Generation of Glycosylated Remnant Epitopes from Human Collagen Type II by Gelatinase B[†]

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ABSTRACT: Gelatinase B/matrix metalloproteinase-9 (MMP-9) is an inflammatory mediator and effector. Considerable amounts of gelatinase B are released by neutrophils in the synovial cavity of patients with rheumatoid arthritis, and gelatinase B-deficient mice are resistant against antibody-induced arthritis. Native human collagen type II is susceptible to cleavage by various collagenases (MMP-1, MMP-8, and MMP-13), which cleave at a single position in the triple helix. Although the triple-helical structure may persist after this single cleavage, we show that gelatinase B degrades the resulting fragments into small remnant peptides. These were identified by mass spectrometry and Edman degradation. Localization of 31 cleavage sites shows that the immunodominant epitopes remain intact after cleavage and may become available, processed as antigens and presented in MHC-II molecules. Furthermore, most post-translational modifications were identified on the fragments, including nine glycosylation sites. In particular, it is shown for the first time by structural analysis that in natural human collagen II, lysines in the main immunodominant epitope are modified by partial hydroxylation and partial glycosylation. Determination of T-cell reactivity against such fragments indicates that, besides the two known main immunodominant epitopes, other glyco-epitopes may be present in collagen II. This reinforces the role of glycopeptide antigens in autoimmunity.

Gelatinase B/MMP-9 is a member of the family of matrix metalloproteinases (MMPs),[1] which play important roles in the degradation of the extracellular matrix in various physiological and pathological processes. In addition, gelatinase B and other MMPs process a number of regulatory molecules, including serine protease inhibitors, cytokines such as pro-interleukin-1β and pro-transforming growth factor-β, chemokines, and membrane-bound receptors. Gelatinase B is an inducible protease, prominently expressed and secreted under inflammatory conditions. It has been associated with a variety of diseases and disease processes, including rheumatoid arthritis, multiple sclerosis, bullous pemphigoid, diabetes, cancer cell invasion and metastasis, angiogenesis, and aneurysm formation (*1*). Inhibition of its expression by, for example, ribozyme techniques or gene ablation provided insights into the crucial role of gelatinase B in these processes and indicated that gelatinase B may be a useful therapeutic target (*2*−*9*).

Since therapeutic inhibition of gelatinase B activity is under consideration, understanding its exact functions, including the role of the cleavage of each substrate, is crucial. It is remarkable that the role of the cleavage of its most efficient substrate, gelatin, is not well understood and remains a topic of controversy. Gelatinase B contains a fibronectin domain with a high affinity for gelatin, resulting in efficient gelatinolytic properties (*10*). Gelatin is heat-denatured collagen, but heat denaturation is not a physiological process. Initially, it was thought that cleavage of the triple helix by collagenases at a single site results in denaturation, but recently, this was shown not to be the case (*11*).

A better understanding of the exact role of gelatinase B in the turnover of collagens is particularly important for rheumatoid arthritis. Large amounts of gelatinase B have been found in the synovial fluid of arthritis patients, and the levels correlated with the number of neutrophils and with the level of the main neutrophil chemoattractant, interleukin-8. In addition, upregulation of gelatinase B in synovial fluid was shown to result in net activity, probably because the enzyme was activated and not (predominantly) complexed with its physiological inhibitors such as TIMP-1 (*12*). These data may indicate a role of gelatinase B in the migration of neutrophils to the synovial fluid. The migration of neutrophils toward the main neutrophil chemoattractant of the mouse, truncated granulocyte chemotactic protein (GCP)-2/LIX, was reduced in gelatinase B$^{-/-}$ mice compared to the wild-type

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[1] Abbreviations: APMA, *p*-aminophenylmercuric acetate; GCP-2, granulocyte chemotactic protein-2; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

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controls (*13*). Furthermore, gelatinase B processes neutrophil-attracting chemokines, resulting in a positive feedback loop (*14*, *15*). However, a direct role of gelatinase B in the degradation of the cartilage is also possible. In particular, it was found that gelatinase B generates autoimmune peptides from denatured bovine type II collagen (*12*). Gelatinase B gene ablation in mice results in resistance to arthritis, experimentally induced by the injection of antibodies against collagen type II (*4*). These experiments also suggest that gelatinase B contributes to the generation of collagen II antigens. These may be presented to T-cells and may be recognized by antibodies, inducing acute inflammation via the classical pathway of complement activation. To clarify the exact role of neutrophils and neutrophil enzymes in these processes, we determined in this study whether and how gelatinase B participates in the degradation of native human type II collagen. Furthermore, by protein sequence and mass spectrometry analysis, a considerable number of post-translational modifications were identified and localized on the natural human collagen II, in particular on the major immunodominant epitope. Determination of the T-cell reactivities against the fragments indicates that, besides the two known main immunodominant epitopes, other previously unknown glyco-epitopes are present in collagen II.

EXPERIMENTAL PROCEDURES

Materials. Natural human gelatinase B was purified from neutrophils and activated as described previously (*14*). Recombinant collagenases were from R&D Systems (Poole, Abingdon, U.K.), and stromelysin-1 was from Biogenesis (Poole, U.K.). These enzymes were activated with APMA according to the instructions from the manufacturer, and subsequently dialyzed against assay buffer [100 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 10 mM $CaCl_2$].

Human and bovine collagen type II were purified from cartilage using the differential salt fractionation methods of Miller *et al.* (*16*). Unless otherwise indicated, it was dissolved at a concentration of 0.5 mg/mL in 0.6% acetic acid at 4 °C, and subsequently, the pH was neutralized with 2 M Tris.

Digestion of Collagen Type II with Collagenases and/or Gelatinase B. Human collagen type II (1 μg/μL) was incubated with 16 ng/μL APMA-activated collagenase (MMP-1, MMP-8, or MMP-13) and/or 16 ng/μL activated gelatinase B. Human collagen type II (2 μg/μL) was also incubated with an equal volume of neutrophil degranulate, obtained as follows. Human neutrophils (10^7 cells/mL) were stimulated with 0.5 μM fMLP in degranulation buffer [20 mM Tris-HCl (pH 7.4), 117 mM NaCl, and 15 mM $CaCl_2$] for 20 min at 37 °C. After centrifugation, the supernatant was incubated with 0.58 ng/μL APMA-activated stromelysin-1 for 2 h at 37 °C. Digested human collagen was subsequently analyzed by SDS−PAGE under reducing conditions, immediately after being heated at 95 °C for 2 min in SDS−PAGE loading buffer.

Identification of the Cleavage Sites by Gelatinase B in Denatured Human Collagen Type II. Human collagen type II was dissolved and denatured at 5 mg/mL in 0.1% acetic acid by being heated for 10 min at 65 °C. After neutralization, denatured collagen type II was incubated at 1 mg/mL with 0.7 ng/μL activated gelatinase B in assay buffer. The resulting cleavage products were separated by reversed phase HPLC on a PepMap C18 column with a gradient of CH_3CN in 0.1% acetic acid (0 to 20% CH_3CN over the course of 100 min, followed by 20 to 80% CH_3CN over the course of 10 min). The flow from the column outlet was splitted to a fraction collector and to an EsquireLC ion trap mass spectrometer (Bruker, Bremen, Germany). The cleavage products were identified by their masses and by Edman degradation of the corresponding fractions with a Procise 491 cLC protein sequencer (Applied Biosystems, Foster City, CA), as described for bovine collagen (*12*).

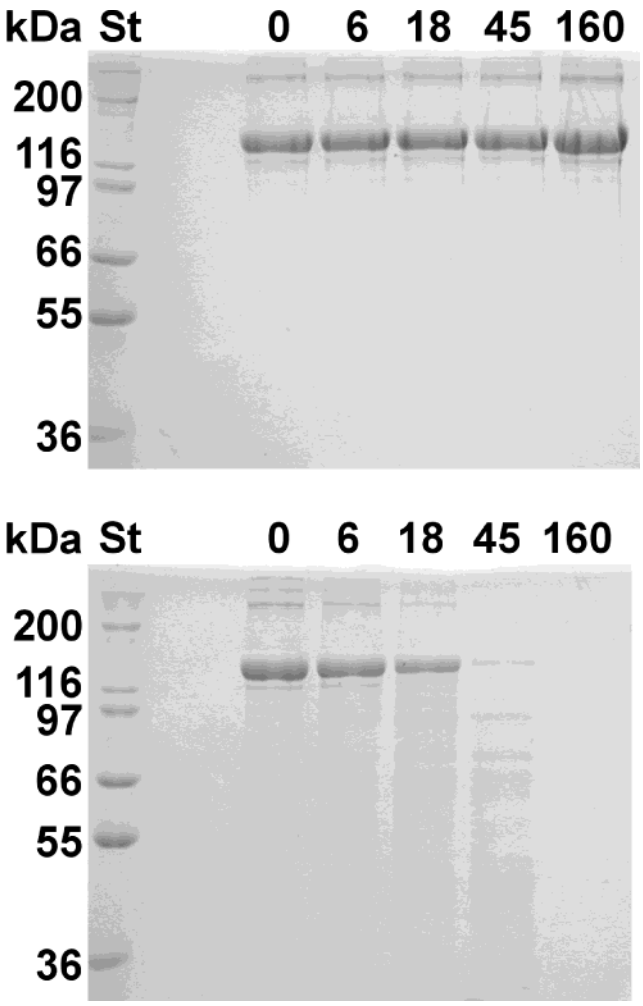

FIGURE 1: Cleavage of human collagen type II by gelatinase B. Native (top panel) or heat-denatured (bottom panel) natural human collagen type II was incubated with activated neutrophil gelatinase B for the indicated time intervals (in hours). Subsequently, the samples were analyzed by reducing SDS−PAGE and Coomassie Brilliant Blue staining. Molecular mass standard (St) proteins are shown (kilodaltons).

Determination of T-Cell Reactivity against Gelatinase B-Generated Peptides from Collagen II. Bovine collagen II was digested with gelatinase B, and the resulting fragments were separated as indicated above for human collagen II. HPLC fractions were made compatible with tissue culture conditions through several rounds of dilution followed by lyophilization. This process drives off the volatile organic reagents, leaving the digest ready for dissolution in tissue culture media. Male DBA/1 mice that were 6−8 weeks of age (Jackson Laboratories, Bar Harbor, ME) were immunized with 50 μL of an emulsion containing an equal volume of bovine CII and Complete Freund's Adjuvant. Ten days after

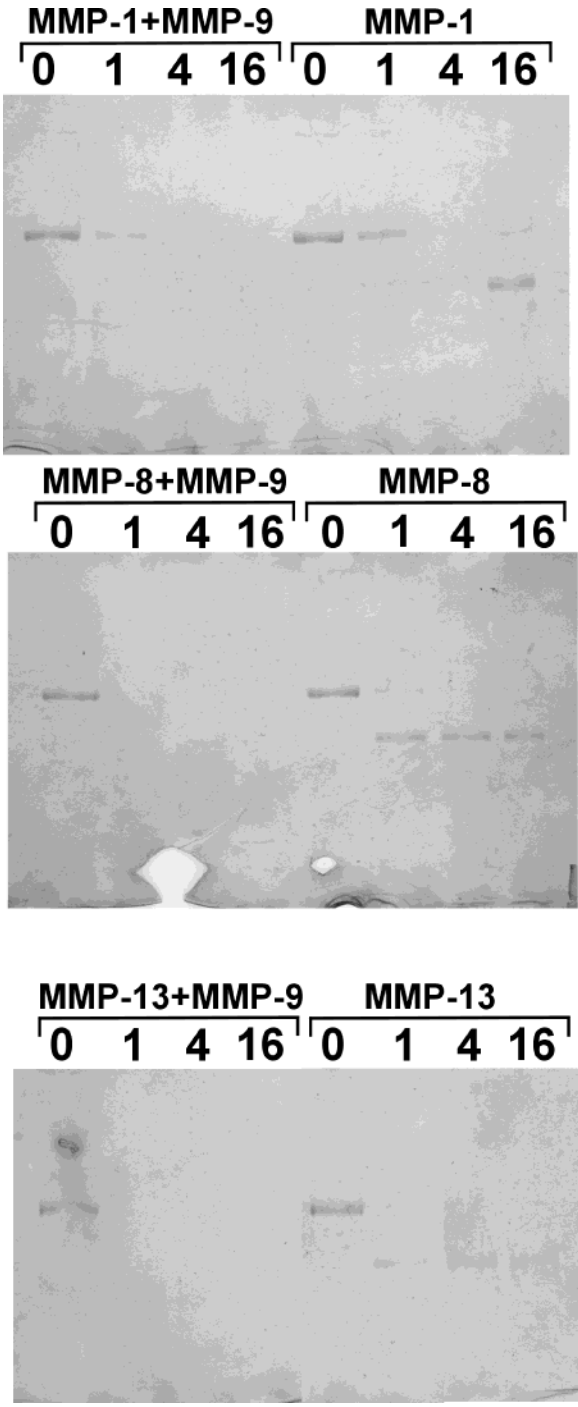

FIGURE 2: Cleavage of human native collagen type II by collagenases and gelatinase B. Human native collagen II was incubated for various time intervals (indicated in hours) with the indicated collagenases, alone or in combination with gelatinase B (MMP-9). Subsequently, the samples were analyzed by reducing SDS−PAGE and Coomassie Brilliant Blue staining.

immunization, draining lymph nodes were removed, disassociated, and washed in HL-1 (Cambrex Corp., East Rutherford, NJ). Lymphocytes and antigen-presenting cells were cultured in 96-well plates to which the HPLC fractions were added, at 4×10^5 cells/well in 300 μL of HL-1 medium supplemented with 50 μM 2-mercaptoethanol, 2 mM glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 0.1% BSA (fraction V, IgG free, low endotoxin, Sigma Chemical Co., St. Louis, MO) at 37 °C in 5% humidified CO_2 for 4 days. Eighteen hours prior to the termination of

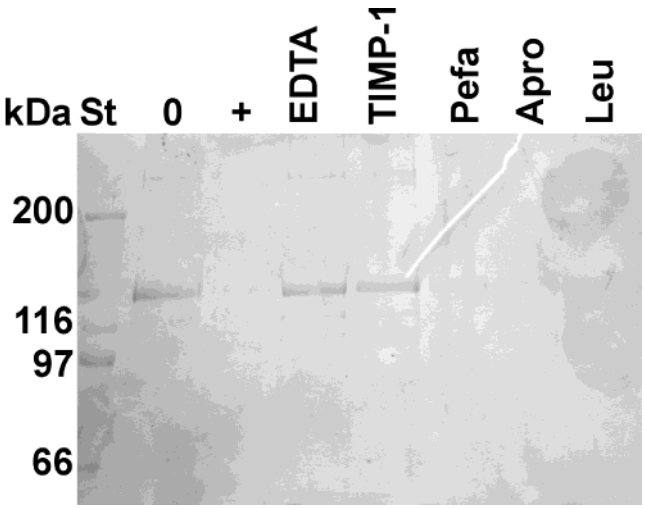

FIGURE 3: Degradation of human native collagen type II by neutrophil degranulate. Purified human native collagen II was incubated for 48 h with stromelysin-1-activated neutrophil degranulate, with the addition of the indicated protease inhibitors. Subsequently, the samples were analyzed by reducing SDS−PAGE and Coomassie Brilliant Blue staining. Lane 0 contained an equivalent amount of collagen II without incubation and (+) after incubation for 48 h with neutrophil degranulate but without inhibitors. St, molecular mass standard proteins; Pefa, Pefabloc; Leu, leupeptin; Apro, aprotinin.

the cultures, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to each well. Cells were harvested onto glass fiber filters, and counted on a Matrix 96 direct ionization β-counter (Packard Instrument Co., Meriden, CT). All proliferation assays using control antigens were cultured in triplicate and expressed as mean disintegrations per minute (dpm).

RESULTS

Cleavage of Collagen Type II from Human Cartilage. Native human type II collagen was purified from cartilage using the differential salt fractionation methods (*16*). Cleavage of human collagen II by gelatinase B was only observed after heat denaturation (Figure 1), which is similar to the degradation of bovine type II collagen (*12*). Since heat denaturation is not a physiologically relevant process, we tried to determine whether gelatinase B can contribute to the degradation of collagen type II under physiological circumstances. Interstitial and neutrophil collagenases and collagenase-3 (MMP-1, -8, and -13, respectively) have been shown to be able to perform a single cleavage in native triple-helical collagens types I−III, resulting in the formation of typical $^3/_4$ and $^1/_4$ collagen fragments (for review, see ref *17*). This does not result in the unwinding of the triple helix, unless further fragmentation occurs (*11*). Therefore, we analyzed whether gelatinase B can further degrade the $^3/_4$ fragment of collagen II generated by collagenases. Incubation of native human collagen II with the collagenases resulted in the expected formation of the $^3/_4$ fragment (Figure 2), neutrophil collagenase/MMP-8 being the most efficient enzyme for this process. Importantly, the combined action of a collagenase and gelatinase B resulted in complete degradation of native collagen II. Further, we analyzed whether the collagenase contributed to the degradation of the $^3/_4$ fragment, or whether gelatinase B can perform this degradation alone. Variation of the collagenase concentration

FIGURE 4: Localization of the cleavage sites of gelatinase B and the post-translational modifications in denatured natural human collagen type II. Denatured human collagen type II was digested with gelatinase B. The resulting fragments were separated by RP-HPLC and analyzed by mass spectrometry and Edman degradation. Thirty-one different cleavage sites were identified (▼), of which seven (●) are new compared with the previously determined cleavage sites in bovine type II collagen. The cleavage site by the collagenases (which is also a cleavage site for gelatinase B when the collagen is denatured) is denoted with an asterisk. In addition, a large number of side chain modifications were localized, including hydroxylation (OH) and glycosylation ($OHex_n$). The two known immunodominant epitopes are boxed. Two differences in the determined amino acid sequence, compared to the translated gene-derived sequence, are in bold. Sequences determined by Edman degradation are underlined.

resulted in variation of the rate of the $^3/_4$ cleavage, but the further degradation of the $^3/_4$ fragment by gelatinase B was unaffected, even when the collagenase concentration was significantly lower than the gelatinase B concentration (data not shown). This indicates that gelatinase B performs the degradation of the $^3/_4$ fragment in a manner independent of the collagenase that is used, and that a complex such as that described for interstitial collagenase and gelatinase B (*18*) may be active, but is not required.

Cleavage of Human Native Collagen Type II by Neutrophil Degranulate. To investigate whether the proposed mechanism for collagen II degradation is relevant for the *in vivo* situation, the cleavage of native human collagen type II by neutrophil degranulate was analyzed. Neutrophil degranulate, from 10^7 stimulated neutrophils/mL, was first incubated with stromelysin-1 to activate gelatinase B and neutrophil collagenase. This activated degranulate was used to digest native human collagen type II, resulting in complete degradation (Figure 3). Addition of MMP inhibitor EDTA or TIMP-1 completely inhibited the degradation, while the serine protease inhibitors Pefabloc, aprotinin, and leupeptin had no effect. These data indicate that the combination of the MMPs neutrophil collagenase and gelatinase B is capable of completely degrading native type II collagen in physiological situations.

Gelatinase B Releases Glycosylated Epitopes from Human Collagen Type II. Human collagen type II was heat-denatured and subsequently digested with activated natural human neutrophil gelatinase B. The resulting fragments were separated by RP-HPLC, and the masses of all of the fragments were determined by on-line mass spectrometry. Parallel fractions were collected, and the amino-terminal sequences of the fragments in the fractions were determined by Edman degradation (Figure 4 and Table 1). Thirty-one cleavage sites were identified, seven of which were previously not detected in bovine denatured collagen type II (*12*). Interestingly, one of the new cleavage sites was found close behind the major immunodominant epitope, namely, at position 305. Our data indicate that cleavage sites are fewer than 20 residues from each immunodominant epitope and show that gelatinase B can release the immunodominant fragments from collagen II. This may cause the onset of and perpetuate an ongoing autoimmune reaction. The technology that was used enabled us to localize in the sequence an extensive number of post-translational modifications, namely, hydroxylations of prolines and lysines and glycosylations of hydroxylysines (*12*). This was possible since hydroxylation of proline and of lysine result in specific signals in the Edman degradation analysis, and the glycosylation of a hydroxylysine results in the absence of signal. The presence of sugars

Table 1: Identification of the Peptides Generated by Gelatinase B Cleavage of Denatured Human Collagen II

fragment	theoretical mass (Da)[a]	mass (Da)[b]	post-translational modifications[c]	fractions
699−713	1348.72	1704.6	OH + $OHex_2$	25−27
		1542.9	OH + OHex	25−27
147−158	1166.61	1182.8, 1166.7	0/1OH	32, 33, 37
927−...[d]				35
147−161	1365.71	1381.8	OH	37
207−224	1588.74	1636.8	3OH	39, 40
519−533	1500.70	1533.0	2OH	41
357−380	2197.12	2586.1, 2602.4	3/4OH + $OHex_2$	43, 47
		2407.8, 2423.5, 2440.5	2/3/4OH + OHex	44, 47, 49
		2230.4, 2246.1, 2262.6, 2278.6	2/3/4/5OH	44, 45, 47, 48, 50
306−326	1973.00	2006.0	2OH	46, 47
363−380	1673.80	1721.9	3OH	47
519−539	2109.04	2142.1	2OH	50
618−650	2942.11	3007.8	4OH	50
381−413	3021.26	3068.6, 3084.9	3/4OH	52−55
327−356	2847.06	2894.2	3OH	53
699−...[d]				53
414−...[d]				53, 55
42−92	4768.17	4864.8, 4883.0, 4897.6	6/7/8OH	55−59
42−...[d]				60
93−146	5038.55	5198.4	10OH	59
93−...[d]		5360.8	9OH + OHex	59
		5522.3	9OH + $OHex_2$	59
		5298.65, 5281.25	4/5OH + OHex	62
				60, 75, 78, 79, 81
846−926	7145.65	7243.2, 7258.0, 7274.3	6/7/8OH	60, 61
447−...[d]				60
699−...[d]				60
147−224	6820.35	7129.1, 7288.3, 7110.2, 7273.2	7/8OH + $OHex_{1/2}$	62, 63
714−...[d]				65, 66, 68
750−794	4072.25	4136.88, 4152.88	4/5OH	66
654−698	3966.17	4030.85, 4046.83, 4062.83	4/5/6OH	66
654−...[d]	7784.44	7944.4, 7926.7, 7912.4	8/9/10OH	65
273−356		8106.7	9OH + OHex	65
		8270.4	8OH + 2OHex	65
		8434.4	8OH + $OHex_2$ + OHex	65
				65
567−617	4675.18	4706.8, 4722.9, 4739.3	2/3/4OH	68−70
		4883.9, 4902.8	2/3OH + OHex	68, 69
		5046.3, 5063.8	2/3OH + $OHex_2$	68, 69
		5224.8	2OH + OHex + $OHex_2$	68
795−845	4752.24	4817.4, 4833.6, 4848.4, 4865.4, 4881.0	4/5/6/7/8OH	70−74
795−...[d]				75
801−845	4169.58	4250.1, 4264.8, 4280.8	5/6/7OH	74−76
801−...[d]				78
483−539	5353.86	5482.2, 5466.1, 5451.0, 5434.2	5/6/7/8OH	77−80
483−533	4745.16	4858.2, 4841.4, 4825.8	5/6/7OH	79−82
16−41	2355.75	2387.3	2OH	82, 83
483−518	3261.60	3357.7, 3341.7, 3326.5, 3310.5	3/4/5/6OH	84−91
954−1038	7527.23	7639.8, 7653.8, 7671.3, 7686.3	7/8/9/10OH	89−91

[a] Without post-translational modifications, the monoisotopic mass < 2200 Da and the average mass > 2200 Da. [b] Only individual peaks with a signal-to-noise ratio of at least three were considered, and for each indicated mass, at least three peaks (with different charges and/or with different isotopes) were found. The maximum mass measurement error was 0.3 Da (m/z) in the used mass range (400−2000 Da/charge). [c] OHex, hexose (162.14 Da) linked to a post-translationally added hydroxyl (total mass of 178.14 Da); $OHex_2$, dihexose (324.28 Da) linked to a post-translationally added hydroxyl (total mass of 340.28 Da); 4/5OH, four to five hydroxylations. [d] Unambiguously detected by Edman degradation, but not by mass spectrometry.

was confirmed by the measured masses of the fragments. Particularly interesting are the modifications of the segment of residues 273−356, which contains the main immunodominant epitope. The lysine at position 283, in this epitope, is partially hydroxylated. Furthermore, the mass spectrometry data indicate partial glycosylation of the fragment, with zero to three different monosaccharides, in approximately equal amounts. Three lysines are present in this peptide, which are positioned before a glycine and therefore susceptible to modification. However, the lysine at position 346 is not hydroxylated, as shown by the Edman degradation (the detection limit is approximately 10% of the actual signal). This lysine is also not glycosylated, as the absence of glycosylation on the fragment of residues 327−356 was shown by mass spectrometry (the detection limit was <5% of the total amount of the fragment). Since the sugars on collagen II contain one or two monosaccharides, our data show for the first time unambiguously that the lysines at positions 283 and 289 in the main immunodominant epitope of human collagen II are partially hydroxylated and also partially glycosylated. This has major consequences for the immunoreactivity of this peptide, since modification of this

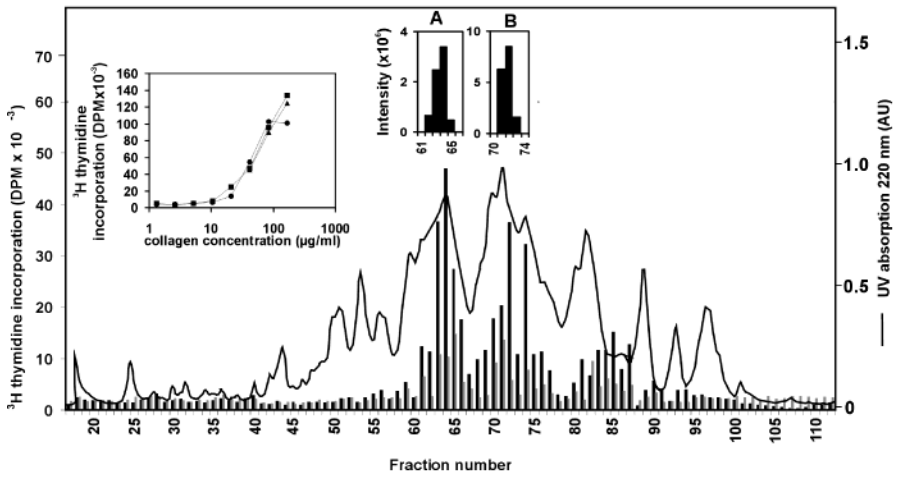

FIGURE 5: T-Cell reactivity against gelatinase B-generated remnant epitopes from collagen type II. Denatured bovine type II collagen was incubated with gelatinase B, and the resulting fragments were separated by RP-HPLC. T-Cell reactivities were determined against the collected fractions (the black and gray histograms document data from two separate experiments), and fragments were identified by mass spectrometry and Edman degradation. The intensities of the signals on the mass spectra that correspond to the peptides containing the immunodominant epitopes [peptide 447−482 (A) and peptide 273−356 (B)] in the different fractions are given at the top. In fraction 72, all the different glycoforms of peptide 273−356 were present, but in fraction 71, only the peptide with two or more hexoses was found. In fraction 73, only the unglycosylated variant was found. In fraction 74, peptide 273−356 was identified by Edman degradation among other fragments, but was not detected by mass spectrometry. In the left inset, the dose-dependent T-cell reactivities against untreated denatured α1(II) collagen (▲) and against denatured collagen II digested with gelatinase B for 0 h (■) and 4 h (●) are compared.

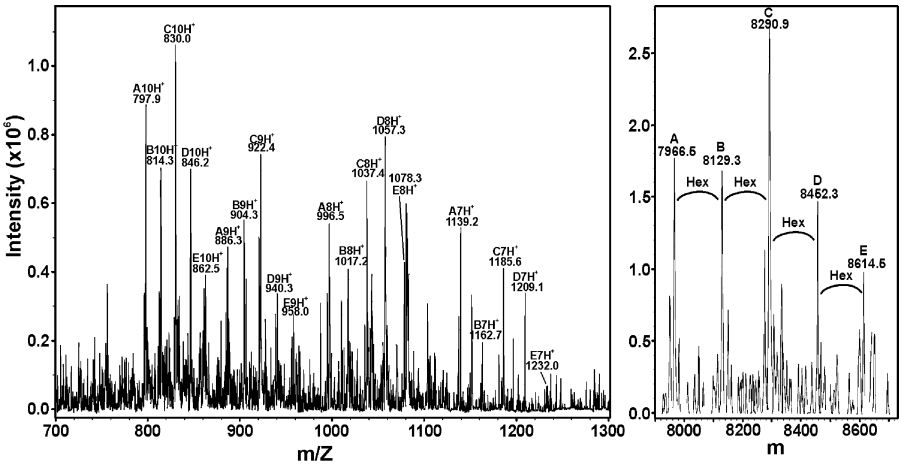

FIGURE 6: Mass spectrum of the immunodominant epitope (273−356). The mass spectrum of fraction 72 of the RP-HPLC separation of digested bovine collagen II is shown as unprocessed data (left panel) and after charge deconvolution (right panel). In the left panel, the charges of the different compounds are indicated by the numbers of protons. Compound A corresponds to peptide 273−356 without sugars, and the other indicated compounds (B−E) correspond to the same peptide with one or more additional hexoses (Hex).

peptide by short O-linked glycans has been shown to influence strongly the binding to T-cell receptors (*19*).

T-Cell Reactivity against Natural Collagen II (Glyco-)-Epitopes. The immunogenicity of gelatinase B-cleaved collagen II was compared with that of uncleaved denatured collagen II by assessment of the induction of T-cell proliferation (Figure 5, inset). The cleavage had no influence on T-cell reactivity, confirming that gelatinase B does not destroy the immunodominant epitopes of collagen II. Instead of synthetic peptide epitope scanning, we determined the T-cell reactivities against natural glycopeptides to confirm the identity of the immunodominant epitopes. Bovine denatured collagen II was digested with gelatinase B, and the fragments were separated by RP-HPLC followed by on-line mass spectrometry analysis. The T-cell reactivities of all fractions were determined, as shown in Figure 5. In addition, mass spectrometry and Edman degradation analysis were performed on the fractions with significant T-cell reactivity. As expected, the immunodominant epitopes [peptide 464−475-(A) and peptide 273−356(B)] were identified in the fractions inducing the strongest T-cell proliferation. For instance, the mass spectrum of fraction 72 is presented in Figure 6, showing peptide 273−356 with zero to four hexoses. Furthermore, a number of other fractions (e.g., fractions 81−87), in which the two known main immunodominant epitopes were not detected, also induced significant T-cell proliferation. This indicates that additional peptides from natural collagen II induce a T-cell response, in contrast with previous epitope scanning data with synthetic peptides (*20*, *21*).

DISCUSSION

Collagens are proteins characterized by the repeated Gly-Xaa-Xaa sequence, in which Xaa is often proline. This sequence allows the formation of a triple-helical chain, formed by three separate peptide chains. A large number of collagens are known (*22*). The triple helices of collagen types

I−III show considerable lateral association after removal of the amino- and carboxy-terminal propeptides, resulting in the formation of large macromolecular fibrils, such as in tendons and cartilage. The degradation of collagens plays a fundamental role in a variety of physiological and pathological processes, since it is required for cell migration, morphogenesis, and tissue repair. However, the triple-helical structure is highly resistant to proteolysis, because the peptide bonds are inaccessibly oriented toward the inner side of the helix.

The classical mammalian collagenases, namely, interstitial collagenase (MMP-1 or collagenase 1), neutrophil collagenase (MMP-8 or collagenase 2), and collagenase 3 (MMP-13), cleave the triple helix of the fibril-forming collagen types I−III at a single site at $^3/_4$ of the length of the molecule. In addition, membrane-type MMP-1 (MT-MMP-1) has similar collagenolytic activity. Collagenase binds to the native triple helix at the $^3/_4$ position through extensive molecular contacts, and these possibly induce a conformational change leading to the local unwinding of the helix (*17*). Before this study, it was relatively unclear how the resulting fragments are further degraded. One hypothesis was that the cleavage leads to denaturation of the whole helix, resulting in the susceptibility to a large number of proteases. However, it has recently been shown that the triple-helical structure persists after the cleavage by a collagenase (*11*, *23*). Here, we show that gelatinase B can be responsible for the further degradation of the $^3/_4$ fragment into small peptides, although it is not capable of cleaving full-length native collagen in the absence of a collagenase. In a more physiological context, these data were confirmed with crude neutrophil degranulate, in which the MMPs and not the serine proteases play an essential role in the degradation of native collagen type II. In addition, we identified all the cleavage sites by gelatinase B by means of mass spectrometry and Edman degradation analysis of the fragments. Our data show that the immunodominant epitopes remain intact and become available for binding to, for example, MHC-II of antigen-presenting cells after digestion with gelatinase B (*12*, *24*). The remnant epitopes may be further processed intracellularly by antigen-presenting cells, or they may be loaded extracellularly in empty MHC-II molecules of the antigen-presenting cells (*25*, *26*). This may lead to activation of autoreactive T-cells, resulting in the enhancement and perpetuation of autoimmune arthritis (*27*).

By the combination of mass spectrometry and Edman degradation, we were also able to pinpoint post-translational modifications in human collagen II. Glycosylation is known to protect against proteolysis, and post-translational modification of collagen II has been shown to influence its immunogenicity, as immunization with underglycosylated collagen II results in a lower incidence and lower severity of arthritis (*28*, *29*). Glycosylated peptides bound to MHC-II were shown to stimulate other T-cell clones than unglycosylated peptides did (*19*). Particularly interesting is the fact that transgenic mice expressing human HLA-DR-4 instead of endogenous MHC-II and expressing human collagen II instead of mouse collagen II were shown to be incompletely tolerant of the glycosylated form of the main immunodominant epitope, and T-cells from patients with rheumatoid arthritis preferentially recognize the glycosylated form (*30*). However, the latter observation may also be the result of cross-reactivity to an unrelated peptide, as this occurs frequently with glycosylated peptides (*31*, *32*). Before this study, it was not known whether the immunodominant epitope in natural human collagen II is effectively glycosylated. Here, we show that K_{283}, in the immunodominant epitope, and K_{289}, at the edge of the epitope, are partially hydroxylated and partially glycosylated in natural human collagen II. Serendipitously, we found that T-cells may also be activated by other modified peptides from collagen II, which were not detected in previous classical epitope scanning experiments with unmodified synthetic peptides.

Together, these data underline the importance of extracellular proteolysis and posttranslational modifications of collagen II in the immunopathogenesis of rheumatoid arthritis. Posttranslationally modified remnant epitopes, released by the combined action of collagenases and gelatinase B during an inflammatory reaction, may assist in breaking the tolerance of collagen II and may also result in the perpetuation of the autoimmune reaction in patients with rheumatoid arthritis.

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