Characterization of Recombinant Human Type IX Collagen

ASSOCIATION OF α CHAINS INTO HOMOTRIMERIC AND HETEROTRIMERIC MOLECULES

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As type IX collagen is a minor cartilage component, it is difficult to purify sufficient amounts of it from tissues or cultured cells to study its structure and function. Also, the conventional pepsin digestion used for fibrillar collagens cannot be utilized for purifying type IX collagen, because it contains several interruptions in its collagenous triple helix. A baculovirus expression system was used here to produce recombinant human type IX collagen by coinfesting insect cells with three viruses containing full-length cDNAs for the α1(IX), α2(IX), and α3(IX) collagen chains together with a double promoter virus for the α and β subunits of human prolyl 4-hydroxylase. Correctly folded recombinant type IX collagen was secreted, consisting of the three α chains in a 1:1:1 ratio and showing the expected biphasic thermal melting profile. When the individual α chains were expressed, disulfide-bonded homotrimers and homodimers of the α chains were observed. When the cells were coinfected with the viruses for all three α chains, heterotrimers of α1(IX), α2(IX), and α3(IX) were detected in cell culture medium, and the other possible combinations were less prominent. When any two of the α chains were co-expressed, in addition to the homodimers and homotrimers, only α1(IX) and α3(IX) chains were disulfide-bonded. The results thus suggest that the most favored molecular species is an α1(IX)2α3(IX) heterotrimer, but the chains are also able to form disulfide-bonded heterotrimers of α1(IX) and α3(IX) chains and (α1(IX))2, (α2(IX))2, and (α3(IX))3 homotrimers.

Type IX collagen, which belongs to the group of fibril-associated collagens with interrupted triple helices, is a component of hyaline cartilage, intervertebral discs, and the vitreous body. The molecule is a heterotrimer consisting of three genetically distinct chains, α1(IX), α2(IX), and α3(IX) (1) and possesses three collagenous domains (COL1 to COL3, numbered from the C terminus) flanked by four noncollagenous domains (NC1 to NC4) (2, 3). Type IX collagen is also a proteoglycan, because a glycosaminoglycan side chain is covalently attached to the NC3 domain of the α2(IX) chain (4).

Hyaline cartilage contains mixed fibrils of types II, IX, and XI collagens, of which type II is the major component. Type XI collagen is an internal component of the fibril, whereas type IX collagen is located on the surface. Covalent lysine-derived cross-links between the central COL2 region of the α3(IX) chain and the C-telopeptide of type II collagen and between the N-terminal ends of the COL2 domains of all the type IX collagen α chains and the N-telopeptide of type II collagen stabilize the interaction between type II and IX collagens (5–8). The flexibility of the NC3 domain of type IX collagen allows the COL3 and NC4 domains to project from the fibril surface, possibly to mediate interactions between cartilage collagens and noncollagenous proteins (6, 9, 10).

The association of α chains in proper stoichiometry and register is a prerequisite for the formation of a stable collagen helix. The mechanism of chain selection and association has been studied most extensively in the fibrillar collagens, for which the crucial role of large C-terminal propeptides in chain selection and association has been demonstrated. These propeptides contain specific recognition sequences that direct the association of α chains in a collagen type-specific manner (11, 12). For example, the fibrillar collagenous polypeptides synthesized by chondrocytes, namely α1(II), α1(XI), and α2(XI), are found only as two trimeric molecules in vitro, (α1(II))3 and α1(IX)α2(IX)α1(II), despite the 10 theoretically possible combinations (13).

The factors responsible for chain selection and assembly in the case of the fibril-associated collagens with interrupted triple helices are likely to be different from those affecting the fibrillar collagens, because their C-terminal NC1 domains are much smaller and there is no appreciable homology with the C-propeptides of the fibrillar collagens. The chain assembly of type IX collagen has been studied both in vitro and in vivo. Labourdette and van der Rest (14) carried out chain association experiments in vitro using the polypeptide components of a pepsin-resistant low molecular weight fragment (15) isolated from bovine cartilage and showed that homotrimers can be formed, especially by the α1(IX) and α2(IX) chains, although the heterotrimer α1α2α3 was the predominant molecule formed when all three chains were present. Similar results were obtained using synthetic peptides containing the complete NC1 domains and five C-terminal Gly-X-Y tripeptide units of the COL1 domain (16). These peptides were able to associate into trimers stabilized by disulfide bonds, thus indicating a significant role of the NC1 domain in chain selection and association. Interestingly, generation of a mouse line harboring an inactivated Col9a1 gene (17) led to a functional knockout of all type IX collagen polypeptides, suggesting that homotrimers or heterotrimers of the α2(IX) and α3(IX) chains do not exist in vivo without the α1(IX) chain (18).
In the present work, we have used a baculovirus expression system to produce recombinant human type IX collagen in insect cells in order to study the structure and chain assembly of type IX collagen. We report here for the first time on the production of type IX collagen, which consists of three different α chains, in insect cells simultaneously with the tetrameric enzyme prolyl 4-hydroxylase, needed for the production of stable collagen.

MATERIALS AND METHODS

Construction of Full-length cDNAs for Type IX Collagen—Three α chains were isolated from human fetal cartilage of several individuals by the guanidinium isothiocyanate method, and about 1 μg of total RNA was reverse-transcribed using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Aliquots of cDNA were used for a single step amplification by polymerase chain reaction (Expand™ long template polymerase chain reaction system; Roche Molecular Biochemicals) using oligonucleotide primers for the 5′- and 3′-ends of the three α chains. Specific oligonucleotide primers for the α1(IX) chain were designed based on the published sequences (19, 20). The oligonucleotide MH-18 (ACT CCC TTG CGG CCG CTT CAT AGG), corresponding to the 5′-noncoding sequence of the α1(IX) cDNA, contained an engineered NcoI cleavage site, and the oligonucleotide MH-19 (TCA TGC AGA CGG CCG TGC AGC AGT AAG), corresponding to the 5′-noncoding sequence, contained an engineered EagI cleavage site. For amplification of the α2(IX) cDNA, the specific oligonucleotides MH-22 (TCT GCC GTC GGT GCG GCC GCG GAC ACG C), corresponding to the 5′-noncoding sequence of the α2(IX) cDNA, and MH-23 (TCA TGC AGA CGG CCG TGC AGT AAG), corresponding to the 3′-noncoding sequence, were designed based on published sequences (21, 22). The oligonucleotide MH-22 contained an engineered NcoI cleavage site, and MH-23 contained an engineered EagI cleavage site. Specific oligonucleotides for the 5′- and 3′-ends of the α3(IX) cDNA were designed based on the published sequences (23). The oligonucleotides MH-40 (TAC TCT GGG CTC GCG CCG CTT CAT AGG), corresponding to the 5′-noncoding sequence of the α3(IX) cDNA, and MH-30 (TGC GGC GTC CTT TGT AGA TTC CTC ACG), corresponding to the 3′-noncoding sequence, contained engineered XbaI cleavage sites. The cDNAs were digested with the enzymes indicated above and ligated into the pVL1392 vector. The cDNAs were digested with the enzymes indicated above and ligated into the pVL1392 vector. The cDNAs were completely sequenced (Sequenase™ reagent kit, Amersham Pharmacia Biotech) using cDNA type sequencing primers. The three recombinant viruses were co-transfected into Spodoptera frugiperda (Sf9; Invitrogen) insect cells with a modified Autographa californica nuclear polyhedrosis virus by means of the BaculoGold transfection kit (Pharmingen), and the resultant viral pools were collected, amplified, and plaque-purified (24).

Expression of Recombinant Type IX Collagen—Sf9 or Trichoplusia ni (High Five) cells in suspension were cultured in monolayers in 96-well plates (TMN-FH medium (Sigma) supplemented with 10% fetal bovine serum (Bioclear), and High Five cells were also cultured in suspension in SF9-100 SFM medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum at 27 °C. Prior to the infection, the insect cells were seeded at densities of 5–6 × 10⁵ cells/ml for the expression of recombinant proteins in monolayers and 1 × 10⁶ cells/ml for expression in suspension. The cells were co-infected with three viruses coding for the α1(IX), α2(IX), and α3(IX) chains and a double promoter virus 4PHab coding for the α- and β-subunits of human prolyl 4-hydroxylase (25), with multiplicities of infection of 2:2:2:4:2, respectively. Ascorbate (80 μM/ml) was added daily to the culture medium.

Isolation of Recombinant Type IX Collagen from Insect Cells—After 72 h of infection, the High Five cells were detached from the culture plates by pipetting and harvested by centrifugation at 1000 × g for 5 min. Those cultured in suspension were also harvested by centrifugation. Intracellular proteins were extracted from the cells by homogenization in 0.27 M NaCl, 0.5% Triton X-100, and 0.07 M Tris-HCl buffer, pH 7.4, as described earlier (26). The supernatant of the homogenate was stored at 4 °C, and the Triton-insoluble pellet was dissolved in 1% SDS at room temperature for 2 h, after which the insoluble remains were discarded. Alternatively, the cells were suspended and homogenized in 0.75 M NaCl, 0.5 M acetic acid, pH 2, on ice (7.5 × 10⁶ cells/ml) for 30 s using a glass-Teflon homogenizer. The homogenate was centrifuged at 12,000 × g for 20 min at 4 °C, and proteins were precipitated from the supernatant by increasing the NaCl concentration to 3 M and mixing at 4 °C for 12–16 h followed by centrifugation and dissolution of the pellet in 50 mM acetic acid (27). Homogenization was performed either without protease inhibitors or in the presence of 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM E-64, 1 μM leupeptin, 1 μM pepstatin, and 75 mM aprotinin, separately or in various combinations.

Purification of Recombinant Protein from Culture Medium—The presence of type IX collagen in the culture medium was assayed by SDS-PAGE and Western blotting with the monoclonal antibody 95D1A (28). To purify the type IX collagen, the proteins were precipitated from the culture medium by adding solid ammonium sulfate to a 25% saturation and placing the mixture on ice for 1 h. The precipitate was collected by centrifugation at 12,000 × g for 20 min at 4 °C and dissolved in 0.5 M NaCl, 0.05 M HCl buffer, pH 7.4, at 4 °C overnight to a concentration of about 1 mg/ml. The dissolved recombinant protein was then purified by gel filtration through Sephacryl S-300HR in the same buffer. Further purification was achieved by cation exchange on a CM-Sepharose fast flow column in a buffer of 2 mM urea, 50 mM PIPES, and 20 mM NaCl at pH 6.5, eluting with an increasing NaCl concentration gradient (0.02–1 M NaCl).

Characterization of Recombinant Type IX Collagen—The recombinant protein isolated was characterized by SDS-PAGE followed by staining with Coomassie Brilliant Blue or Western blotting with the monoclonal antibody 95D1A. The purified material was dialyzed against 50 mM acetic acid, hydrolyzed in 6 M HCl at 110 °C for 16 h and subjected to amino acid analysis in an Applied Biosystems 421 analyzer. The thermal stability of the material was determined by CD analysis at a fixed wavelength (221 nm), raising the temperature linearly at a rate of 6 °C/h (28). For N-terminal sequencing, purified recombinant type IX collagen was electrophoresed under reducing conditions and transferred to Polyvinyldenedifluoride-type membrane, and the excised bands were subjected to Edman degradation with 477/120A liquid-phase protein/peptide sequencer (Applied Biosystems).

RESULTS

Expression of Recombinant Human Type IX Collagen in Insect Cells—Three recombinant viruses, each coding for one of the three α chains of human type IX collagen, were generated and used to infect High Five cells together with a double promoter virus, 4PHab (25), coding for the α and β subunits of human prolyl 4-hydroxylase. The cells were harvested after 72 h of culture and homogenized in a buffer containing Triton X-100, as well as to isolate other recombinant collagens from insect cells (26, 29) and to extract type IX collagen from tissues. No recombinant protein could be detected in the Triton X-100 soluble protein fraction by Coomassie staining, but individual α chains were clearly detectable in the insoluble fraction (not shown). Selective salt precipitation, which had been used previously to extract type IX collagen from tissues (27), was therefore used to isolate intracellular type IX collagen. The amount of intracellular type IX collagen produced by the insect cells was estimated by comparison with known amounts of Coomassie-stained recombinant human type II collagen to be 4–8 mg/liter of culture (not shown). However, analyses of the isolated material by Western blotting with the 95D1A antibody repeatedly revealed that the material was partially degraded, an effect that was not significantly reduced by the use of various protease inhibitors (not shown).

Purification of Type IX Collagen from the Culture Medium—The presence of type IX collagen in the culture medium was shown by SDS-PAGE and Western blotting of dialyzed medium using the 95D1A antibody (Fig. 1). In comparison with the intracellular material extracted by acid/NaCl and precipitated with 3 M NaCl, the recombinant type IX collagen present in the medium appeared to be less seriously degraded (Fig. 1), and

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; Tm, midpoint temperature of thermal transition; PIPES, 1,4-piperazineethanesulfonic acid.

2 This antibody was generated using a collagenous fragment of recombinant human type XIII collagen as an antigen. It was found to recognize the collagen domains of various denatured collagen chains (A. Snellman and T. Pihlajaniemi, unpublished observations).
therefore a protocol for isolating and purifying type IX collagen from the culture medium was designed. Addition of solid ammonium sulfate to a 25% saturation resulted in specific precipitation of the type IX collagen (Fig. 2), and analysis of the supernatant from this precipitation by SDS-PAGE followed by Coomassie staining or Western blotting showed that only a minor amount of type IX collagen had remained in solution (results not shown). Up to 10 mg of type IX collagen was obtained from 1 liter of culture medium. The precipitated type IX collagen was dissolved in a buffer containing 0.5 M urea to enhance the solubilization of the protein, and the solution was chromatographed on a Sephacryl S-300HR column in the same buffer. Analysis of the fractions containing most of the type IX collagen indicated removal of low molecular weight contaminants, e.g. the remaining bovine serum albumin (Fig. 3, lane 3). Further purification was achieved by cation exchange chromatography on a CM-Sepharose fast flow column (Fig. 3, lane 4).

**Analysis of the Purified Recombinant Human Type IX Collagen**—The results of amino acid analysis of the purified material corresponded well with calculated values for human type IX collagen (Table I), and using these values, a purity of over 90% was estimated for the recombinant type IX collagen. The melting behavior of the recombinant type IX collagen was analyzed by CD. The profile was biphasic, with about 2/3 of the transition centering at $T_m = 37.5$ °C and about 1/3 of the transition centering at $T_m = 46.0$ °C (Fig. 4).

The amino acid sequence at the N terminus of the $\alpha$(IX) chain, as identified by Edman degradation, was AVKRRPR, corresponding to the predicted signal peptide cleavage site (19). The sequences for the $\alpha$(II) and $\alpha$(III) chains could not be determined, apparently because of N-terminal blocking. The tendency of the N termini of these two chains to get blocked may be explained by possible presence of glutamine (22, 23) at the N terminus of the polypeptides (30).

**Association of $\alpha$ Chains into Disulfide-bonded Molecules**—The formation of disulfide-bonded homomeric and heteromeric molecules by the $\alpha$ chains was studied by expressing each chain individually and in all possible combinations together with prolyl 4-hydroxylase. Analyses of samples of the culture media by SDS-PAGE followed by Western blotting with the 95D1A antibody showed that all three $\alpha$ chains of type IX collagen appear to be capable of forming disulfide-bonded homodimers. Whereas $\alpha$(IX) chains showed clear homotrimer formation, the heterotrimers of $\alpha$(II) and $\alpha$(III) chains were not readily detectable (Fig. 5). When all three chains are expressed simultaneously, the heterotrimer $\alpha$(IX)$\alpha$(II)$\alpha$(III) is the dominant molecular species. However, it is possible that also other disulfide-bonded trimers are formed, but in quantities that are below the detection limit. Interestingly, co-expression of any two $\alpha$ chains results in formation of detectable amounts of disulfide-bonded heterodimeric molecules only when the chains concerned are $\alpha$(I)(IX) and $\alpha$(III) (Fig. 5). Any other combination of two $\alpha$ chains appears to result only in the formation of disulfide-bonded homodimers (Fig. 5). Abundance of monomeric molecules seen in Fig. 5 is likely to reflect the release of the monomers into the culture medium due to cell
Recombinant Human Type IX Collagen

Amino acid analysis of the purified recombinant human type IX collagen

| Amino acid      | Recombinant human type IX collagen | Expected values from cDNA deduced amino acid sequence of type IX collagen |
|-----------------|-----------------------------------|--------------------------------------------------------------------------|
| Aspartic acid   | 60.7 ± 0.2                        | 54.9                                                                     |
| Glutamic acid   | 102 ± 0.7                          | 92.7                                                                     |
| 4-Hydroxyproline| 69.5 ± 0.4                        | —                                                                        |
| Serine          | 39.4 ± 0.4                        | 36.4                                                                     |
| Glycine         | 258 ± 5.7                         | 282                                                                     |
| Histidine       | ND                                | 9.0                                                                     |
| Arginine        | 49.6 ± 1.8                        | 49.9                                                                     |
| Threonine       | 30.5 ± 0.7                        | 22.5                                                                     |
| Alanine         | 60.3 ± 1.1                        | 53.1                                                                     |
| Proline         | 103 ± 0.7                         | 190                                                                     |
| Valine          | 39.6 ± 0.1                        | 36.0                                                                     |
| Methionine      | ND                                | 11.7                                                                     |
| Tyrosine        | ND                                | 6.3                                                                      |
| Isoleucine      | 25.0 ± 0.1                        | 32.4                                                                     |
| Leucine         | 58.6 ± 0.1                        | 53.1                                                                     |
| Hydroxylysine   | 8.67 ± 0.3                        | —                                                                        |
| Phenylalanine   | 15.8 ± 0.2                        | 11.7                                                                     |
| Lysine          | 43.6 ± 0.6                        | 48.1                                                                     |
| Cysteine        | ND                                | 8.1                                                                      |
| Tryptophan      | ND                                | 1.8                                                                      |

*The values are given as mean ± S.D. per 1000 residues, n = 2. a The values for nonrecombinant human type IX collagen are not known. b ND, not determined.

Fig. 4. CD analysis of denaturation of the purified recombinant type IX collagen. Denaturation was monitored by the change in mean molar ellipticity in 0.05 M acetic acid at a fixed wavelength (221 nm). The estimated Tm values were 37.5 °C (about ½ of transition) and 46.0 °C (about ¼ of transition).

Fig. 5. Analysis of type IX collagen chain association. The recombinant α chains were expressed individually and in all possible combinations together with prolyl 4-hydroxylase in an adherent culture of High Five cells. Samples of the culture media were analyzed by SDS-PAGE under nonreducing conditions and Western blotting with the antibody 95D1A. Molecular species were identified by differences in electrophoretic mobility. Lane 1, prolyl 4-hydroxylase expressed alone; lane 2, α1(IX); lane 3, α2(IX); lane 4, α3(IX); lane 5, a1(IX) and a2(IX); lane 6, α1(IX) and α3(IX); lane 7, α2(IX) and α3(IX); lane 8, all three α chains co-expressed.

The results presented here show that all recombinant type IX collagen α chains are able to form disulfide-bonded homodimers, whereas only the α1(IX) chains show homotrimer formation. These findings support previous observations that the C-terminal fragments of α1(IX) chains exhibit the highest potential for homotrimer formation and α3(IX) chains have the lowest ability for self-association (16). Homomers and disulfide-bonded dimeric molecules were also detected in the culture medium. Because collagen is secreted as trimeric molecules, these species may originate from secreted trimeric molecules that were not fully disulfide-bonded. Alternatively, the monomers and dimers could represent immature molecules that are released into the culture medium as a result of cell lysis.

In a reassociation study using pepsin-resistant C-terminal low molecular weight fragments of bovine type IX collagen, Labourdette and van der Rest (14) found some formation of homomeric molecules in addition to the expected α1α2α3 heterotrimer upon mixing of fragments of all three α chains. Our results obtained using full-length α chains indicate that the

Discussion

As type IX collagen is a minor cartilage component that is covalently cross-linked to type II collagen fibrils and has interruptions in its triple helix, it has been difficult to isolate intact type IX collagen molecules from tissues. We have now produced and isolated intact heterotrimeric human type IX collagen for the first time using a baculovirus expression system. To obtain stable type IX collagen, a double promoter virus for the α and β subunits of prolyl 4-hydroxylase (25) was co-expressed with the three viruses for the α chains of type IX collagen itself.

Our initial attempts to purify intracellular type IX collagen failed because the protein was insoluble in a buffer containing Triton X-100 and was easily degraded after acid extraction and selective salt precipitation. Unlike the intracellular material, type IX collagen purified from the medium migrated as a single band of over 200 kDa in SDS-PAGE under nonreducing conditions, and reduction indicated that the material consisted of three α chains in a 1:1:1 ratio. It is likely that the three viruses for the α chains and the virus for prolyl 4-hydroxylase do not infect all the insect cells with equal efficiency, which in turn may lead to the intracellular accumulation of underhydroxylated and improperly folded molecules that are insoluble or susceptible to degradation.

Amino acid analysis of the secreted trimeric type IX collagen showed that the composition of the material was in agreement with composition expected based on the cDNA deduced amino acid sequence. An adequate degree of 4-hydroxylation of the Y-position prolines in the Gly-X-Y sequences is required to stabilize the collagen triple helix (31), but the extent of prolyl 4-hydroxylation in type IX collagen is currently not known. That in the recombinant type IX collagen was about 80% of the theoretical maximum. CD analysis indicated that this degree of hydroxylation is adequate, because the Tm values of the recombinant type IX collagen corresponded well to the values reported for type IX collagen isolated from tissues (32). The transition profile was biphasic because the COL3 domain has a higher thermal stability than the rest of the molecule (32). The results of CD analysis also indicate proper folding of the recombinant type IX collagen.

Lysis during the expression. Second, some of the monomers may originate from secreted trimeric molecules that were not fully disulfide-bonded. Also, the abundance of monomers is in part an artifact, because the efficiency of electroblotting is inversely related to the size of the blotted molecules.

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The results presented here show that all recombinant type IX collagen α chains are able to form disulfide-bonded homodimers, whereas only the α1(IX) chains show homotrimer formation. These findings support previous observations that the C-terminal fragments of α1(IX) chains exhibit the highest potential for homotrimer formation and α3(IX) chains have the lowest ability for self-association (16). Homomers and disulfide-bonded dimeric molecules were also detected in the culture medium. Because collagens are secreted as trimeric molecules, these species may originate from secreted trimeric molecules that were not fully disulfide-bonded. Alternatively, the monomers and dimers could represent immature molecules that are released into the culture medium as a result of cell lysis.

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heterotrimer is the predominant product formed. It is therefore possible that the C termini of the NC1 domains that are missing in the low molecular weight fragments enhance formation of the heterotrimer in preference to homotrimers. Because our results were obtained using secreted material, it is possible that homotrimeric molecules are also formed but remain mostly intracellular. It is also possible that homotrimeric molecules are present in the culture medium, but in very low quantities.

Two cysteine residues located at the COL1/NC1 junction in all three α(IX) chains are involved in interchain disulfide bond formation. The functionality of these cysteines in connecting together any two of the α chains has been demonstrated by Labourdette and van der Rest (14), who were able to detect all possible dimeric combinations in minor quantities. In the present case, however, α1α3 was the only heterodimeric molecular structure detected. It is possible that the other two disulfide-bonded heterodimeric species were also formed but in quantities that were below the detection limit of the experimental system. Similarly, it is possible that in a reassociation study using synthetic NC1 domains, heterodimeric molecules were formed but remained undetected. Even though our results were obtained with a semiquantitative analysis, it appears that co-expression of the α1(IX) and α3(IX) chains results in formation of the heterodimer, which dominates the respective homodimers. The results thus suggest that in the association of full-length type IX collagen a chains the most favored trimeric form is an α1(IX)α2(IX)α3(IX) heterotrimer, although α1(IX) chains are capable of forming disulfide-bonded homotrimeric molecules.

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