The α1(VIII) and α2(VIII) Chains of Type VIII Collagen Can Form Stable Homotrimeric Molecules*

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Type VIII collagen is a short chain collagen. Two chains have been described, α1(VIII) and α2(VIII), but the chain composition of type VIII collagen is far from resolved. To address this question, we have expressed full-length α1(VIII) and α2(VIII) chains in an in vitro translation system supplemented with semipermeabilized cells. Both chains gave a translation product of ~80 kDa that could be shown to produce a chymotrypsin/trypsin-resistant product of ~60 kDa, indicating that both chains could form homotrimers. Hydroxylation of proline residues was a prerequisite for stable trimer formation. The melting temperature for the α1(VIII) homotrimer was 45 °C, whereas that for α2(VIII) was 42 °C. The ability of both chains of type VIII collagen to form stable triple helices suggests that there may be different forms of this collagen and that cells may modulate the chain composition in response to different biological conditions.

Collagen VIII is a member of the short chain class of collagens that was initially detected as a product of bovine aortic and rabbit corneal endothelial cells (1–4) and has subsequently been shown to be produced by smooth muscle cells, mast cells, and certain tumor cell lines (5–8). Two distinct chains have been identified, α1(VIII) and α2(VIII). Pepsin digestion of Descemet’s membrane suggested that heterotrimers of two α1(VIII) chains to one α2(VIII) chain are formed (9–13), although the chain composition of type VIII collagen is far from resolved.

Type VIII collagen has a short triple helix, and both the α1(VIII) and α2(VIII) chains are similar to the α1(X) chain of type X collagen. Sequence analysis has shown each chain to contain a collagenous domain of 454 amino acids, a short N-terminal non-triple helical region (NC2), and a longer C-terminal non-triple helical region (NC1) (9–10). Studies in Descemet’s membrane suggest that type VIII collagen is a major component of the hexagonal lattice seen in that tissue, and a similar lattice structure can be elaborated by cultured endothelial cells (12, 13).

Type VIII collagen is thought to play a key structural role in the vasculature, where it is found both in the subendothelium and in the elastin-rich tunica media (14–16). Recent evidence indicates that it is up-regulated in response to injury (5, 17). Despite the importance of this molecule, little is known about chain composition or supramolecular organization in the vasculature. It has recently been suggested that α1(VIII) chains can form homotrimers in vitro (18), although no evidence was presented about stability of the helix. In this report, we describe the expression of full-length constructs of α1(VIII) and α2(VIII), in an in vitro translation system supplemented with a semipermeabilized fibroblast cell line, and show that both α1(VIII) and α2(VIII) are capable of forming stable homotrimers.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all chemicals were obtained from BDH/Merck (Poole, Dorset, United Kingdom). The medium and all other cell culture reagents were obtained from Life Technologies, Ltd. ( Paisley, Renfrewshire, Scotland). HT-1080 fibroblasts were obtained from the European Collection of Cell Cultures Cell Bank. Restriction enzymes, nuclease S7, RNasin, and the Expand High Fidelity PCR kit were obtained from Boehringer (Mannheim, Germany). The Flexi® reticulocyte lysate translation kit, the in vitro transcription kit, and nucleotides were obtained from Promega (Madison, WI). Protein A-Sepharose beads, phenylmethylsulfonyl fluoride, N-ethylmaleimide, chymotrypsin, proteinase K, trypsin, high purity collagenase type III, soybean trypsin inhibitor, and α,α′-dipyridyl were purchased from Sigma (St. Louis, MO). RNA purification spin columns were obtained from Qiagen Inc. (Crawley, Sussex, UK). The TA Cloning® kit was purchased from Invitrogen (Leek, The Netherlands). Prestained molecular mass markers were obtained from Biocytex (Saint Cloud, France). Tran35S label was obtained from ICN Biomedicals Ltd. (Thame, Oxon, UK), and 14C-methylated proteins were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). High purity digitonin was obtained from Calbiochem (La Jolla, CA). The pBlu-HaI8 plasmid was obtained from Invitrogen (Leek, The Netherlands). Polyacylamide gel electrophoresis.

Preparation of Permeabilized Cells—Trypsinized confluent HT-1080 cells were washed in phosphate-buffered saline and permeabilized with digitonin (40 μg/ml) as described (10).

Generation of Recombinant Plasmids—A full-length clone of α1(VIII) was previously isolated from a λgt11 human aorta cDNA library. Briefly, primary screening was carried out with probes designed to the NC1 domain. Positive clones underwent a secondary screening using a 496-base pair probe designed according to the NC2 domain and part of the helix. Subsequent positives were sequenced using λgt11 forward and reverse primers; one clone was found to contain ~90 base pairs of 3’-untranslated region, the entire coding sequence of α1(VIII), and ~200 base pairs of 5’-untranslated region. This was excised from λgt11 and subcloned into EcoRI-cut pBluescript(+) and designated pBlu-HaI8.

An α2(VIII) clone containing the entire coding sequence was generated by overlap extension PCR from a mouse genomic clone containing exons 3 and 4, pNP-m129-21 (gift from Prof. B. Olsen, Boston, MA). First round proofreading hot start PCR was used to generate each exon separately. Combination of the two products in a second round allowed fusion and elongation of a full-length product. Reactions were carried out with the Expand High Fidelity PCR kit using the manufacturer's
A pair fragment was purified and cloned into TA Cloning® vector pCR 2.1-cation of the 2.1-kilobase pair overlap extension product. A 2.1-kilobase exon 3 forward primer and the exon 4 reverse primer allowed amplification to self-prime for six thermal cycles. Subsequent addition of the recommended cycling conditions. Reaction mixtures (50 μl) comprised 30 pmol of each primer, −10 ng of template DNA, 2.6 units of enzyme mixture (thermostable Taq and Pwo DNA polymerases), 2 mM MgCl2, 200 mM nNTPs and 1× kit buffer. Exon 3 primers were designed to sequence supplied with the pNF-m129-21 clone.2 The forward primer (5’-CGAATTCATGCCTGCAGG16GCTCTG) included the initial ATG start site (boldface), and the reverse primer (5’-GGCTCCGAA12(TG)ATTACGTCGGCCTTTGC) represented a chimera of exon 3 and 4 (italics) and the last base from a 3'-end of exon 3. These were used to generate an expected fragment of 215 base pairs. An expected product of 1.9 kilobase pairs was generated using a chimera forward primer complementary to the exon 3 reverse primer (5’-CAGCAGGCCATCGGTA-GAOGCCATTCAATGATGGGCGCTTTGC) with the first 26 bases based on exon 3 (italics) and the remainder based on the published exon 4 sequence (positions 29–46), and a reverse primer (5’-GGCTCCGATTATGCCTGGGCGACAGC) to the 3'-end of exon 4 (positions 1923–1938) including the a2(VIII) stop codon (boldface).

Purified PCR products from each reaction were combined and allowed to self-prime for six thermal cycles. Subsequent addition of the exon 3 forward primer and the exon 4 reverse primer allowed amplification of the 2.1-kilobase pair overlap extension product. A 2.1-kilobase pair fragment was purified and cloned into TA Cloning® vector pCR 2.1 and was designated pCR 2.1-MFa28. Sequencing showed successful exon fusion with the correct reading frame being maintained across the boundary (data not shown). Comparison with the known mouse exon 3 and NC1 sequence showed that two base changes were present, resulting in a methionine to leucine change in the NC2 domain (position 66) downstream of the predicted signal peptide cleavage and a serine to threonine change at position 239 in the NC1 domain (10). These changes were not considered to be a problem because both amino acids are present in human a2(VIII). A full-length clone of bovine α1(X) in the vector pGEM-3Z (designated pGEM-BX) was kindly donated by Dr. N. Fukai, personal communication.

2 N. Fukai, personal communication.

**Fig. 1.** Autoradiograph of translation products synthesized in the presence and absence of collagen VIII mRNA. Aliquots of isolated cells from translations in the presence of α1(VIII) RNA (lanes 3, 4, 9, and 10), α2(VIII) RNA (lanes 5, 6, 11, and 12), and controls of sterile water (lanes 2 and 8) were analyzed on an 8% gel by SDS-PAGE. Samples were denatured at 60 or 100 °C under reducing (lanes 1–6) or nonreducing (lanes 7–12) conditions. Sizes of 14C-methylated marker proteins are shown (lanes 1 and 7).

**Table:**

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------|---|---|---|---|---|---|---|---|---|----|
| RNA  | o1 | o1 | o1 | o2 | o2 | o1 | o1 | o2 | o2 |
| Denaturing temp | 60 | 100 | 60 | 100 | 60 | 100 | 60 | 100 | 60 |

**Fig. 2.** Autoradiograph demonstrating translocation of protein products into semipermeabilized cells by proteinase K digest. Isolated cells from α1(VIII) and α2(VIII) translations were analyzed directly (lanes 2 and 8), subjected to proteinase K digestion (lanes 3, 6, and 9) or subjected to proteinase K digestion following pretreatment with Triton X-100 (lanes 4, 7, and 10). Samples were analyzed by SDS-PAGE on a 10% gel under reducing (lanes 1–4) and nonreducing (lanes 5–10) conditions. Sizes of 14C-methylated marker proteins are shown (lanes 1 and 5).

inserts: *HindIII* (for both collagen VIII clones) and *SalI* (type X collagen clone). Transcriptions were carried out on 10 μg of the linearized clones for 3–4 h at 37 °C in a reaction mixture containing >100 units of T3 RNA polymerase (for pBlu-HaI8) or T7 RNA polymerase (for pGEM-BX with PCR 2.1-MFa28), ~70 units of RNasin, 10 mM dithiothreitol, 3 mM NTPs, and 1× transcription kit buffer. RNA was purified by binding to a commercially available spin column and eluted in diethyl pyrocarbonate-treated water containing 1 mM dithiothreitol and ~35 units of RNasin.

**Cell-free Translation—**Translations were carried out using a rabbit reticulocyte lysate system. Reaction mixtures (volume of 51.5 μl) containing 35 μl of lysate, 20 μM amino acids (minus methionine), 50 μg/ml sodium ascorbate, 25 μCi of [35S] methionine, ~140 units of RNasin, 2.5 or 5 μl of transcribed RNA (substituted for sterile H2O in controls), 50 mM KCl, and 4 × 10^5 permeabilized HT-1080 cells were translated for 1.5 h at 30 °C. After this time, N-ethylmaleimide was added to a concentration of 25 mM and stored on ice. Translations were also carried out in the presence of a,a'-dipyridyl (89 μg/ml). Translation mixtures were diluted five times with 0.1 mM potassium acetate, 20 mM HEPES, and 2 mM magnesium acetate, pH 7.2; cells were isolated by centrifugation in a microcentrifuge at 14,000 × g for 5 min; and appropriate aliquots were used in subsequent investigations.

**Chymotrypsin/Trypsin Digests—**Aliquots of isolated cells from translation mixtures were treated with chymotrypsin/trypsin mixtures as described (20), except that digestion was carried out for 5 min. Digestion was stopped by the addition of 2 μl of soybean trypsin inhibitor (50 mg/ml) and 2 volumes of boiling 2× SDS-PAGE loading buffer.

**Collagenase Digestion—**Aliquots of isolated cells from translation mixtures were digested for 4 h with 1 unit of high purity bacterial collagenase type III as described (20). The reaction was terminated by the addition of 2× SDS-PAGE loading buffer containing 5 mM EDTA.

**Proteinase K Digests—**Aliquots of isolated cells from translation mixtures were digested with 250 μg/ml proteinase K as described (21). Digestion was stopped with 1× phenylmethylsulfonyl fluoride and 2× SDS-PAGE loading buffer.

**Heat Denaturation—**Aliquots of isolated cells from translation mixtures were reuspended in 10 μl of 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, pH 8.0, and 1% Triton X-100 and left on ice for 10 min. Samples were added to a thermal cycling block and subjected to a range of temperatures from 36 to 50 °C, increasing by 1 °C every 2 min.
Samples were removed every 2 min and subjected to a chymotrypsin/trypsin digest as described above before analysis by SDS-PAGE.

SDS-PAGE—Samples were taken up in 2× SDS-PAGE loading buffer, with or without 0.7 M β-mercaptoethanol, and analyzed on either 8 or 10% (digested samples) gels following the method of Laemmli (22). Samples were denatured at either 100 °C for 3 min or 60 °C for 10 min. Gels were fixed, vacuum-dried onto filter paper, and placed against Kodak X-Omat AR film for analysis. Relative molecular masses of translated products were determined by reference to 14C-methylated proteins (myosin, 220 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; and lysozyme, 14.3 kDa) (Calbiochem).

RESULTS

Major Translation Products—Translation of RNA derived from both the recombinant full-length α1(VIII) and α2(VIII) constructs in an in vitro translation system supplemented with a semipermeabilized fibroblast cell line gave major products of ~80 kDa (Fig. 1). Control reactions in which RNA was substituted for sterile water yielded no major products or background bands (Fig. 1, lanes 2 and 8). The α1(VIII) 80-kDa bands corresponded approximately to the predicted molecular mass of single monomeric chains (73 kDa). As expected, α2(VIII) ran slightly faster due to a shorter NC2 domain. Both α1(VIII) and α2(VIII) chains associated to form high molecular mass products (Fig. 1, lanes 3 and 9 and lanes 5 and 11, respectively), which were stable at 60 °C in the presence of SDS, but were susceptible at 100 °C. This is similar to the results observed for collagen X, where it is thought that strong noncovalent interactions within the NC1 domain are responsible for oligomer stability under denaturing conditions (23–25). These high molecular mass products were not susceptible to reducing conditions. The use of semipermeabilized cells has been shown to allow the formation of disulfide bonds (19); however, interchain disulfide bonds do not appear to be involved in oligomerization of either chain.
Differing intensities were observed between the translation products of α1(VIII) and α2(VIII). This was a reproducible finding and can be attributed to differing amounts of label incorporation (22 methionines present in α1(VIII) and 7 in PCR 2.1-MPa28).

Protease K digests confirmed translocation of protein products to the cellular rough endoplasmic reticulum (Fig. 2). Both α1(VIII) and α2(VIII) showed evidence of compartmental protection. Intact 80-kDa bands were present after treatment (Fig. 2, lanes 3 and 9). Only when isolated cells were treated with Triton X-100 prior to digestion were the monomeric chains degraded.

**Collagenase Digests**—The translation products were shown to be collagenous by their susceptibility to high purity bacterial collagenase type III (Fig. 3). α1(VIII) collagenase-resistant bands were seen migrating at ~22 kDa (assumed to represent the NC1 domain), and a diffuse band at ~50 kDa. The latter was stable at 60 °C (Fig. 3, lanes 3 and 10, NC1x arrow), but was eliminated at 100 °C, resulting in a corresponding increase in intensity of the 22-kDa band (lanes 4 and 11, α1 NC1 arrow). In the case of α2(VIII), a similar collagenase-resistant band was observed, although it had a lower molecular mass of ~18 kDa that ran at or near the dye front (Fig. 3, lanes 6, 7, 13, and 14, α2 NC1 arrow).

**Chymotrypsin/Trypsin Digests**—Chymotrypsin/trypsin digestion has previously been demonstrated as a reliable assay for the formation of stable triple helical molecules of collagen X (20) and is based on the premise that incompletely folded or incorrectly folded helices of collagen are sensitive to proteolytic cleavage. Only the non-collagenous domains NC1 and NC2 of correctly folded collagen VIII heterotrimers will be degraded, leaving the helical domain intact. When both the α1(VIII) and α2(VIII) translation products were digested with chymotrypsin/trypsin, a 60-kDa band was evident (Fig. 4, lanes 3, 6, 10, and 14, 60kDa arrow) in both the reduced and nonreduced samples, indicating the ability of both chains to form a triple helix. Considerable differing band intensity could be seen between the α2(VIII) 80- and 60-kDa bands despite equal amounts of translation product in each lane (Fig. 4, lanes 5 and 6 and lanes 13 and 14, respectively). This was also observed with the α1(VIII) samples, albeit to a lesser extent. This principally reflects a reduction in methionine label upon removal of the non-collagenous domains (45% for α1(VIII) and 87.5% for α2(VIII)). Phosphoimage analysis of the undigested and digested bands showed that both the α1(VIII) and α2(VIII) chains exhibited a similar efficiency for triple helix formation.

The stability of the helix, and therefore its resistance to proteolytic attack, is dependent on the degree of hydroxylation of proline residues as shown by translations carried out in the presence of α,α′-dipyridyl. A chymotrypsin/trypsin digest carried out on the translation products of either α1(VIII) or α2(VIII) did not produce the 60-kDa fragment (Fig. 4, lanes 4 and 12 and lanes 7 and 15, respectively), indicating that hydroxylation of procollagen chains was necessary for the formation of correctly aligned stable triple helices at 30 °C.

**Melting Temperatures**—The stability of the α1(VIII) and α2(VIII) heterotrimers produced in vitro was assessed by measuring chain denaturation temperature (T_m). Translation products of both chains were subjected to a variety of temperatures and digested with chymotrypsin/trypsin (Fig. 5). Resistant helical band intensities were quantified by phosphoimage analysis, and a 50% loss was seen at 45 °C for α1(VIII) (Fig. 5A, lane 6) and at 42 °C for α2(VIII) (Fig. 5B, lane 9). The latter produced a major product of ~60 kDa and a slightly smaller minor fragment. A control T_m determination was carried out on bovine α1(X) and found to be 44 °C (data not shown).

**DISCUSSION**

Despite the fact that type VIII collagen has been known for over 16 years and the gene structure has been delineated, there is still a dearth of information about the protein and its supramolecular structure. The best available information suggests that in Descemet’s membrane, type VIII collagen is a heterotrimer with a chain composition of (α1(VIII))₂(α2(VIII)), which appears to aggregate to form a hexagonal lattice structure (12–13).

Similarity to type X collagen supports comparisons between these two molecules, and whereas they have similar gene structures and collagen helices (9), type X collagen appears to be a product of the hypertrophic chondrocyte and epiphyseal cartilage, and it is becoming increasingly clear that a variety of cell types can synthesize and secrete type VIII collagen (16). This raises the question as to whether all cells produce type VIII collagen with the same chain composition and how these variations in chain composition affect the supramolecular structure and function. We have been trying to address the question of chain composition by looking at the ability of the α-chains to form stable triple helices in an in vitro translation system.

The experiments described clearly show that full-length α1(VIII) and α2(VIII) can be successfully translated in vitro...
and that these chains associate into stable homotrimeric molecules. The translated chains were of the correct size and were susceptible to collagenase. These chains appeared to form higher molecular mass aggregates that were stable to denaturation at 60 °C, although they disappeared when heated to 100 °C. By analogy to type X collagen, the association is presumably mediated by the NC1 domain. It has been shown that both full-length α1(X) oligomers and collagenase-resistant NC1 aggregates are stable under denaturing conditions (23–25). In support of this, the presumed NC1 domain of α1(X) VIII produced by collagenase digestion could be shown to aggregate into a 50-kDa complex (Fig. 3, NC1x arrow). The two collagen types share a high degree of homology within this domain, and current thinking on folding of both collagens VIII and X centers on common hydrophobic groupings within the NC1 domain acting as nucleation centers for zipper-like trimerization in a carboxyl- to amino-terminal direction (26). Such homology maybe responsible for the strong noncovalent interactions shown here. However, the absence of an α2[VIII] collagenase-resistant NC1 aggregate implies that there may be other requirements for full-length α2[VIII] chain oligomerization.

Both the α1[VIII] and α2[VIII] chains were shown to be triple helical molecules by their resistance to chymotrypsin/trypsin mixture and the appearance of a 60-kDa proteinase-resistant fragment. The presence of a second slightly smaller chymotrypsin/trypsin-resistant fragment of α2[VIII] could be due to the presence of a Gly-X-Y imperfection 41 amino acids from the start of the helical domain; unlike all of the α1[VIII] imperfections that represent Gly-X-Gly, this is a Gly-X-X-X imperfection and may constitute a cleavage site in the triple helix. Unlike previous reports, our results show that a stable triple helix is only formed if hydroxylation of proline occurs, as no proteinase-resistant fragments were obtained after incubation in the presence of α,α′-dipyridyl, a prolyl hydroxylase inhibitor (27).

The stability of a triple helix is determined by hydrogen bonds between chains, a high imino acid content, and possibly side chain interactions. The importance of hydroxylation of proline to helix stability has been clearly shown (28). In addition, recent work has highlighted the importance of Gly-Pro-Arg triplets and appropriate charged residues in the X and Y positions (29, 30). Stabilization by glycosylated threonine appears to be significant only in vent worm cuticle collagen (31). The use of semipermeabilized cells allowed maximum modification of prolyl residues. In this system, bovine α1(X) homopolymers had a \( T_m \) of 44 °C, which is higher than that reported in a translated system supplemented with isolated HT-1080 micromes (\( T_m = 41 ^\circ C \)), an alternative source of endoplasmic reticulum (20). In the case of α1(VIII) homopolymers, a \( T_m \) of 45 °C was found. A previous report (18) has presented evidence that α1(VIII) chains can form homotrimers in a cell-free system; this is the first proof that stable trimers are formed and that hydroxylation of proline is a prerequisite. The \( T_m \) of 42 °C reported here for the α2[VIII] homopolymers is less than that for both α1(VIII) and α1(X), which was a surprising finding since in human α2[VIII], there are more prolines present in the Y position of the Gly-X-Y triplet than there are in α1(VIII) (76 versus 73) and more Gly-Pro-Arg triplets (11 versus 6). There are no available data at present on the mouse sequence, but these results appear to indicate that not all prolines are hydroxylated in the helix or that there are less potential proline substrates in the mouse than in the human. Further work is required to clarify these apparent differences in stability.

Although the function of type VIII collagen is not known, it is becoming clear that it is expressed in many situations associated with inflammation and repair, where it may form a provisional matrix for cell attachment and migration (5, 17, 32, 33). Clearly, the ability to form strong, stable molecular assemblies will be a major determinant of that function. The present results suggest that molecules with different chain assemblies may be one way of modulating provisional matrices produced by different cell populations.

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