Recombinant Human Type II Collagens with Low and High Levels of Hydroxylysine and Its Glycosylated Forms Show Marked Differences in Fibrillogenesis in Vitro*

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Type II collagen is the main structural component of hyaline cartilages where it forms networks of thin fibrils that differ in morphology from the much thicker fibrils of type I collagen. We studied here in vitro the formation of fibrils of pepsin-treated recombinant human type II collagen produced in insect cells. Two kinds of type II collagen preparation were used: low hydroxylysine collagen having 2.0 hydroxylysine residues/1,000 amino acids, including 1.3 glycosylated hydroxylysines; and high hydroxylysine collagen having 19 hydroxylysines/1,000 amino acids, including 8.9 glycosylated hydroxylysines. A marked difference in fibril formation was found between these two kinds of collagen preparation, in that the maximal turbidity of the former was reached within 5 min under the standard assay conditions, whereas the absorbance of the latter increased until about 600 min. The critical concentration with the latter was about 10-fold, and the absorbance/microgram collagen incorporated into the fibrils was about one-sixth. The morphology of the fibrils was also different, in that the high hydroxylysine collagen formed thin fibrils with essentially no interfibril interaction or aggregation, whereas the low hydroxylysine collagen formed thick fibrils on a background of thin ones. The data thus indicate that regulation of the extents of lysine hydroxylation and hydroxylysine glycosylation may play a major role in the regulation of collagen fibril formation and the morphology of the fibrils.

The collagens are a family of extracellular matrix proteins that play a dominant role in maintaining the structural integrity of various tissues. A well coordinated deposition of the components of the extracellular matrix is essential to achieve and maintain their physiological function (for reviews, see Refs. 1–3).

Type II collagen is the main structural component of hyaline cartilages and forms their fibrous scaffold, which interacts with various types of proteoglycan. It forms networks of thin fibrils that differ in morphology from the much thicker fibrils of type I collagen, the main fibril-forming collagen in most other tissues. Many factors such as the presence of various proteoglycans (4–6) and interactions between collagen types (7–9) influence collagen fibril formation and the architecture of the resulting fibrils formed. Thus the differences in fibril architecture between tissues are not necessarily dependent on differences between the various collagen types themselves. However, fibril formation experiments with purified collagens in vitro have demonstrated that the differences in structure between type II and type I collagen molecules appear to be sufficient to explain many of the characteristic differences between these two types of fibril present in tissues (10). The kinetics for the assembly of type II collagen fibrils in such experiments differed markedly from those for the assembly of type I collagen, and the critical concentration for type II collagen at 37 °C was about 50 times greater (10). In addition, the type II collagen fibrils formed in vitro were much thinner than those of type I collagen and formed three-dimensional networks (10).

The extents of hydroxylation of lysine residues and glycosylation of hydroxylysine residues in type II collagen are much higher than those in the other two major fibril-forming collagens, types I and III (11). Thus the differences in fibril morphology between types II and I might be related to these differences. The functions of the hydroxylysine-linked carbohydrate units are nevertheless poorly understood at present. As these are the most extrusive groups on the surface of the collagen molecule, it has been suggested that they may influence fibril assembly (12). Fibril formation experiments with type I collagen in vitro have demonstrated that this collagen with an experimentally produced increased degree of lysine hydroxylation and hydroxylysine glycosylation formed thinner fibrils than the same protein with a normal degree of these modifications, but only a minor extent of overglycosylation could be achieved in these experiments (13). In agreement with these data, type II collagen from the annulus fibrosus, which has a slightly higher extent of lysine hydroxylation and hydroxylysine glycosylation than that from articular cartilage, formed fibrils with a smaller diameter (14). The possibility has not been excluded, however, that the presence of minor amounts of proteoglycan in the collagen preparations may have contributed to these differences.

To allow study in detail of the effects of the extents of lysine hydroxylation and hydroxylysine glycosylation on fibril formation, molecular collagen species are required that are genetically identical but differ markedly with respect to these modifications. It has recently been reported that recombinant human collagens with stable triple helices can be produced in insect cells by coinfection with baculoviruses coding for the polypeptide chains of the collagen to be produced and the two types of subunit of prolyl 4-hydroxylase, the key enzyme of collagen synthesis (15, 16). The use of an additional baculovi-

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rus coding for lysyl hydroxylase (17) markedly increased the level of lysine hydroxylation and the amount of glycosylated hydroxylysine residues, and it was possible to obtain pepsin-treated recombinant human type II collagen preparations in which the hydroxylysine residue content ranged from 1 to 21/1,000 and that of glycosylated hydroxylysine residues from less than 1 to 9/1,000 (18). We used such recombinant type II collagen preparations here to study their in vitro fibrillogenesis.

MATERIALS AND METHODS

Production and Isolation of Recombinant Type II Collagens with Low and High Hydroxylysine and Glycosylated Hydroxylysine Content—High Five insect cells (Invitrogen) were cultured in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) in shaker flasks at 27 °C. For the production of recombinant type II collagen with a low hydroxylysine content, the cells were infected with viruses coding for rhproCINIII and 4PHβ as described previously (18), whereas for a high hydroxylysine content they were coinfected with the above viruses and an additional virus coding for human lysyl hydroxylase (17, 18). The cells were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, incubated on ice for 30 min, and centrifuged at 16,000 g for 30 min (18). The supernatant was chromatographed on a DEAE cellulose column (DE-52, Whatman), equilibrated and eluted with a 0.2 M NaCl and 0.05 M Tris buffer, pH 7.4, the void volume being collected. The pH of the sample was lowered to 2.0–2.5, and the sample was digested with a final concentration of 150 μg/ml of pepsin for 1 h at 22 °C. The pepsin was irreversibly inactivated by neutralization of the sample followed by overnight incubation on ice. The recombinant collagen was precipitated by adding solid NaCl to a final concentration of 4 M and centrifuging at 16,000 g for 1 h. The pellet was dissolved in a 0.5 M NaCl, 0.5 M urea, and 0.05 M Tris buffer, pH 7.4, for 1 day. The sample was chromatographed on a Sephadryl HR-500 gel filtration column (Amersham Pharmacia Biotech), eluted with a solution of 0.2 M NaCl, and 0.05 M Tris, pH 7.4, dialyzed against 0.1 M acetic acid, and lyophilized.

In Vitro Fibril Formation—To prepare stock solutions, the collagen preparations were dissolved in 0.05% acetic acid at a concentration of 1 mg/ml and centrifuged at 200,000 g for 1 h at 4 °C. The upper half of the supernatant was withdrawn and adjusted to a final concentration of 200 μg/ml, checked with a Jasco J-500A spectropolarimeter. The collagen self-assembly conditions followed a modification of the method described by Williams et al. (19): briefly, 100 μg/ml of type II collagen, 30 mM K2HPO4, and 135 mM NaCl, pH 7.4. The samples were then transferred to a thermostatically controlled quartz cuvette. Fibril formation was triggered by increasing the incubation temperature to 34 °C. Optical density at 313 nm was monitored in steps of 2 min with a Perkin-Elmer Lambda2 spectrophotometer (connected to a personal computer). Each recording was limited to 900 min. The sample was centrifuged at 5,000 × g for 30 min after the experiment, and the concentration of the supernatant was measured by CD spectroscopy to determine the amount of aggregated collagen. The experiments to study the effects of collagen concentration were performed as above except that the collagen concentration was varied, and the time was 600 min.

Electron Microscopy—Aliquots of the assembly mixtures for electron microscopy were incubated in parallel with the turbidity time assay. After 1,000 min, samples of 4 × 5 μl were transferred to formvar-coated copper grids using a micropipette, and the fibrils were allowed to settle for 30 min. The buffer was then drained cautiously with a filter paper, and three washing steps were performed. The fibrils were stained with freshly prepared 1% uranyl acetate, dissolved in distilled water for 2 min, washed 3 times more, and dried. The grids were examined using a Zeiss EM 109 electron microscope. Fibril width was measured with an image analysis system (Optoquant, Lübeck, Germany) using an internal distance of 10 periods in the collagen banding pattern.

Low Angle Rotatory-shadowing Electron Microscopy—Recombinant type II collagen preparations were dialyzed against a solution of 50% glycerol in 0.05% acetic acid for 16 h at 4 °C. The samples were sprayed onto freshly cleaned mica using an air brush. The droplets on the mica were dried at room temperature at 10–12 mm Hg for 12 h in a vacuum coater (Edwards 306). The dried specimens were rotatory-shadowed with platinum using an electron gun positioned at 6° to the mica surface and then coated with a film of carbon generated by an electron gun positioned at 90° to the mica surface. The replica was floated on distilled water and collected on a grid covered with a formvar film. The specimens were studied with a Zeiss 109 transmission electron microscope.

Other Assays—Protein samples hydrolyzed in 6 M HCl for 24 h at 110 °C under N2 were used for amino acid analyses performed in an Applied Biosystems 421 or Beckman system 6300 analyzer. The glycosylated hydroxylysine content was determined by hydrolyzing collagen samples in 2 M KOH for 24 h at 110 °C in 2-ml polypyrrole reaction vials. Hydroxylysine and its glycosides were separated by cation exchange chromatography on Dowex 50WX-8 as described previously (18, 20). A glucosylgalactosylhydroxylysine standard (20) was used to quantify the glucosylgalactosylhydroxylysine and galactosylhydroxylysine. For the analysis of pepsinized collagen chains, the samples were studied by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions followed by Coomassie staining.

RESULTS AND DISCUSSION

Content of Hydroxylysine and Its Glycosides in the Type II Collagen Preparations—The recombinant human type II pro-collagen preparations produced in insect cells were converted to collagens by digestion with pepsin at 22 °C for 1 h, and the pepsin-treated collagens were purified. Two kinds of recombinant human type II collagen preparation were used: low hydroxylysine collagen, produced without recombinant lysyl hydroxylase, and high hydroxylysine collagen, produced with recombinant lysyl hydroxylase. Both types were purified when studied by SDS-polyacrylamide gel electrophoresis under reducing conditions followed by Coomassie staining (Fig. 1) and also by amino acid analysis (details not shown).

The recombinant low hydroxylysine type II collagen preparation had low levels of total hydroxylysine and its glycosides.

**Table I**

| Amino acid                        | Recombinant Low Hydroxylysine Type II Collagen | Recombinant High Hydroxylysine Type II Collagen | Nonrecombinant Type II Collagen from Articular Cartilage |
|-----------------------------------|-----------------------------------------------|-----------------------------------------------|----------------------------------------------------------|
| Total hydroxylysine               | 2.0                                           | 19.0                                          | 15.3                                                     |
| Nonglycosylated hydroxylysine     | 0.7                                           | 10.1                                          | 9.2                                                      |
| Galactosylhydroxylysine           | 0.5                                           | 6.3                                           | 4.2                                                      |
| Glucosylgalactosylhydroxylysine  | 0.8                                           | 2.6                                           | 1.9                                                      |

* Ref. 14.
(Table I), whereas the recombinant high hydroxylysine preparation had levels that were even higher than those in nonrecombinant human type II collagen from articular cartilage (Table I). As discussed elsewhere (18), the marked increase in the quantity of glycosylated hydroxylysine residues obtained by coexpression with lysyl hydroxylase must be due to the presence of a high level of endogenous collagen glycosyltransferase activities in the High Five insect cells.

Fibril Formation in Vitro—Formation of fibrils of the two kinds of human recombinant type II collagen was studied using an assay based on the increase in absorbance at 313 nm in a 100 μg/ml collagen solution at 34 °C as a function of time. A marked difference was found between the low and high hydroxylysine collagens, in that the maximum absorbance of the former was reached within 5 min, whereas the absorbance of the latter increased for about 600 min (Fig. 2). The amount of low hydroxylysine collagen incorporated into the fibrils was slightly smaller than that of high hydroxylysine collagen but was more than 90% in both cases (details not shown). Thus the maximum absorbance/μg collagen incorporated into the fibrils of the low hydroxylysine collagen was about 6 times that observed with the high hydroxylysine collagen (Fig. 2).

The critical concentrations for fibril formation also differed markedly between the two types of recombinant human type II collagen, the apparent critical concentration for the low hydroxylysine collagen being less than 10 μg/ml, whereas that for the high hydroxylysine collagen was about 70 μg/ml (Fig. 3).

Electron Microscopy—Electron micrographs of the two kinds of recombinant type II collagen when visualized by rotatory shadowing showed predominantly monomers of uniform length (details not shown). No differences were detected between the low and high hydroxylysine collagens with this technique.

Electron microscopy of the fibrils formed by the high hydroxylysine collagen showed that they were typically very thin with essentially no interfibril interaction or fibril aggregation (Fig. 4A). A few short, slightly thicker fibrils could also be seen with sharp tips on both ends (Fig. 4A). In contrast, the low hydroxylysine collagen formed thick fibrils on a background of thin ones (Fig. 4B).
To study the distribution of the fibril diameters in more detail, a histogram of 200 measured fibril diameters was plotted against their relative frequency (Fig. 5). Fibril aggregates were excluded from these measurements, and thus the histogram shows only diameters of single fibrils. The high hydroxylysine collagen showed the highest frequency of fibrils with a diameter of about 10 nm, whereas the low hydroxylysine collagen had the highest frequency of fibrils with a diameter of about 20 nm, and some fibrils had diameters exceeding 60 nm (Fig. 5).

All experiments reported here were carried out with type II collagen samples prepared from the corresponding procollagens by removing the N and C propeptides by digestion with pepsin at 22 °C for 1 h. Although the collagen triple helix is resistant to proteolytic enzymes, such treatment is likely to degrade part of the N and C telopeptides, the short nontriple-helical sequences at the ends of the collagen molecules (1–3). Many previous studies have shown that pepsin-treated collagens tend to form fibrils with a smaller diameter and a lesser degree of a highly resolved cross-striation pattern than collagens produced from procollagens by the cleavage with procollagen N and C proteinases (see Ref. 8). Thus the fibrils studied here are not identical to those formed in vivo, but the pepsin treatment cannot explain any of the differences in fibril assembly and morphology between the recombinant low and high hydroxylysine type II collagens.

The glucosylgalactose moiety is highly hydrophilic and has a length of about 1 nm (14). This moiety is oriented parallel to the backbone of the collagen molecule and shields three or four amino acid residues (14). The glucosylgalactose moiety can thus be expected to inhibit lateral growth of collagen fibrils because it reduces the surface available for hydrophobic interactions. This hypothesis is supported by our morphological findings and also by the data on fibril formation, because the final turbidity of the high hydroxylysine collagen was only about one-sixth of that of the low hydroxylysine variety.

Turbidity is related to molecular mass per unit length in the case of very long molecules. The difference in turbidity between the low and high hydroxylysine collagens accounts for a threefold difference in fibril diameter. This is not fully in accordance with the morphometric analysis of single fibrils, however, because only a portion of the fibrils deviated in diameter. It is likely that interfibrillar interactions or banding of unit fibrils contributed to the increased light scattering, even though the electron micrographs seem to suggest that the thick fibrils appear to be formed from thin unit fibrils.

Conclusions—The data indicate the presence of marked differences in fibril formation between recombinant human type II collagens containing low and high amounts of hydroxylysine and its glycosylated forms. The maximal absorbance of the low hydroxylysine collagen was reached within 5 min, whereas the absorbance of the high hydroxylysine collagen increased for about 600 min and was only about one-sixth of that obtained with the former. Furthermore, the critical concentration for fibril formation with the high hydroxylysine collagen was about 10 times that with the low hydroxylysine collagen. The morphology of the fibrils formed was also different, in that the high hydroxylysine collagen formed very thin fibrils with essentially no interfibril interaction or fibril aggregation, whereas the low hydroxylysine collagen formed thick fibrils on a background of thin ones.

The hydroxylation of lysine residues and the glycosylation of hydroxylysine residues vary markedly in extent between collagen types and even within the same collagen type, between tissues and in a given tissue in various physiological and pathological states (11, 12). The collagen molecules present in the thin fibrils found in embryonic tissues, for example, have higher extents of these modifications than the thicker fibrils found in adult tissues. The present data indicate that regulation of the extents of these modifications may play a major role in governing collagen fibril formation and the morphology of the fibrils formed in vivo.

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Fig. 5. Distribution of diameters of fibrils formed by the recombinant high (dark gray) and low (light gray) hydroxylysine type II collagens.
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