed fibroblast, HT 1080 fibrosarcoma cells, U937 myelomonocytic cells, and human monocytes (8–10). Neutrophil gelatinase is thus unique in being TIMP-free and in part covalently associated with this 25-kDa protein, which is not a homologue of TIMP-1. As proposed by others (8), the fact that neutrophil gelatinase is TIMP-free may explain its high specific activity compared to gelatinases from other sources. This could reflect the need for a specialized function of neutrophil gelatinase, in providing the environment necessary for diapedesis of the cell.

Only a part of neutrophil gelatinase is covalently complexed to NGAL. Also, we find that NGAL can be purified in a monodermal form not complexed with gelatinase. The partial association of NGAL with neutrophil gelatinase suggests that NGAL may exert modulatory actions in providing the environment necessary for diapedesis of the cell. Neutrophil gelatinase remains to be determined.

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Note Added in Proof—While this paper was under editorial evaluation, a report appeared that confirmed the existence of a complex of neutrophil gelatinase with a lipocalin (Triebel, S., Blaser, J., Reinke, H., and Tschesche, H. (1992) FEBS Lett. 314, 386–388).

REFERENCES

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