Glycosylation of Natural Human Neutrophil Gelatinase B and Neutrophil Gelatinase B-Associated Lipocalin†

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ABSTRACT: Gelatinase B is a matrix metalloproteinase (MMP-9) involved in tissue remodeling, development, cancer, and inflammation. Neutrophils produce three major forms of (pro)gelatinase B: 92 kDa monomers, homodimers, and complexes of gelatinase B covalently bound to neutrophil gelatinase B-associated lipocalin (NGAL). In contrast to the case for other proteinases, little information about the glycosylation of any natural human MMP is available. Here, both gelatinase B and NGAL were purified from human peripheral blood neutrophils, and the entire contents of the released *N*- and *O*-glycan pools were analyzed simultaneously using recently developed high-performance liquid chromatography-based technology. The results are discussed within the context of the domain structure of gelatinase B and a molecular model of NGAL based on data from this study and the three-dimensional nuclear magnetic resonance (NMR) structure of the protein. More than 95% of the N-linked glycans attached to both gelatinase B and NGAL were partially sialylated, core-fucosylated biantennary structures with and without outer arm fucose. The O-linked glycans, which were estimated to comprise approximately 85% of the total sugars on gelatinase B, mainly consisted of type 2 cores with Galβ1,4GlcNAc (lactosamine) extensions, with or without sialic acid or outer arm fucose. This paper also contains the first report of O-linked glycans attached to NGAL. Although both proteins were isolated from neutrophils and contained O-linked glycans mainly with type 2 cores, the glycans attached to individual serine/threonine residue(s) in NGAL were significantly smaller than those on gelatinase B. In contrast to NGAL, gelatinase B contains a region rich in Ser, Thr, and Pro typical of O-glycosylated mucin-like domains.

Remodeling of the extracellular matrix (ECM)[1] is achieved by both de novo synthesis and enzymatic modifications of ECM components. The matrix metalloproteinases (MMPs) (collagenases, stromelysins, matrilysins, gelatinases, and membrane-type metalloproteinases) are a family of enzymes which control this process (*1*, *2*). Among these, gelatinase B (MMP-9, EC 3.4.24.35) is the most complex in terms of protein domain structure. The regulation of expression and activity of gelatinase B is also more complex than those of most other MMPs. For instance, in contrast to gelatinase A, gelatinase B is not produced constitutively by most cells, but its activity is induced by different stimuli depending on the cell type, thus providing a means of raising the local concentration of gelatinolytic activity in response to specific

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[1] Abbreviations: 2-AB, 2-aminobenzamide; ABS, *Arthrobacter ureafaciens* sialidase; AMF, almond meal fucosidase; A(*n*), number (*n*) of antennae linked to the trimannosyl core; BKF, bovine kidney α-fucosidase; BTG, bovine testis galactosidase; CLF, *Charonia lampas* fucosidase; CV, collagen type V domain; DHB, dihydroxybenzoic acid; EβGal, *Bacteriodes fragillis* endo-β-galactosidase; ECM, extracellular matrix; ESMS, electrospray mass spectrometry; F and Fuc, fucose; FN, fibronectin; G and Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; gu, glucose unit; HPLC, high-performance liquid chromatography; LPS, lipopolysaccharide; M and Man, mannose; MALDI, matrix-assisted laser desorption/ionization; MMP, matrix metalloproteinase; MS, mass spectrometry; N, neutral (glycan) or HexNAc when used in a formula; NA, sialylated N-linked (glycan); NDV, Newcastle disease virus sialidase; NeuNAc, *N*-acetylneuraminic acid; NGAL, neutrophil gelatinase B-associated lipocalin; NMR, nuclear magnetic resonance; NP, normal phase; OA, sialylated O-linked (glycan); OGS, Oxford GlycoSciences; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide *N*-glycanase F; PSD, post source decay; QTOF, quadrupole time-of-flight; S, sialic acid; SDS, sodium dodecyl sulfate; SPG, *Streptococcus pneumoniae* β-galactosidase; SPH, *Streptococcus pneumoniae* β-hexosaminidase; TFA, trifluoroacetic acid; TIMP, tissue inhibitor of metalloproteinase; TOF, time-of-flight; t-PA, tissue plasminogen activator; u-PA, urokinase; WAX, weak anion exchange; Zn, zinc binding domain.

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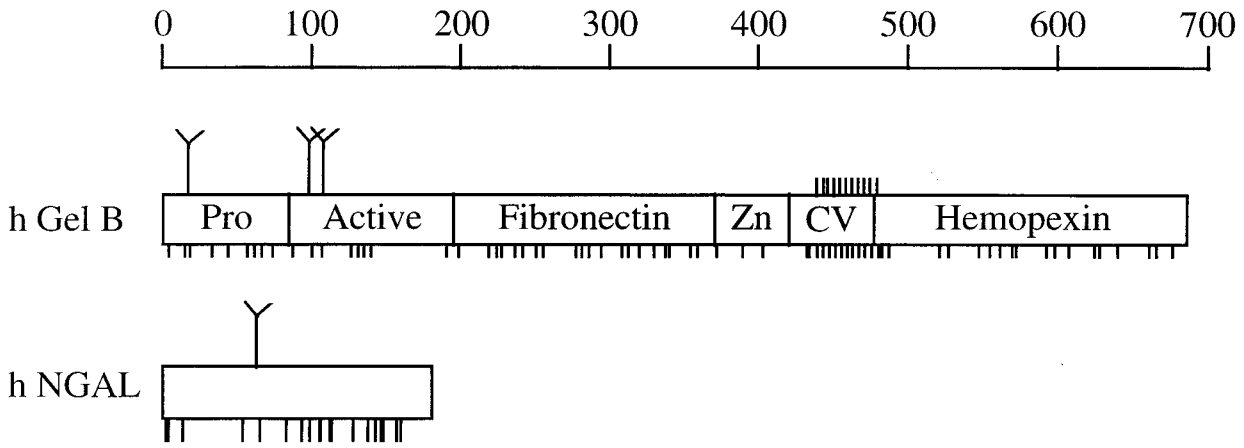


FIGURE 1: Domain structure and glycosylation sites. h Gel B represents human gelatinase B, and h NGAL represents human NGAL. The scale at the top shows the number of amino acids in the proteins. Gelatinase B consists of seven domains, some of which are homologous with previously characterized domains in other proteins such as FN (fibronectin), Zn (zinc binding domain), CV (collagen type V domain), and hemopexin. N-Linked glycosylation sites are denoted by the symbol Y, and serine and threonine residues which are potential sites for O-glycosylation are denoted by the symbol I both above and below the protein domain.

physiological events. Gelatinase B has been implicated in cytokine-mediated inflammatory reactions and in autoimmune processes. Depending on post-translational modifications in different cell types, various molecular forms have been identified. (i) Neutrophils stimulated with IL-8 secrete a 91 kDa gelatinase (calculated molecular mass from the amino acid sequence is 75 kDa) which is found in high concentrations in synovial fluid of rheumatoid arthritis patients (*3*, *4*). In addition, neutrophils produce covalently linked heterodimers of MMP-9 and neutrophil gelatinase B-associated lipocalin (NGAL) (130 kDa) and homodimers of MMP-9 [~200 kDa (*5*)]. (ii) Human peripheral blood monocytes stimulated with IL-1 and monocytic cell lines secrete 85 and 96 kDa gelatinase B forms, respectively, that may be noncovalently complexed with the tissue inhibitor of metalloproteases (TIMP-1) (*6*−*9*). (iii) The leukocytic 91 and 85 kDa forms have been characterized as amino-terminally truncated forms in comparison with those from human tumor cells. In all these cases, gelatinase B was identified by protein sequencing analysis.

In addition to degrading ECM substrates directly, the serine proteases, tissue plasminogen activator (t-PA), urokinase (u-PA), and plasmin, together with matrix metalloproteinases, form a protease cascade in which a series of proenzymes are cleaved to form active enzymes (reviewed in ref *10*). Each enzyme activates the next proenzyme in the cascade until the final step, during which the proenzyme of gelatinase B is cleaved by stromelysin-1 to the active form. Although structural and functional studies of the oligosaccharides of serine proteases have been performed (*11*, *12*) and presumably all MMPs are glycoproteins, only one study about any natural human MMP has been published so far (*13*). In addition to its normal role in tissue remodeling, gelatinase B is also involved in tissue repair and wound healing. Together with elastase (*14*) and collagenase (*15*), which are also located in granules, gelatinase B, located in a specific third subcellular set of granules (*16*), solubilizes the ECM. In this way, leukocytes are assisted in entering the circulation from the bone marrow and in diapedesis, processes which enable the white blood cells to reach the inflammatory focus (*17*).

Gelatinase B is a marker of inflammatory diseases, including rheumatoid arthritis (*4*) and multiple sclerosis (*18*). In the central nervous system, gelatinase B may contribute to the enzymatic degradation of the blood−brain barrier and generate autoimmune peptides from intact myelin constituents (*19*, *20*), suggesting that it may be the basis of autoimmunity in multiple sclerosis (*21*). It has also been suggested (*22*) that secretion of gelatinase B may be an important feature of tumor cell metastasis by providing the basis for a mechanism for invasion (*23*). Therefore, it is relevant to determine whether the glycosylation of the gelatinase B molecule is different in these physiological and pathological functions.

Gelatinase B is the product of a single-copy gene on human chromosome 20 q12-q13 (*24*). The heterogeneity of the gene product is attributed in part to proteolytic processing and to differences in glycosylation. The murine gelatinase B gene and protein structurally resemble the human one, although the contribution of glycosylation to its total molecular mass (110 kDa) may be larger (*25*, *26*). Neutrophils do not produce gelatinase A, but release preformed gelatinase B by degranulation after activation. This degranulation is part of the aspecific immune response to infections. Chemotactic factors, including IL-8, from infected tissues attract leukocytes and stimulate them to degranulate and release the inactive precursors to generate active gelatinase B (*3*).

Neutrophil gelatinase B is a soluble glycoprotein, and is also found in a covalent complex with neutrophil gelatinase B-associated lipocalin (NGAL), a 25 kDa protein with which it forms a complex via Cys^{87} on NGAL (*27*). NGAL is a member of a family of lipocalins which include β-lactoglobulin and α-1 acid glycoprotein. The site via which gelatinase B interacts with NGAL is presumably in the hemopexin domain. NGAL is also present in neutrophils in a monomeric and dimeric form, and it contains a single N-glycosylation site at Asn^{65}-Val-Thr (Figure 1). NGAL was shown not to have an effect on the enzyme activity of gelatinase B (*28*, *29*). Neutrophils have granules containing only NGAL, others which contain both NGAL and NGAL−gelatinase B, and a third group whose members contain only gelatinase B (gelatinase B granules). This last subset of granules, which

contains higher concentrations of gelatinase B, is more rapidly mobilized than the others since it requires only low levels of intracellular Ca^{2+}. These granules may therefore have a function different from the others (*16*).

Gelatinase B is a multidomain enzyme (Figure 1) for which there are, as yet, no NMR or X-ray crystallography data, although the crystal structure of the recombinant catalytic domains of other MMPs has been resolved (*30*, *31*). Likewise, no structural data about the carbohydrates linked to human gelatinase B exist. There are three possible N-linked glycosylation sites in human gelatinase B, all of which are located in the amino-terminal part of the molecule at sequons Asn^{19}-Leu-Thr, Asn^{101}-Ile-Thr, and Asn^{108}-Tyr-Ser [numbering based on the amino-terminal residue in the fibrosarcoma and myelomonocytic (THP-1) cell-derived enzyme and on the human cDNA-derived protein sequence (*8*, *9*)]. The gelatinases B, produced by stimulated human peripheral blood monocytes and neutrophils, are amino-terminally truncated and have Leu^9 or Leu^{11} as the first residues (*3*, *4*, *6*) and, thus, in the pro form contain all three N-linked sequons. The active form of the enzyme contains only two N-glycosylation sites since 78 amino-terminal amino acids, which include Asn^{19}, are cleaved when the proenzyme is converted to the active form by stromelysin-1 or gelatinase A (*32*). The amino acid sequence of human and mouse gelatinase B, as deduced from molecular cloning experiments (*8*, *26*), indicates that the molecule contains many Ser and Thr residues in the vicinity of proline. In particular, in both murine and human gelatinase B there is a section of the primary sequence that is rich in Pro, Ser, and Thr residues (between residues 452 and 503 in human gelatinase B, Figure 1). Such a section of amino acids is characteristic of a peptide region which may contain a cluster of O-linked glycans, such as the hinge region of IgA1 (*33*). Interestingly, this region, which is homologous with a portion of the helical region of α2(V) collagen (*8*), is not present in other members of the secreted matrix metalloprotease family, including gelatinase A.

Recently, the N-linked and O-linked sugars of recombinant murine gelatinase B, expressed in the yeast *Pichia pastoris*, were analyzed. In accordance with this expression system, these consisted of oligomannose structures (*34*). Here we report the major structural features of the N- and O-linked carbohydrates of normal human neutrophil gelatinase B and the covalently bound lipocalin NGAL.

MATERIALS AND METHODS

NGAL Purification. Human neutrophils were isolated from freshly prepared buffy coats (*35*). After disruption of the neutrophils with nitrogen cavitation (*36*) the postnuclear supernatants were centrifuged at 37000*g* for 30 min on a three-layer Percoll (Pharmacia, Uppsala, Sweden) gradient (*5*). The specific granules were harvested, and Percoll was removed by ultracentrifugation at 180000*g* for 90 min. The granules were lysed in 1% octyl glucoside, and NGAL was purified on a monoclonal anti-NGAL column (*37*)

Preparation and Analysis of Gelatinase B from Human Neutrophils. Human neutrophils were isolated from the buffy coats of blood donations (Red Cross, Antwerp, Belgium) as described previously (*3*). After stimulation for 24 h with lipopolysaccharide (LPS) from *Escherichia coli* (0111.B4, Difco Laboratories, Detroit, MI) (2 μg/mL) at 37 °C in serum-free Dulbecco's minimal essential medium, the cell supernatants were harvested, filtered, and purified by affinity chromatography on gelatin−Sepharose (*3*). The material was checked for purity by amino-terminal sequence analysis on an automated pulsed liquid sequencer (Applied Biosystems, model 477A). The phenylthiohydantoins were identified by high-performance liquid chromatography (HPLC) analysis on a PTH C18 5 μm column (Applied Biosystems PTH analyzer model 120A).

Deglycosylation Experiments and Zymography. To determine the degree of glycosylation of the neutrophil gelatinase B, enzymatic deglycosylation was used and the reaction products were analyzed by gelatin zymography (*38*). Aliquots of gelatinase B were deglycosylated with sialidase (5 milliunits) and *O*-glycosidase (endo-*O*-acetylgalactosaminidase) (1 milliunits) or PNGase F (20 milliunits) (Oxford GlycoSciences Ltd., Abingdon, U.K.) for various time intervals at 30 or 37 °C. Treated samples and appropriate controls were analyzed by gelatin substrate zymography to detect alterations in the molecular mass by enzymatic deglycosylation. This technique provides information about the molecular size and the catalytic activity of the enzyme and its variant forms. Experiments can be performed with both crude and purified samples (in this study, only data with purified gelatinase B are shown). Quantification of the gelatinase B activity was achieved by scanning densitometry.

In-Gel Enzymatic Release of N-Linked Glycans. The gelatinase B preparation was subjected to reducing SDS−PAGE, resulting in the dissociation of the dimers and heteromers into monomers; the variants in the gel slices were reduced and alkylated, and N-linked glycans were released by incubation with PNGase F in the presence of *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18) as described in ref *39*. The glycans were extracted with water and acetonitrile, purified by passage through a microcolumn containing (from bottom to top) Dowex AG3X 4A (OH^-), AG50X 16 (H^+), and C-18, and the solvent was removed. They were reconstituted in 2 μL of water for analysis by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).

In-Gel Digestion of Residual Proteins with Trypsin. After removal of the glycans, the remaining proteins were incubated with trypsin as described previously (*39*). Peptides were extracted with three changes of 200 μL of 50% acetonitrile in water with sonication for 30 min. The extracted peptides were dried, redissolved in water, and passed through a column containing about 30 μL of C-18 resin. They were eluted with 100 μL of 50% aqueous acetonitrile and dried.

Release, Re-N-Acetylation, and Labeling of N- and O-Labeled Glycans. Approximately 50 μg of each protein was dialyzed against 0.1% TFA and lyophilized. N-Linked glycans were released by hydrazine at 85 °C and re-N-acetylated using a GlycoPrep 1000 instrument (Oxford GlycoSciences Ltd.). These hydrazinolysis conditions represent a compromise between achieving nonselective release, maximization of the yield, and minimization of degradation of released sugars (*40*). The recovery procedures may result in the loss of some sialic acid residues. O-Linked glycans were released from neutrophil gelatinase B (60 μg) and NGAL (40 μg) via manual hydrazinolysis as described previously (*33*). Glycans were recovered from the origin following paper chromatography by elution with water.

Fluorescent Labeling of the Reducing Terminus with 2-Aminobenzamide (2-AB). The free glycan solutions were evaporated to dryness using a vacuum centrifuge. 2-AB labeling was carried out by reductive amination using the Oxford GlycoSciences signal labeling kit (*41*) and excess label removed by ascending paper chromatography.

Simultaneous Exoglycosidase Sequencing of the Released Glycan Pool. Glycan solutions were evaporated to dryness in a vacuum centrifuge. Ten microliter of standardized enzyme solutions was added, and the mixtures were incubated for 16−24 h at 37 °C in 100 mM citrate/phosphate buffer (pH 5) containing 0.2 mM zinc acetate and 0.15 M sodium chloride. Conditions for the individual enzymes in the arrays were as follows: *A. ureafaciens* sialidase (EC 3.2.1.18, ABS), 1−2 units/mL; almond meal α-fucosidase (EC 3.2.1.111, AMF), 3 milliunits/mL; *Charonia lampas* α-fucosidase (EC 3.2.1.51, CLF) (Oxford Glycobiology Institute), 10 units/mL in 1 mg/mL BSA; bovine testes β-galactosidase (EC 3.2.1.23, BTG), 1−2 units/mL; *Streptococcus pneumoniae* β-hexosaminidase (EC 3.2.1.30, SPH), 2 units/mL; Newcastle disease virus neuraminidase (Hitchner B1 strain) (EC 3.2.1.18, NDV), 0.2 unit/mL in 50 mM sodium acetate; and a substrate concentration of 20 μM. Samples were purified from protein and salts prior to MALDI-TOF MS by passing them through mixed bed resins of Chelex100 (Na^+)/Dowex AG50X 12 (H^+)/Dowex Ag3X 4A (OH^-)/QAE Sephadex A-25. Samples were purified from the exoglycosidases before HPLC analysis by passing them through a microcentrifuge tube inset with a protein binding filter (Microspin 45 μm CN, Pro-Mem, R. B. Radley and Co Ltd., Shire Hill, Saffron Walden, Essex, U.K.). The filter was washed with 15 μL of 5% acetonitrile.

Separation of Glycans by Charge and Normal Phase High-Performance Liquid Chromatography (HPLC) Separations of Neutral and Acidic Oligosaccharides. Weak anion exchange (WAX) chromatography was carried out using a GlycoSep-C column [Oxford GlycoSciences Ltd. (OGS)] according to the method described in ref *42*. Normal phase (NP) HPLC separations were performed on a Glycosep-N chromatography column (OGS) using the low-salt conditions and structures assigned as described in ref *43*.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Positive ion MALDI-TOF mass spectra were recorded with a PerSeptive Biosystems Voyager Elite Biospectrometry Workstation operated in reflectron mode with delayed extraction. The extraction voltage was 20 kV, the grid voltage 65%, and the delay 75 ns. Two hundred fifty-six shots of the nitrogen laser (337 nm) were fired to acquire each spectrum. Samples were prepared by mixing 0.5 μL of the glycan solution on the stainless steel MALDI target, together with 0.5 μL of the matrix solution [a saturated solution of 2,5-dihydroxybenzoic acid (2,5-DHB) in acetonitrile] and allowing it to dry at room temperature. Peptides were examined on the same instrument using α-cyano-4-hydroxycinnamic acid [1 μL from a solution of 10 mg/mL in 7/3 (v/v) acetonitrile/0.1% TFA] as the matrix. The resulting molecular masses were used to search the sequence database maintained at the European Molecular Biology Laboratory (Heidelberg, Germany).

Quadropole Time-of-Flight Mass Spectrometry (QTOF). The fragmentation data relating to the analysis of the O-linked glycans were recorded on a QTOF mass spectrometer at Micromass Ltd. (Floats Road, Wythenshaw, Manchester, U.K.).

Post-Source Decay (PSD) MALDI Spectra. PSD MALDI spectra were acquired with the instrument described above for selected ions. The delay was switched to 0 ns to maximize the yield of PSD ions, and the spectra were acquired by reflectron-ratio stepping.

Tritium Labeling of the Released Glycan Pool. A fraction of the automated hydrazine-released glycans was labeled at the reducing terminal with tritium according to a method described previously (*44*).

RESULTS

Purification of NGAL and Neutrophil Gelatinase B. NGAL was purified by monoclonal antibody affinity chromatography, and the eluate consisted of a mixture of monomers (25 kDa) and dimers (46 kDa). The purified material reacted with both polyclonal and monoclonal anti-NGAL antibodies and was more than 95% pure as estimated by SDS−PAGE.

Gelatinase B was secreted by human peripheral blood neutrophils and purified by affinity chromatography on gelatin−Sepharose. From the analysis after electrophoresis in SDS−PAGE gels, the sample was estimated to contain approximately 50% monomeric gelatinase B, 35% dimeric gelatinase B, and 15% gelatinase B−NGAL heterodimer. A fraction of the protein was further purified by SDS−PAGE (*3*). Amino-terminal sequence analysis (after SDS−PAGE) yielded the sequences Leu-Val-Leu-Phe-Pro-Gly-Asp... and Leu-Phe-Pro-Gly-Asp-Leu-Arg..., consistent with the structure of full-length gelatinase B and gelatinase B truncated by two amino acid residues. The purified material was able to cleave gelatin, did not contain gelatinase A or the tissue inhibitor of metalloproteases, and, using zymography, was shown to migrate at a relative molecular mass of 90−95 kDa (Figure 2a). A homodimer of gelatinase B was also visualized by zymography at ∼200 kDa, in addition to a heterodimer of gelatinase B with NGAL (*27*) which migrated at approximately 130 kDa (Figure 2a).

Enzymatic Deglycosylation of Purified Human Neutrophil Gelatinase B. Purified gelatinase B was enzymatically deglycosylated with PNGase F. It contained at least 5 kDa of N-linked carbohydrates as visualized by a molecular shift after digestion with the *N*-glycosidase (Figure 2a). The extent of the contributions of the N-linked sugars to the entire molecule and the stepwise deglycosylation (Figure 2a) are compatible with the occupancy of at least two of the three N-glycosylation sequons. The bandwidth of the monomer is indicative of heterogeneity of the gelatinase B which originates from both amino-terminal truncation and glycosylation. The kinetics of enzymatic removal of N-linked sugars from neutrophil gelatinase B forms were reproduced with different batches of PNGase F. No significant alteration in gelatinolytic activity was observed after PNGase F treatment in multiple experiments. Accordingly, quantitative analysis of serial dilutions by scanning densitometry did not yield significant differences in gelatinolysis between the natural and the de-N-glycosylated samples.

MALDI-TOF MS Analysis of Gelatinase B Glycans in Gel Bands. After SDS−PAGE (Figure 2b), the 91 kDa band was excised and the sugars were released and desialylated in situ by incubating with PNGase F and *A. ureafaciens* sialidase.

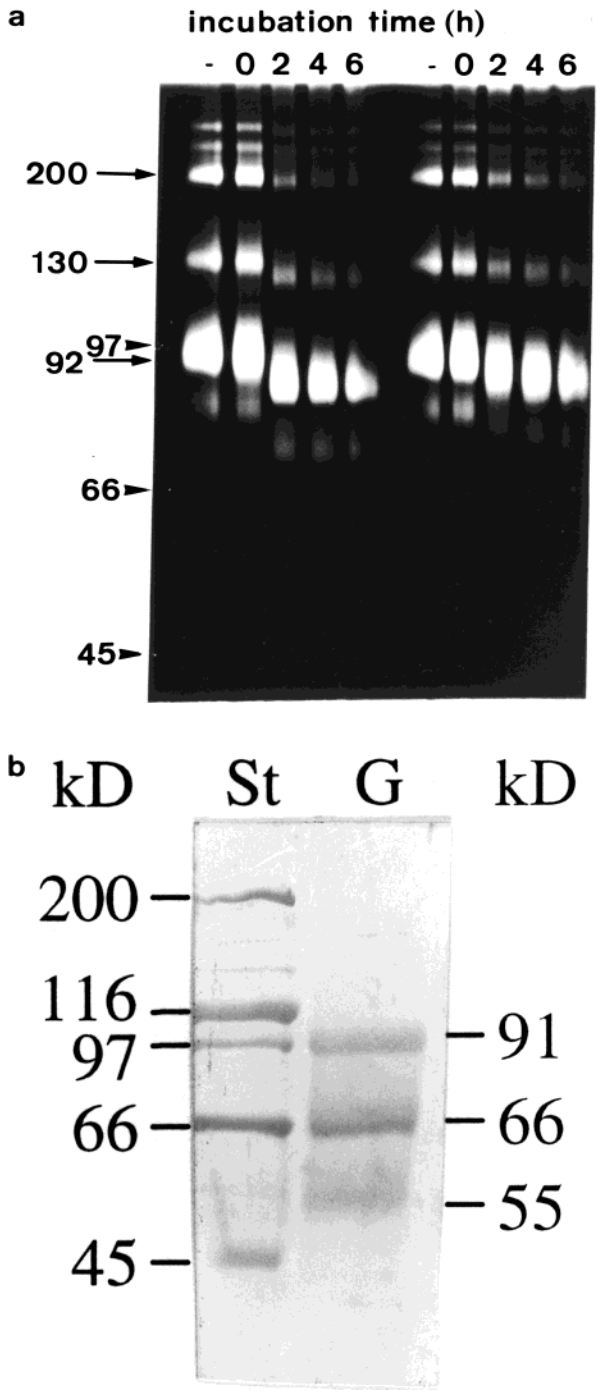


FIGURE 2: Gelatinase B zymography and staining analysis. Panel a depicts PNGase F deglycosylation of neutrophil gelatinase B and zymography analysis. Purified gelatinase B from human neutrophils was analyzed by SDS−PAGE gelatin zymography and consisted of a monomer (arrow at 92 kDa), a heterodimer with NGAL (arrow at 130 kDa), and a homodimer (arrow at 200 kDa). Incubation with 2 (left panel) and 0.2 milliunits (right panel) for various periods of time resulted in a gradual decrease in the relative molecular mass of the monomer and in the appearance and size of the homo- and heterodimer. Lane (−) contained the gelatinase B preparation in incubation buffer without PNGase F, whereas lane 0 contained all the constituents of the reaction mixture, including the PNGase F. The molecular mass standardization was obtained by separating size markers in the zymography gel. These markers (in kilodaltons) are indicated by the arrowheads. Panel b shows the SDS−PAGE gel of a neutrophil gelatinase B preparation (91 kDa). The band at 91 kDa was excised, and the sugars were released in situ by PNGase F. Two bands at 55 and 66 kDa were identified as degradation fragments of gelatinase B.

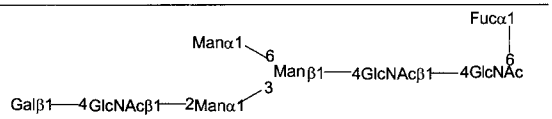
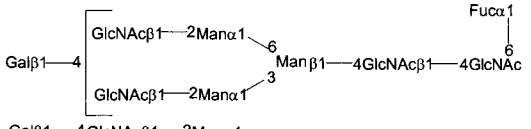
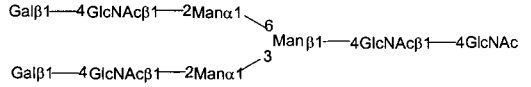
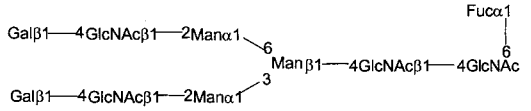
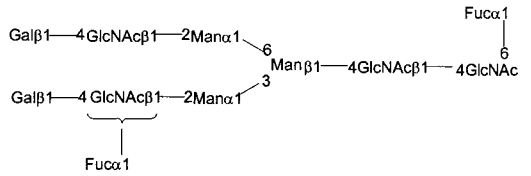
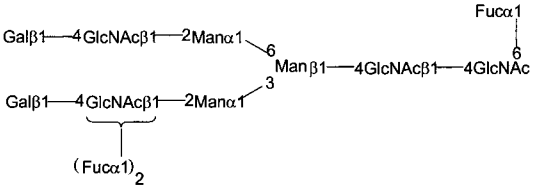
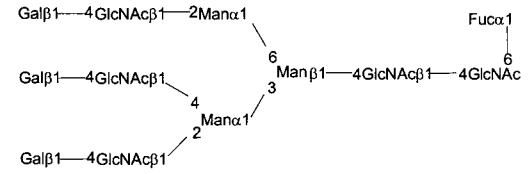
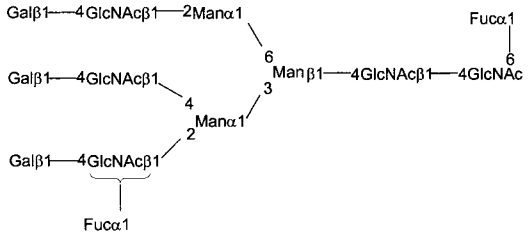
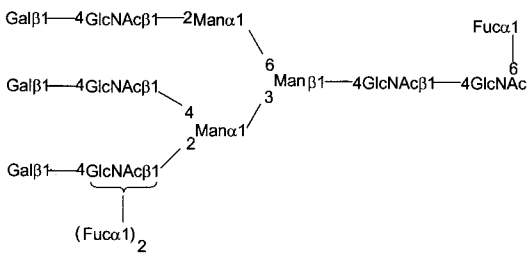
The isolated neutral sugars were analyzed by MALDI-TOF MS. The glycans recovered from the 91 kDa band are shown in Table 1 and consisted mainly of the biantennary compounds with one and two fucose residues ($H_5N_4F_1$ and $H_5N_4F_2$, where H is hexose, N is hexosamine, and F is fucose). Consistent with the NP-HPLC analysis of neutrophil gelatinase B containing neutrophil gelatinase B complexed with NGAL, some mono-, bi-, or trifucosylated triantennary structures were also detected. PSD analysis confirmed that these structures had one core fucose at the reducing terminus. Finally, the specificity of exoglycosidase digestions yielded information about the linkages. Almond meal α-fucosidase (1 unit/mL, specific for Fucα1−3/4GlcNAc) digestion showed that the second fucose of $H_5N_4F_2$ is α1−3-linked to an outer arm GlcNAc, and this was confirmed by digestion with *S. pneumoniae* galactosidase (1 unit/mL, specific for galactose linked to a GlcNAc which is not substituted with fucose) when no digestion of the fucose-substituted antennae occurred. Digestion with almond meal α-fucosidase (1 unit/mL), *S. pneumoniae* galactosidase (1 unit/mL), and *S. pneumoniae* hexosaminidase (10 milliunits/mL, at this concentration, specific for GlcNAcα1−2Man with the condition that the mannose is not substituted on C6) together showed that the third antenna of the triantennary sugars is α1−4-linked to the α1−3-linked mannose of the core (and not α1−6-linked to the α1−6-linked mannose).

The 66 and 50 kDa bands from the SDS gel were identified after in-gel digestion with trypsin as fragments of the 91 kDa band by peptide mapping and sequencing as described in Materials and Methods. Interestingly, the only glycans associated with these fragments were biantennary, galactosylated glycans with and without core fucose. This may suggest that the larger glycans are better able to protect the molecule from protease digestion.

Charge Analysis. The total carbohydrates (N-linked and O-linked) were released from the neutrophil gelatinase B preparation (containing NGAL) by hydrazinolysis. The sample was re-N-acetylated, reduced, radiolabeled with tritium, and analyzed by WAX chromatography (Figure 3a). The glycan pool contained a mixture of neutral (N), sialylated N-linked (NA), and O-linked (OA) carbohydrates. These pools were assigned by comparison with the analysis of fetuin sugars on the same system. After neuraminidase treatment, 90% of the sugars became neutral (Figure 3b). The remaining 10% of the charged glycans were not further analyzed and are expected to be artifacts associated with radiolabeling. However, the possibility that they contain sulfated or phosphated glycans or glucuronic acids cannot be totally ruled out.

Analysis of the N-Linked Glycans of Purified Neutrophil Gelatinase B (Containing NGAL). N-Linked glycans were released from neutrophil gelatinase B by incubation with hydrazine at 95 °C under conditions optimized for maximum release and recovery of N-linked glycans. The 2-AB-labeled glycan pool was resolved by normal phase HPLC (Figure 4). The oligosaccharide structures were assigned from their elution positions which were expressed in glucose units (gu) by comparison with a standard dextran hydrolysate ladder (top of Figure 4). The gu values were assigned with reference to the gu values of known standards and from the predetermined incremental values for the addition of particular monosaccharide residues to common glycan cores (for full

Table 1: Masses, Composition, and Structures of the N-Linked Glycans from Gelatinase B

Mass[a]		Comp.[b]	Enzyme digests[c]		Formula[d]	Structure
Found	Calc.		1	2		
1444.9	1444.5	H_4N_3F	H_3N_3F	H_3N_3	-	
1647.8	1647.6	H_4N_4F	H_3N_4F	H_3N_4	A2G1F	
1663.7	1663.6	H_5N_4	H_3N_4	H_3N_4	A2G2	
1809.8	1809.7	H_5N_4F	H_3N_4F	H_3N_4	A2G2F	
1955.9	1955.7	$H_5N_4F_2$	$H_4N_4F_2$	H_3N_4F	A2G2F2	
2101.9	2101.8	$H_5N_4F_3$	$H_5N_4F_3$	$H_5N_4F_3$	A2G2F3	
2174.9	2174.8	H_6N_5F	H_3N_5F	H_3N_5	A3G3F	
2321.0	2320.8	$H_6N_5F_2$	$H_4N_5F_2$	H_4N_5F	A3G3F2	
2467.1	2466.9	$H_6N_5F_3$	H_5N_5F3	H_5N_5F	A3G3F3	

[a] Monoisotopic mass of the MNa^+ ion. [b] H is hexose, N HexNAc, and F fucose. [c] Digest 1 is the *A. ureafaciens* sialidase and bovine testis β-galactosidase, and digest 2 is *A. ureafaciens* sialidase, bovine testis β-galactosidase, and bovine kidney α-fucosidase. [d] A1−3 represent the number of antennae linked to the trimannosyl core. G0−3 represent the number of terminal galactose residues in the structure. Gal is galactose, M or Man mannose, and F core fucose.

details, see ref *43*). The preliminary assignments were confirmed using a series of parallel exoglycosidase digestions in which aliquots of the glycan pool were digested with the four enzyme arrays shown in Figure 4 and Table 2. Peaks which are present in one profile and missing in the subsequent profile contained glycans with terminal residues which were susceptible to digestion by the additional enzyme shown in the lower profile (for full details, see refs *43* and *45*). In common with the glycans enzymatically released from neutrophil gelatinase B excised from the SDS−PAGE gel

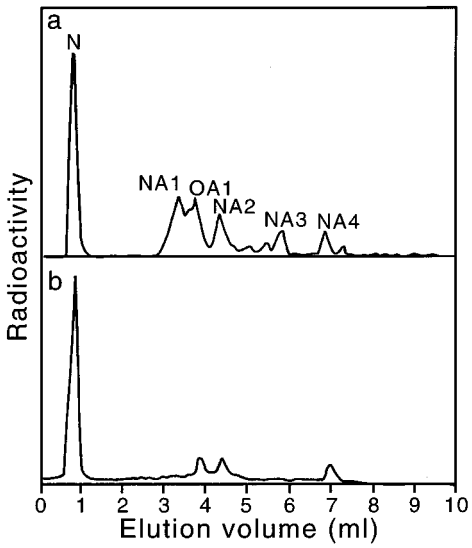


FIGURE 3: Panel a depicts a WAX analysis of the total glycan pool released by hydrazine from neutrophil gelatinase B. The fluorescently labeled sugars were resolved by anion exchange chromatography and charges assigned by comparison of their elution times with those of standard fetuin sugars. Panel b is a WAX profile of the gelatinase B N- and O-linked glycan pool after digestion with *A. ureafaciens* sialidase. Eighty percent of the glycan pool became neutral after incubation with the enzyme, indicating that the charge on these sugars was due to sialic acid: N, neutral glycans; NA, sialylated N-linked glycans; OA, O-linked glycans. Numbers denote the charge state.

bands (see above) and with those released from NGAL (see below), mainly complex-type biantennary structures were detected. These were core-fucosylated and, in addition, sometimes contained outer arm fucose linked to GlcNAc. A limited quantity of triantennary structures was also found.

Analysis of the O-Linked Glycans of Neutrophil Gelatinase B. O-Linked glycans were released by manual hydrazinolysis at 60 °C for 4 h under conditions designed to minimize degradation. The sequential enzymatic digestions of the O-linked glycan pool of neutrophil gelatinase B are shown in Figure 5a−c and Table 3. The data indicate that neutrophil gelatinase B contains a series of O-linked glycans which vary in size from the neutral core 1 disaccharide (Galβ1−3GalNAc) to elongated core 2 structures modified with sialic acid and/or fucose (Figure 5d and Table 3a−c). The major component was Galβ1−4GlcNAcβ1−6[NeuNAcα2−3Galβ1−3]GalNAcα1-R (20%).

Other structures include NeuNAcα2−6[NeuNAcα2−3Galβ1−3]GalNAcα1-R (disialylated core 1, 9.6%), NeuNAcα2−3Galβ1−3GalNAcα1-R (monosialylated core 1, 6.4%), and Galβ1−4[Fucα1−3]GlcNAcβ1−6[NeuNAcα2−3Galβ1−3]GalNAcα1-R (4.1%, containing the Lewis X epitope). The glycan structures were determined by comparison with the elution positions of standard sugars and the results of digestions with specific exoglycosidases. For example, the authentic galactosylated core 2 structure (Galβ1−4GlcNAcβ1−6[Galβ1−3]GalNAc) isolated from bovine fetuin coeluted with peak 9 on both normal phase and reverse phase HPLC (data not shown). Consistent with the proposed structure of peak 9 was the degradation of peak 13 (Galβ1−4GlcNAcβ1−6[NeuNAcα2−3Galβ1−3]GalNAc) to peak 7 following digestion with SPG, SPH, and BTG (data not shown). These structural assignments were all consistent with on-line NP-HPLC/ESMS (QTOF) analysis

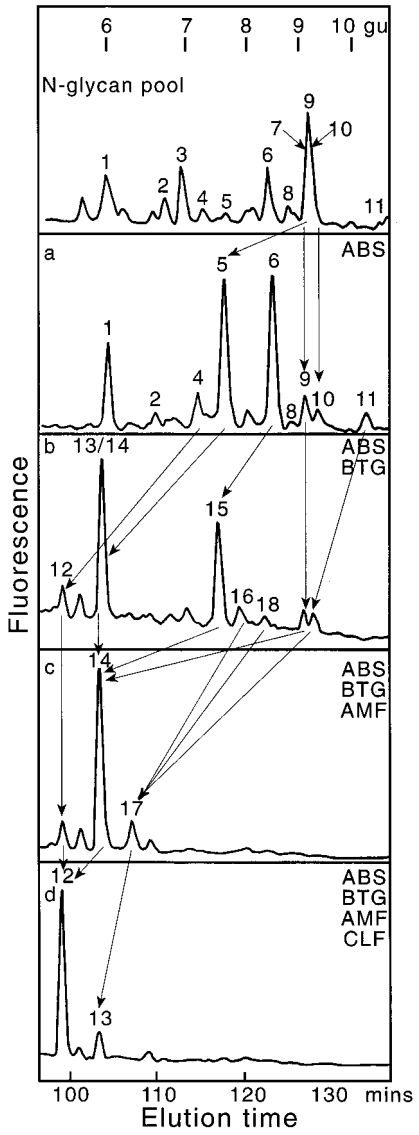


FIGURE 4: Simultaneous exoglycosidase sequencing of the neutrophil gelatinase B N-linked glycan pool, which also contained NGAL, released by hydrazinolysis. The major peaks have been annotated and assigned (Table 2). The arrows indicate the shift of peaks following digestion with the enzyme arrays. The structures of the oligosaccharides were determined from their elution positions measured in gu, the known incremental values for monosaccharide additions to glycan cores, and the specificity of the exoglycosidase enzymes in the arrays. The assignments were consistent with the MALDI MS analysis of NGAL and the PNGase F-released glycan pool (Tables 1 and 4). The enzymes that were used are indicated on the right of the panels.

of the total O-linked glycan pool which produced the expected fragmentation pattern (data not shown). However, it was noted that the Galβ1−3 residue identified on the glycan in peak 9 was resistant to BTG. This was in contrast to the authentic galactosylated core 2 structure isolated from bovine fetuin (data not shown). In addition, analysis of the material eluting at >6 gu indicated that approximately 3% of the glycans contain the sialyl Lewis X epitope.

These O-linked glycans contribute to the observed additional molecular mass (>10 kDa) of the enzyme, which cannot be ascribed to the amino acid composition (76 kDa) and the N-linked glycans (5 kDa). More than 85% of the glycans were characterized as O-linked sugars. The location of the O-linked glycans has not been determined. However,

Table 2: N-Linked Glycan Pool and Exoglycosidase Digestions of Neutrophil Gelatinase B Containing NGAL

			gu values of components of the glycan pool				
peak	formula[a]	asialoglycan pool MS composition[g]	hydrazine-released glycan pool	digest a (ABS[f])	digest b (ABS/BTG[f])	digest c (ABS/BTG/AMF[f])	digest d (ABS/BTG/AMF/CLF[f])
1	O-link[c]		5.93[c]	5.93	5.92	4.15[e]	off scale
2	A2G1(1,3)F[b]	H4N4F	6.68	6.68	5.92	5.92	5.49
3	O-link[d]		7.15[d]	5.93	5.92	4.15[e]	off scale
4	A2G2[b]	H5N4	7.15	7.15	5.49	5.49	5.49
5	A2G2F[b]	H5N4F	7.57	7.57	5.92	5.92	5.49
6	A2G2F2[b]	H5N4F2	8.37	8.37	7.71	5.92	5.49
7	A2G2FS2		8.82	7.57	5.92	5.92	5.49
8	A3G3F[b]	H6N5F	8.76	8.76	6.31	6.31	5.91
9	A2G2F3[b]	H5N4F3	9.33	9.33	9.33	5.92	5.49
10	A3G3F2[b]	H6N5F2	9.6	9.6	8.34	6.31	5.91
11	A3G3F3		10.49	10.49	9.6	6.31	5.91
12	A2G0				5.49	5.49	5.49
13	A3G0				5.91	5.91	5.91
14	A2G0F				5.92	5.92	5.49
15	A2G1(1,3)F2				7.71	5.92	5.49
16	A3G1F				8.05	6.31	5.91
17	A3GOF				6.31	6.31	5.91
18	A3G1F2				8.34	6.31	5.91

[a] A2 and -3 represent the number of antennae linked to the trimannosyl core. G0−3 represent the number of galactose residues in the structure. S is sialic acid, G galactose, and F fucose. Oligosaccharide structures are shown in Table 1. [b] Identified in MS and HPLC profiles of the N-linked asialoglycan pool. [c] The 5.93 gu peak contains O-linked glycan Fucα1,3/4Galβ1,4GlcNAcβ1−6Galβ1−4GlcNAcβ1−6Galβ1−3GalNAc. [d] The 7.15 peak contains an unassigned O-linked glycan. [e] The 4.15 peak (off scale) contains O-linked glycan GlcNAcβ1−6Galβ1−4GlcNAcβ1−6Galβ1−3GalNAc. [f] ABS is *A. ureafaciens* sialidase, BTG bovine testes galactosidase, AMF almond meal fucosidase, and CLF *C. lampas* fucosidase. [g] H is hexose, N HexNAc, and F fucose.

gelatinase B contains a type V collagen domain, consisting of a stretch of repeating Ser/Thr-Xaa-Xaa-Pro units (Figure 1). This domain is a potential site for O-glycosylation and is conserved in mouse gelatinase B (*26*). Interestingly, it is absent in gelatinases A from various mammalian species.

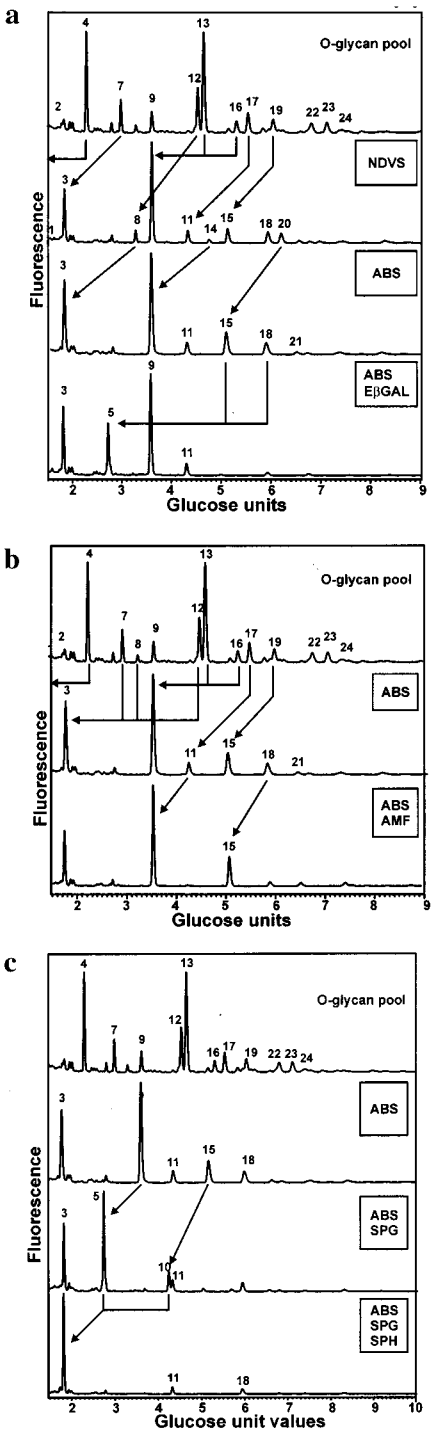
Characterization of the N-Linked Glycans Attached to NGAL. Figure 6 shows the hydrazine-released 2-AB-labeled glycan pool from NGAL resolved by NP-HPLC together with the parallel digestions of the pool with arrays of exoglycosidase enzymes. The digests were monitored by HPLC and MALDI-TOF MS, and the data are analyzed in Table 4. Almost 80% of the sugars were monosialylated, and many contained an outer arm fucose residue linked to GlcNAc. Unusually, NGAL contains a population of glycans carrying a single fucose on one antenna, but none on the core.

Characterization of the O-Linked Glycans Attached to NGAL. O-Linked glycans have not been reported previously on NGAL. Nevertheless, NGAL was subjected to hydrazinolysis under conditions which optimally release O-linked glycans. Material was recovered which was 2-AB labeled, and Figure 7 shows that some of the peaks were digested by exoglycosidases. The majority of the material that was recovered eluted on NP-HPLC in the region of di- and trisaccharides (Figure 7, upper panel, gu values of 1.5−2.5). However, less than 20% of the labeled material could be assigned with the available exoglycosidase enzymes. The major component of the unassigned material, which may include noncarbohydrate contaminants, eluted at 1.78 gu which is characteristic of a disaccharide. The technology developed to sequence 2-AB-labeled O-linked glycans by NP-HPLC relies on the use of specific exoglycosidases to cleave every monosaccharide linkage. Many of the NGAL glycans were resistant to all the available glycosidases, and this, together with the limited amount of material, meant that structures could only be assigned to some of the peaks.

Molecular Modeling of NGAL. A molecular model of NGAL (Figure 8) was constructed from the NMR structure (*46*), the glycan analysis described above, and the oligosaccharide structural database which provides the dimensions and torsion angles of the sugars [Glycobiology Institute oligosaccharide structure database (*47*)]. Cys^{87}, the residue which has been suggested to be involved in the homodimerization of NGAL, is shown in black. In common with many other glycoproteins, the N-linked sugar (about 25 Å) is relatively large in comparison to the protein domain to which it is attached.

DISCUSSION

Purification and Glycan Analysis of Natural Matrix Metalloproteinases. MMPs are a family of enzymes that modify the extracellular matrix. In this study, we have defined the structures of (i) the N- and O-linked glycans associated with affinity-purified gelatinase B (MMP-9), which is a mixture of dimers (200 kDa), monomers (92 kDa), and the copurifying NGAL−gelatinase B complex (130 kDa), (ii) the N-linked glycans attached to purified monomers of gelatinase B in an SDS−PAGE gel band, and (iii) the N- and O-linked glycans attached to affinity-purified NGAL (25 kDa). To date, the only other published glycan analysis of any natural MMP is that of MMP-1 (*13*). For MMP-9, structural data are only available for recombinant mouse gelatinase B expressed in the yeast *P. pastoris* (*34*). Several reasons may explain this lack of information. The sources of natural MMPs from normal cells are restricted in comparison with recombinant expression of cDNAs or with materials from transformed cell lines. In addition, since multistep chromatographic purification and sometimes preparative SDS−PAGE are needed to purify these enzymes, they are generally recovered in low yields. Finally, the analytical tools for glycan analysis have been refined only recently (*33*, *39*, *43*, *45*) to a level that enables the analysis



d

Peak Number	GU	Glycan Structure
3	1.81	2AB
4	2.29	2AB
5	2.74	2AB
7	2.98	2AB
8	3.29	2AB
9	3.57	2AB
10	4.24	2AB
11	4.32	2AB
12	4.52	2AB
13	4.63	2AB
15	5.12	2AB
17	5.51	2AB
18	5.93	2AB
20	6.19	2AB

FIGURE 5: O-Linked oligosaccharides from gelatinase B. Panels a−c show the exoglycosidase sequencing of the neutrophil gelatinase B O-linked glycan pool using the enzymes shown in each panel. Aliquots of the total 2-AB-labeled O-linked glycan pool were incubated with different exoglycosidases. Following digestion, the products were analyzed by NP-HPLC and the data (time on the *x*-axis) linearized to the glucose unit scale on the *x*-axis using a new computer program (Peak Time, E. Hart, unpublished data). The major peaks have been annotated and assigned (Table 3a−c). The arrows indicate the movement of peaks following digestion with the enzyme(s). Structures were determined from the elution position of the peaks, measured in gu, relative to known standards, and the specificity of the exoglycosidases that were used. Digestions were performed using each enzyme alone and then in combination with others. The figures show only a selection of the digestions that were performed. The enzymes that were used are indicated on the right of the panels. Panel d contains the schematic structures of the O-linked glycans attached to neutrophil gelatinase B. The structures of the O-linked glycans (peak numbers) are consistent with Table 3: (♦) GalNAc, (■) GlcNAc, (◊) galactose, (△) fucose, and (★) sialic acid. Linkages are represented by the angles of attachment of each monosaccharide.

of 1 nmol of glycoprotein. In-gel enzymatic release for N-linked sugars (*39*) has been used here to provide a final purfication step in which NGAL was dissociated from monomers of gelatinase B and also to provide a level of sensitivity that is not available with earlier methods. In the absence of a generic *O*-glycanase, such a technique is not yet available for O-linked oligosaccharides. Therefore, O-linked glycans were released from human neutrophil gelatinase B by hydrazinolysis optimized for maximum O-linked glycan release with minimum degradation of glycans and

Table 3: Analysis of the O-Linked Glycans from Human Neutrophil Gelatinase B[a]

			panel a				panel b		panel c
peak	gu	glycan	pool	NDVS	ABS	ABS/ EβGAL	ABS/AMR	ABS/SPG	ABS/SPG/ SPH
1	1.42	nd	−	1.4	0.7	−	2.7	4.1	4.5
2	1.73	nd	−	0.9	1.8	0.9	1.7	1.2	4.5
3	1.81	Galβ1−3GalNAc	2.3	16.9	23.0	20.3	20.4	21.0	63.4
4	2.29	NeuNAcα2−3Gal	18.4	−	0.5	−	−	−	−
5	2.74	GlcNAcβ1−6(Galα1−3)GalNAc	−	0.9	1.0	20.2	1.0	38.3	1.3
6	2.78	nd	1.8	2.3	2.8	*	2.3	*	2.4
7	2.98	NeuNAcα2−3Galβ1−3GalNAc	6.4	0.6	−	0.7	0.7	−	−
8	3.29	NeuNAcβ2−6(Galβ1−3)GalNAc	1.6	4.4	−	−	−	−	−
9	3.57	Galβ1−4GlcNAcβ1−6(Galβ1−3)GalNAc	4.0	36.0	36.9	34.6	41.5	0.6	−
10	4.24	GlcNAcβ1−6Galβ1−4GlcNAcβ1−6(Galβ1−3)GalNAc	−	−	−	−	−	7.0	−
11	4.32	Galβ1−4(Fucα1−3)GlcNAcβ1−6(Galβ1−3)GalNAc	−	5.1	4.7	4.6	0.6	4.6	5.1
12	4.52	NeuNAcα2−6(NeuNAcα2−3Galβ1−3)GalNAc	9.6	−	−	−	−	−	−
13	4.63	Galβ1−4GlcNAcβ1−6(NeuNAcα2−3Galα1−3)GalNAc	20.1	−	−	−	−	−	−
14	4.75	nd	−	1.3	−	−	−	−	−
15	5.12	Galβ1−4GlcNAcβ1−6Galβ1−4GlcNAcβ1−6-(Galβ1−3)GalNAc	1.1	5.3	8.3	−	12.2	−	−
16	5.28	nd	2.7	−	−	−	−	−	−
17	5.51	Galβ1−4(Fucα1−3)GlcNAcβ1−6(NeuNAcα2−3-Galβ1−3)GalNAc	4.1	−	−	−	−	−	−
18	5.93	Galβ1−4(Fucα1−3)GlcNAcβ1−6Galβ1−4GlcNAcβ1−6-(Galβ1−3)GalNAc	0.7	4.2	4.4	−	−	3.5	3.9
19	5.96	nd	3.1	−	−	1.2	1.9	−	−
20	6.19	NeuNAcα2−6Galβ1−4GlcNAcβ1−6Galβ1−4-GlcNAcβ1−6(Galβ1−3)GalNAc	0.7	3.8	−	−	−	−	−
21	6.55	nd	−	0.9	1.0	−	1.6	0.8	−
22	6.77	nd	2.7	0.6	0.7	0.6	−	0.6	0.6
23	7.08	nd	2.2	−	−	−	−	−	−
24	7.35	nd	1.1	−	−	−	−	−	−

[a] The table shows the peaks numbered as in Figure 5 (column 1), gu values (column 2), assigned structures (column 3), and the percentages of O-linked glycans B (column 4) released from human neutrophil gelatinase. The analysis of the products following exoglycosidase digestion(s) of the pool (Figure 5a, columns 4−7; Figure 5b, columns 4, 6, and 8; and Figure 5c, columns 4, 6, 9, and 10). Percentages of the glycan populations were calculated from the total integrated area, which includes all nondetermined (nd) structures. Percentages that could not be calculated with confidence due to peak overlap are shown as asterisks. The enzymes that were used are at the top of the each column.

allowing fluorescent labeling of the reducing terminus.

N-Glycosylation of Gelatinase B. In common with NGAL, mainly complex-type biantennary structures were detected on gelatinase B. These were core-fucosylated with or without sialic acid and outer arm fucose linked to GlcNAc. The sugars are large (30 nm) in comparison to the protein domains to which they are attached. Various functions have been attributed to N-linked glycans (*48*, *49*, *51*−*53*). In the protease cascade of which gelatinase B is a member, glycosylation plays a role in the specific activity of t-PA and plasminogen (*12*). By analogy with other glycoproteins, the N-linked glycans attached to gelatinase B might, for example, influence the catalytic activity, the stability, the protease resistance, or the interactions of the protein with its substrate, with specific inhibitors, or with ternary complexes consisting of processing enzymes or ECM components. Here we have established that the N-linked sugars of gelatinase B do not alter the catalytic activity via gelatin substrate zymography analysis by using a combination of enzymatic deglycosylation with protease-free PNGase F and substrate zymography.

O-Glycosylation of Gelatinase B. The proportion of O-linked glycans attached to gelatinase B is high in comparison with the N-linked sugars (85/15). This ratio may be even greater than the data here suggest since the conditions used for release and recovery allow a higher proportion of the total N-linked glycans to be recovered, compared with the O-linked sugars. The analysis indicates that human neutrophil gelatinase B contains a mixture of O-linked glycans with core type 1 (Galβ1−3GalNAcα1-R) and core type 2 (GlcNAcβ1−6[Galβ1−3]GalNAcα1-R). O-Linked glycans are synthesized by stepwise addition of monosaccharide residues to the GalNAc core. Elongation of the O-linked glycan cores can proceed along a number of pathways, the most common of which is the addition of a lactosamine extension (Galβ1−4GlcNAcβ), and the results of the above analysis are consistent with this mechanism. A common feature of O-linked glycans is the presence of α-linked terminal residues, such as α2−3- or α2−6-linked sialic acid and α1−2 (terminal)- and α1−3-linked (subterminal) fucose, at the reducing terminus. Consistent with this, WAX chromatography (Figure 3a) and normal phase HPLC (Figure 5) indicated that gelatinase B contains α2−3- and α2−6-sialylated O-linked glycans which can be removed by incubation with Newcastle disease virus sialidase (specific for the α2−3 linkage) or *A. ureafaciens* sialidase (which will cleave both α2−3- and α2−6-linked sialic acid).

Location of O-Linked Glycans in Gelatinase B. Most O-linked glycans are attached either to the hydroxyl groups in the side chains of threonine or, less often, to serine residues through GalNAc. The amino acid sequence of human and mouse gelatinase B, as deduced from molecular cloning

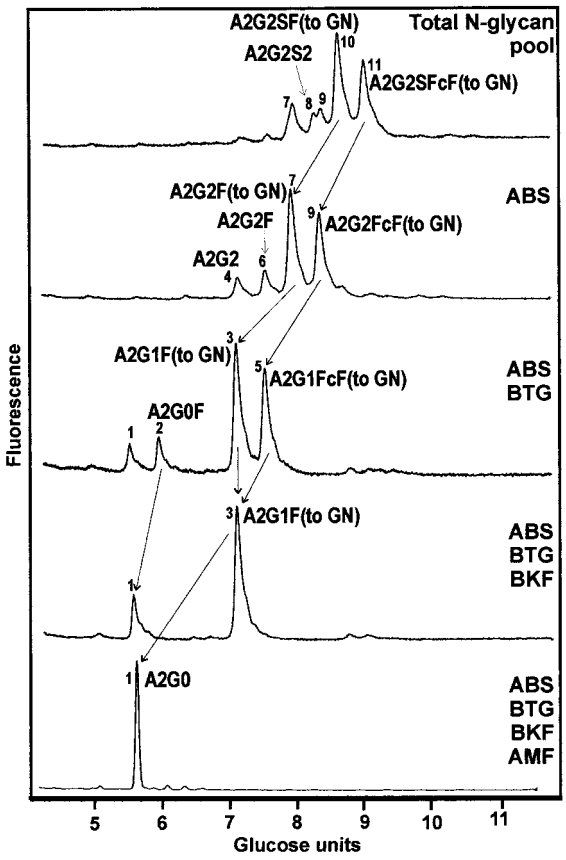


FIGURE 6: Simultaneous exoglycosidase sequencing of the NGAL N-linked glycan pool using enzyme arrays. The major peaks of the NP-HPLC chromatogram have been annotated and assigned (Table 4). The arrows indicate the shift of peaks following digestion with the enzyme arrays. The structures of the oligosaccharides were determined from their elution positions measured in gu, the known incremental values for monosaccharide additions to glycan cores, and the specificity of the exoglycosidase enzymes in the arrays. The assignments were consistent with the MALDI MS analysis (Table 4).

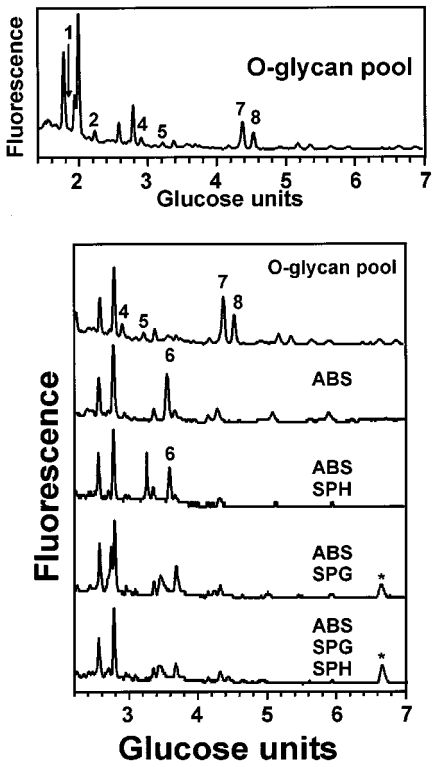


FIGURE 7: Exoglycosidase sequencing of the NGAL O-linked glycan pool using enzyme arrays. Aliquots of the total 2-AB-labeled O-linked glycan pool (top panel) were incubated with different exoglycosidases (lower panel). The digestion products were analyzed by NP-HPLC and the data (elution time on the *x*-axis) linearized to a glucose unit scale using a new computer program (Peak Time, E. Hart, unpublished data). Peaks (Table 5) were assigned from their elution positions, measured in gu, and by comparison with known oligosaccharide standards, and the specificity of the exoglycosidases. The figures show only a selection of the digestions that were performed. In the bottom two profiles of the lower panel, all assigned peaks were reduced to 1.8 gu (peak 1 on the top panel). Asterisks denote N-linked glycans.

experiments (*8*, *26*), indicates that the molecule contains many serine and threonine residues and that a number are in the vicinity of proline residues. In particular, in human gelatinase B, there is a proline-rich section (Pro^{426}-Arg-Pro-Glu-Pro-Glu-Pro-Arg-Pro-Pro) of the primary sequence followed by eight Pro-Thr and three Pro-Ser couples (which are all spaced by two residues) in the region of Pro^{442}−Ser^{483}. This region coincides with the type V collagen domain, which is unique for gelatinase B and is not present in gelatinase A or other MMPs. Molecules which contain a

Table 4: HPLC and MALDI Analysis of the Exoglycosidase Digestions of the PNGase F-Released N-Linked Glycans from NGAL

					exoglycosidase digests[b]						
					1		2		3		4
peak	% assigned undigested peaks	assignment	calcd mass[a]	undigested peaks (gu)	gu	mass (Da)	gu	mass (Da)	gu	mass (Da)	gu
1	−	A2G0	1339.5	−	−	−	5.50	1339.4	5.5-	1339.5	5.53
2	−	A2G0F	1485.5	−	−	−	5.93	1485.5	−	−	−
3	−	A2G1F (to GN)	1647.6	−	−	−	7.07	1647.4	7.06	1647.6	−
4	−	A2G2	1663.6	−	7.15	1663.5	−	−	−	−	−
5	−	A2G1F(core)F (to GN)	1793.6	−	−	−	7.50	1793.5	−	−	−
6	−	A2G2F	1809.6	−	7.57	1809.6	−	−	−	−	−
7	14.7	A2G2F (to GN)	1809.6	7.96	7.96	1809.6	−	−	−	−	−
8	6.2	A2G2S2	2245.8	8.28	−	−	−	−	−	−	−
9	8.9	A2G2F(core)F (to GN)	1955.7	8.39	8.38	1955.5	−	−	−	−	−
10	38.2	A2G2SF (to GN)	2100.7	8.64	−	−	−	−	−	−	−
11	32.0	A2G2SF(core)F (to GN)	2246.8	9.04	−	−	−	−	−	−	−

[a] Monoisotopic mass of the MNa^+ ion of nonlabeled glycan. [b] Exoglycosidase digests: (1) *A. ureafaciens* α2−6 > 3 sialidase, (2) *A. ureafaciens* α2−6 > 3 sialidase and bovine testis β1−3-galactosidase, (3) *A. ureafaciens* α2−6 > 3 sialidase, bovine testis β1−3-galactosidase, and bovine kidney α1−6-fucosidase, and (4) *A. ureafaciens* α2−6 > 3 sialidase, bovine testis β1−3-galactosidase, bovine kidney α1−6-fucosidase, and almond meal α1−3/4-fucosidase.

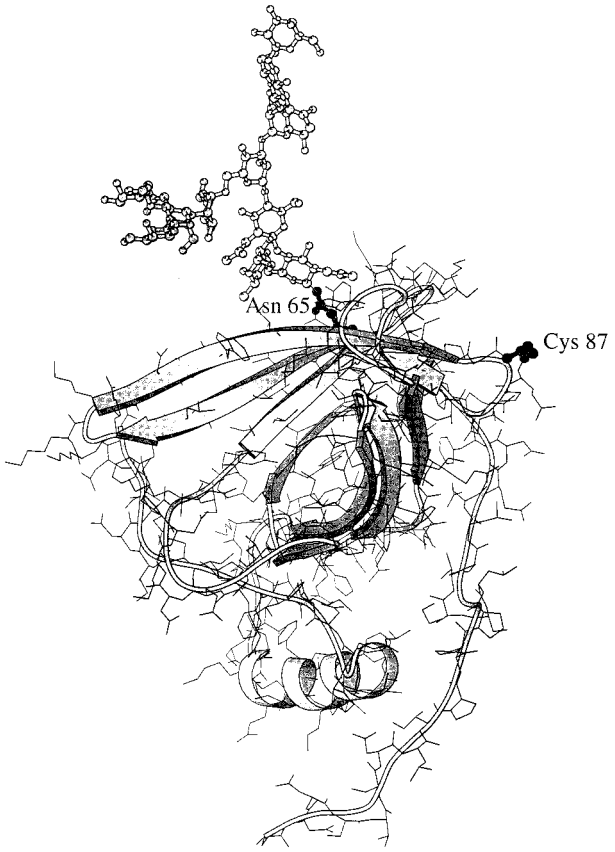


FIGURE 8: Molecular model of NGAL constructed from the NMR structure (*46*) with a biantennary glycan with core and outer arm fucosylation attached at Asn[65]. The glycan structure was modeled using the database of glycan linkages obtained from crystallography (*47*). Cys[87], the residue suggested to be involved in the homodimerization of NGAL and the formation of the heterodimer with neutrophil gelatinase B, is shown in black.

significant proportion of O-linked glycans, such as mucins, frequently contain stretches of peptide sequence with repeating Ser, Thr, and Pro residues. The type V collagen domain is, therefore, a highly probable attachment region for clustered O-linked glycans. Some other rules are emerging which characterize the sites preferred by the family of GalNAc transferases (*54*) which indicate that there is no single consensus sequence in the peptide for GalNAc O-linked to Thr or Ser since O-glycosylation involves a family of related transferases with different specificities. However, there is a clear preference for some amino acids in close proximity, especially for proline.

Functions of O-Linked Glycans in Gelatinase B. Multiple functions have been described for O-linked glycans (*55*), and some general rules are emerging. Clustered O-linked glycans extend and increase the rigidity of the polypeptide chain (*56*−*58*). They also have important roles in recognition, for example, of selectins by their ligands (*59*), in fertilization (*60*), and in the immune system (*61*, *62*). O-Linked sugars also modulate the activity of signaling molecules (*63*) and of enzymes (*64*). A function of the O-linked glycans attached to neutrophil gelatinase B has not been established. In common with other O-glycosylated proteins, the O-linked sugars of gelatinase B would be expected to provide protection from proteolytic enzymes and contribute to the stability of the enzyme. This may be an important function since gelatinase B is released from granules at inflammatory sites where proteases are likely to be abundant. The functional role of the unique type V collagen-like domain of gelatinase B is not yet known, but a similar domain in decay-accelerating factor provides a rigid, protease-resistant spacer. Other domains are involved in substrate binding [fibronectin domains (*65*)] and in interactions with TIMP-1 [hemopexin-like domain (*66*)]. We suggest that the role of the O-linked glycans may be to extend the polypeptide chain (*57*) separating the hemopexin from the other domains and to protect the protein from proteases.

N- and O-Glycosylation of NGAL. The N-linked glycans attached to NGAL were mainly biantennary complex glycans with or without outer arm fucose and sialic acid. O-Linked sugars have not been reported previously on NGAL and were identified as simple core 1 and core 2 structures. This finding may reflect the increased sensitivity of the optimized methodology for O-linked glycan analysis used in this paper. Kjeldsen et al. (*27*) reported the absence of jacalin binding to NGAL and found that NGAL was not sensitive to *O*-glycanase digestion. Therefore, although there was no evidence from the analysis of the NGAL protein, we considered the possibility that the O-linked glycans released from the NGAL preparation may be derived from low levels of gelatinase B which had been coisolated with the NGAL. However, the predominant O-linked glycans found in gelatinase B are absent from those recovered from NGAL, suggesting that this result is not an artifact arising from cross-contamination of NGAL with gelatinase B.

Location of O-Linked Glycan(s) on NGAL. The biosynthesis of (mucin-type) O-linked sugars is highly complex (*67*) and is initiated by the addition of GalNAc to exposed Thr or Ser residues on the fully folded protein in the Golgi. A molecular model of NGAL was constructed (Figure 8) and examined for potential O-linked glycan sites. This indicated that the hydroxyamino acids within the first 15 residues were accessible and therefore potential sites for O-glycosylation. Analysis of the entire NGAL sequence by NetOGlyc 2.0 (*68*), a computer program which predicts

Table 5: O-Linked Oligosaccharides from NGAL[a]

peak	glycan	pool	ABS	ABS/SPH	ABS/SPG	ABS/SPG/SPH
1	Galβ1−3GalNAc			1.82	1.82	1.82
2	NeuNAcα2−3Gal	2.23				
3	Galβ1−3[GlcNAcβ1−6]GalNAc		2.71	2.71	2.71	
4	Galβ1−3[NeuNAcα2−6]GalNAc	2.90				
5	NeuNAcα2−3Galβ1−3GalNAc	3.22				
6	Galβ1−3[Galβ1−4GlcNAcβ1−6]GalNAc	3.57	3.57	3.57	3.57	3.57
7	NeuNAcα2−3Galβ1−3[NeuNAcα2−6]GalNAc	4.37				
8	NeuNAcα2−3Galβ1−3[Galβ1−4GlcNAcβ1−6]GalNAc	4.53				

[a] The glucose unit values and structures assigned to the O-linked glycans released from human NGAL as shown in Figure 7.

positions of O-glycosylation from primary sequence, identified serine residues at positions 5 and 14 as potential substrates for the UDP−GalNAc:polypeptide *N*-acetylgalactosaminyltransferases. Partial occupancy of one or both of these potential O-glycosylation sites on NGAL with the small O-linked glycans (Table 5) may not be expected to exhibit strong jacalin binding or an observable change in molecular mass on SDS−PAGE following digestion with *O*-glycanase.

Molecular Modeling. After analysis of the NGAL N- and O-linked glycans, a molecular model (Figure 8) based on the NMR structure (*46*) and the Glycobiology Institute oligosaccharide database (*47*) was prepared. This was used to search for possible sites of O-glycosylation. In addition, the model highlights the relatively large size of the sugar in comparison with the protein domain and indicates that the N-linked sugar can only partially cover the funnel-like hydrophobic hole of NGAL.

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