Expression and supramolecular assembly of recombinant α1(VIII) and
α2(VIII) collagen homotrimers

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SUMMARY

Collagen VIII is an extracellular matrix macromolecule comprising two polypeptide chains, α1(VIII) and α2(VIII), that can form homotrimers in vitro and in vivo. Here, recombinant collagen VIII was expressed in order to study its supramolecular assembly following secretion. Cells transfected with α1(VIII) or α2(VIII) assembled and secreted homotrimers that were stable in denaturing conditions and had a molecular mass of ~180 kDa on SDS-PAGE gels. Co-transfection with prolyl 4-hydroxylase generated homotrimers with stable pepsin-resistant triple-helical domains. Size fractionation of native recombinant collagen VIII molecules expressed with or without prolyl 4-hydroxylase identified urea-sensitive high-M₉ assemblies eluting in the void volume of a Superose 6HR 10/30 column, and urea-resistant assemblies of ~700 kDa, all of which were composed of homotrimers. Immunofluorescence analysis highlighted the extracellular deposition of recombinant α1(VIII)₃, α2(VIII)₃ and co-expressed α1(VIII)₃/α2(VIII)₃. Microscopy analysis of recombinant collagen VIII identified rod-like molecules of 134 nm in length that assembled into angular arrays with branching angles of ~114°, and extensive networks. Based on these data, we propose a model of collagen VIII assembly in which four homotrimers form a tetrahedron stabilised by central interacting C-terminal NC1 trimers. Tetrahedrons may then act as building blocks of three-dimensional hexagonal lattices generated by secondary interactions involving terminal and helical sequences.
INTRODUCTION

Collagen VIII was initially identified in the culture medium of endothelial cells and designated endothelial cell collagen (EC collagen) (1,2). It is a non-fibrillar collagen with a short triple helical domain that is flanked by non-collagenous C-terminal NC1 and N-terminal NC2 domains (3,4). It is a prominent component of Descemet’s membrane, the basement membrane separating corneal endothelial cells from corneal stroma (5), and of blood vessel walls where it occurs within the vascular subendothelium and in association with medial elastic fibres (6).

Immunohistochemistry has demonstrated that collagen VIII is also present in cornea, optic nerve and umbilical cord (7). In addition to endothelial cells, it is expressed by mesangial cells (8), smooth muscle cells (9,10), mast cells (11) and macrophages (12). Expression of collagen VIII by smooth muscle cells is strongly upregulated following vascular injury (6,10,13).

Collagen VIII comprises two distinct polypeptide chains designated α1(VIII) and α2(VIII) (14,15). In vitro studies have shown that homotrimers of α1(VIII)₃ and α2(VIII)₃ can form that are highly stable (16,17), and that heterotrimers [α1(VIII)₂ α2(VIII) and α1(VIII) α2(VIII)₂] can also occur (18). Some tissue extraction studies have suggested that collagen VIII exists in vivo as heterotrimers (2,5). In another study, data from biochemical, immunohistochemical and molecular biological experiments showed that α1 and α2 chains of collagen VIII exist as homotrimers in cells and tissues (8). Both α1(VIII) and α2(VIII) were present in equimolar concentrations in Descemet’s membrane, immunoprecipitation of collagen VIII from Descemet’s membrane extracts using chain-specific antibodies yielded fractions that contained either α1(VIII) or α2(VIII), transcription of both genes was not always co-ordinated, and both
polypeptides were present in cornea, optic nerve, aorta and umbilical cord but did not always co-localise.

Collagen VIII is structurally closely related to collagen X (19). Both collagens form unusually stable trimers through strong non-covalent interactions of their C-terminal NC1 domains (20-23), and supramolecular hexagonal lattice assemblies (24). Modeling of the crystal structure of collagen X NC1 domain revealed a buried Ca\(^{2+}\) cluster that may contribute to trimer stability, and an aromatic surface patch that may be involved in supramolecular assembly (25). This Ca\(^{2+}\) cluster involves Asp626 and Asp634 residues forming coordinated interactions with the Ca\(^{2+}\) ions. Three hydrophobic residues 611Trp, 625Tyr and 667Tyr in collagen X NC1 domain comprise a hydrophobic external face on collagen X trimers that can bind the steroid moiety of the zwitterionic non-denaturing detergent CHAPS. These residues may be responsible for supramolecular assemblies of collagen X by NC1 hydrophobic interactions (25). The resolution of the \(\alpha_1(\text{VIII})_3\) NC1 trimer crystal structure has shown that, whilst lacking a buried Ca\(^{2+}\) cluster, the hydrophobic region interacts with CHAPS and may contribute to the organization of collagen VIII supramolecular assembly (26). These hydrophobic residues are conserved in both \(\alpha_1(\text{VIII})\) and \(\alpha_2(\text{VIII})\) and may regulate assembly of collagen VIII by a similar mechanism.

In this study, we have expressed recombinant \(\alpha_1(\text{VIII})_3\) and \(\alpha_2(\text{VIII})_3\) in mammalian cells, and investigated their supramolecular assembly. Stable secreted triple-helical collagen VIII homotrimers were obtained after co-transfection with prolyl 4-hydroxylase that were deposited in the extracellular matrix. A novel intermediate of higher-order extracellular assembly has been identified that suggests a tetrahedron model of collagen VIII lattice formation.
EXPERIMENTAL PROCEDURES

Antibodies and reagents

Unless otherwise stated, all reagents were purchased from BDH/Merck, UK. Taq DNA polymerase, T4 DNA ligase calf intestinal alkaline phosphatase, restriction enzymes and hygromycin B were purchased from Boehringer Mannheim, Germany. Plasmid purification kits were purchased from Qiagen Ltd., UK. TA cloning kit, pCEP4, pREP8, 293EBNA cells, INVαF’competent cells, polyacrylamide gels, transfer buffer, dithiothreitol (DTT), SDS protein standards and SDS running buffers were purchased from Invitrogen, UK. A monoclonal antibody against collagen VIII was purchased from AMS Biosciences, UK. A polyclonal antibody to α2(VIII) was kindly provided by Dr P. Davies, New Zealand (8). Horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody was purchased from Dako, UK. DMEM (Dulbecco’s modified Eagles medium), phosphate buffered saline (PBS), fetal calf serum, antibiotics and L-glutamine was purchased from Gibco, UK. T6 defined media was purchased from JRH Biosciences, UK. Pepsin, L-ascorbate-2-phosphate and chymotrypsin were purchased from Sigma, UK. Dialysis membrane was purchased from Medicell International Ltd., UK. Filter paper was purchased from Whatman Laboratory Division, UK. Nitrocellulose was purchased from Pall Corporation, UK. Gel filtration columns and enhanced chemiluminescence reagents were purchase from Amersham Pharmacia, UK.

Expression constructs

Human full length α1(VIII) was isolated from a λgt11 aorta cDNA library, subcloned into pBluescript(+) and designated pBlu-Hal8. It contained a 210bp 5’UTR region, the entire coding
sequence including the putative translation start and termination signals and 24bp of 3’UTR. The α1(VIII) cDNA was isolated from pBlu-Ha18 by restriction digestion with Not1/Xho1 and directly subcloned into pCEP4 for transfection into 293-EBNA cells. This clone was designated pCEP4α1(VIII).

A 6kb α2(VIII) murine genomic clone containing the entire coding sequence (kindly supplied by Dr B. R. Olsen, Boston, USA) was used as a template for overlap PCR. This clone contained exons 3 and 4, which contain the entire coding sequence of α2(VIII). The 6kb clone was cloned into Bluescript(+) BamH1 and Not1 restriction sites. Exon 3 was generated by PCR using a 25bp forward primer for exon 3 with an engineered EcoR1 restriction site inserted 3 bases downstream from the ATG initiation site [(A2E3F) 5’CGAATTCCATGCAGGGGGCTCTG 3’] and a 41bp reverse primer [(A2E3R) 3’CGTTTCCCGGTATGAACCTTTACGGAGATGGCGACGGACGGC 5’]. Exon 4 was generated by PCR using a 44bp forward primer A2E4F containing an 18bp sequence complementary to the 5’ end of the α2(VIII) human exon 4 coding region position 29-46 [(A2E4F) 5’CCGAGAGGGCAAAGGCCAGTACTTGGAAATGCCTCTCTACGGCTGC 3’], and a 25bp reverse primer including the stop codon TTA [(A2E4R) 3’GGCTCCGAATTATGTGGGGCGAGACGC 5’].

The overhang primers (A2E4F and A2E3F) were engineered to terminate on a base preceding a thymine in the complementary strand to prevent addition of further adenine bases by Taq polymerase and subsequent disruption of the reading frame during the second round of PCR when products were used to self prime. Products generated from these PCR reactions were
isolated using agarose gel electrophoresis, excised, purified then used in a second PCR reaction to generate the full length sequence. The products were self primed for six PCR cycles then A2E3F and A2E4R primers were added to amplify the fusion product. The fusion product was gel-purified then subcloned into TA cloning vector pCR 2.1. Correct insertion into a clone designated pCR 2.1 MFa28 was confirmed by EcoR1 digestion and sequencing. The α2(VIII) cDNA was isolated from pCR 2.1 MFa28 by restriction digestion with NotI and directly subcloned into pCEP4 for transfection into 293-EBNA cells. This clone was designated pCEP4α2(VIII).

The full length α subunit of prolyl 4 hydroxylase (αSU P4H) was inserted within a pBluescript(+) (supplied by Dr Kari Kivirikko, Oulu, Finland) vector designated PA59. The αSU P4H cDNA was isolated from PA59 by restriction digestion with KpnI and BamHI and directly subcloned into pREP8 for transfection into 293EBNA cells. This clone was designated pREP8αP4H.

**Recombinant expression of collagen VIII**

293-EBNA cells were maintained in Dulbecco’s modified Eagle’s medium with 0.11 g/l sodium pyruvate and pyroxidine, and supplemented with 10 % (v/v) fetal calf serum, 100 µg/m streptomycin, 100 U/ml penicillin, 2 mM L-glutamine and 1 mM L-ascorbate-2-phosphate. The Epstein Barr Nucleating Antigen (EBNA) cassette was maintained in the 293-EBNA cells using 500 µg/ml geneticin. Stable cell lines expressing α1(VIII) or α2(VIII), respectively, were obtained by transfecting these cells with pCEP4α1(VIII), pCEP4α2(VIII) by lipofectin transfection then selecting with 500 µg/ml hygromycin B. Control cells were produced by
transfecting the pCEP vector into 293-EBNA cells. Recombinant protein expression and secretion into the cell medium was confirmed by western blotting. Confluent 293-EBNA expression cells were washed with PBS then cultured for 48 hours in defined T6 medium containing 2 mM L-glutamine and 1 mM L-ascorbate-2-phosphate. Cell medium was then removed to collect secreted protein. Control cells lines and cells expressing α1(VIII) or α2(VIII) were designated pCEP4293E, α1(VIII)293E and α2(VIII)293E, respectively. Cell lines co-expressing α1(VIII) and α2(VIII) were designated α1α2(VIII)293E.

The clone pREP8αP4H was transfected into α1(VIII)293E and α2(VIII)293E cells using lipofectin in order to increase hydroxylation of proline residues and thereby increase helical stability. Cell lines containing the P4H construct were designated α1(VIII)P4H293E and α2(VIII)P4H293E. The pREP8 vector was also transfected into α1(VIII)293E and α2(VIII)293E cell lines to produce controls lacking the P4H construct. These were designated α1(VIII)PREP8293E and α2(VIII)PREP8293E. Cells were grown to confluence in Dulbecco’s modified Eagle’s medium with 0.11 g/l sodium pyruvate, pyridoxine, 10 % (v/v) fetal calf serum, 100 µg/ml streptomycin, 100 U/ml penicillin, 2mM L-glutamine and 1mM L-ascorbate-2-phosphate. Cells were then washed with PBS then cultured in defined T6 medium containing 2 mM L-glutamine and 1 mM L-ascorbate-2-phosphate. Cell medium was removed after 48 hours to collect secreted protein.

**Smooth muscle cell cultures**

Primary umbilical vein smooth muscle cells (SMC) were isolated from umbilical vein tissue by collagenase digestion, then maintained in Dulbecco’s modified Eagle’s medium with 4500 mg/L
glucose supplemented with 10 % (v/v) fetal calf serum, 100 µg/m streptomycin, 100 U/ml penicillin, 2 mM L-glutamine and 1 mM L-ascorbate-2-phosphate. Only SMC below passage 6 were used. Collagen VIII expression and secretion into the cell medium was confirmed by western blotting. SMC were then washed with PBS and cultured in defined T6 medium containing 2 mM L-glutamine and 1 mM L-ascorbate-2-phosphate. Cell medium was removed after 48 hours to collect secreted protein.

**Immunofluorescence**

The cell lines $\alpha_1$(VIII)293E, $\alpha_2$(VIII)293E, $\alpha_1$(VIII)P4H293E, $\alpha_2$(VIII)P4H293E and $\alpha_1\alpha_2$(VIII)293E were cultured on glass chamber slides. Untransfected 293-EBNA cells were grown as controls for the transfected cells, and SMC were grown to compare collagen VIII immunolabelling with the transfected 293-EBNA cell lines. Cells were seeded at a density of $1 \times 10^4$ cells per chamber, then grown in Dulbecco’s modified Eagle’s medium with 4500 mg/L glucose, 10 % (v/v) fetal calf serum, 100 µg/m streptomycin, 100 U/ml penicillin, 2 mM L-glutamine and 1 mM L-ascorbate-2-phosphate until confluent. Cells were washed in PBS then fixed in 95 % v/v methanol for 10 minutes before further washing in PBS. Cells were then blocked with 5 % BSA for 30 minutes at room temperature, washed in PBS, then incubated with primary antibody against collagen VIII, either monoclonal antibody at 1:200 v/v (10,27) or polyclonal antibody at 1:400 v/v (8), overnight at 4°C. Cells were washed three times with PBS, then incubated with conjugated anti-mouse or anti-rabbit FITC antibodies (1:200 v/v), respectively. Antibodies were prefiltered prior to use through a 0.22 µM membrane to remove any aggregated material. Cells were then washed 3 times with PBS and covered with DAPI mounting fluid. Coverslips were placed over the cells which were then sealed with nail varnish.
and stored at 4°C in darkness. Cells were visualized with a Leica immunofluorescence microscope. Images of DAPI stained cell nuclei and FITC stained collagen VIII were overlaid using Adobe Photoshop 6.0.

**Isolation and SDS-PAGE analysis of recombinant collagen VIII**

Transfected 293-EBNA cell culture medium was concentrated then denatured by boiling in SDS-PAGE loading buffer (50mM Tris.HCl pH 6.8, 10 % (w/v) SDS, 50 % (v/v) glycerol, 0.05 % bromophenol blue). Samples were run at 150V on 4-12 % Bis-Tris gels. In some experiments, 0.05M DTT was added.

Samples resolved on SDS-PAGE gels were transferred to nitrocellulose membranes. Transfer of proteins to the membranes was confirmed by visualisation of pre-stained protein standards. Invitrogen SeeBlue Plus-2 markers (Invitrogen L C5925) were run on all SDS-PAGE gels in this study. Previous reports of collagen VIII and X trimers on SDS-PAGE indicate varying Mr's on SDS-PAGE (within 180-220 kDa range) (17,21,28). A comparison of commercial prestained markers on 4-12% Bis-Tris gels revealed that the high-Mr Invitrogen marker (188 kDa) migrated slower than both the Biorad Kaleidoscope 201 kDa marker and the Biorad broad range standard 250 kDa marker. Thus, apparent discrepancies in trimer mass may reflect the use of different markers and SDS-PAGE gel systems.

Nitrocellulose membranes were washed with TBST (0.01M Tris HCl pH 7.4, 0.15M NaCl and 0.5% polyoxyethylene-sorbitan monolaurate), then blocked using 5% Marvel (w/v) TBST overnight at 4°C. Subsequent wash or antibody incubation steps were supplemented with 2% (w/v) Marvel to maintain blocking of any non-specific antibody binding sites. Membranes were
incubated with primary antibodies in Marvel-TBST overnight at 4°C, washed x3 with TBST, then
incubated with HRP-conjugated anti-mouse antibody for 1 hour at 4°C. Membranes were washed
x3 in TBST before addition of enhanced chemiluminescence reagents and exposure to BIORAD
MR film.

**Size exclusion chromatography**

Transfected cell medium from the pCEP4 collagen VIII transfections was concentrated in 50mM
Tris/HCl pH 7.4, 150mM NaCl, 2mM CaCl₂ and chromatographed on gel filtration columns
(Sephacryl 200 16/60 and Superose 6 10/30) using AKTA Prime and AKTA Explorer systems
(Amersham Pharmacia, U.K). Fractions obtained following size fractionation of medium samples
on a Superose 6 10/30 column were analysed by immuno-slot blots (probed as outlined above for
western blotting), which provided a means of monitoring the molecular mass of recombinant
collagen VIII preparations in native conditions. Samples were also analysed by SDS-PAGE and
western blotting. Elution positions of native collagen VIII assemblies were compared with three
other non-globular extracellular matrix molecular weight standards of laminin (900 kDa),
fibronectin (500 kDa) and thrombospondin (450 kDa), as well as IgG (167 kDa). Recombinant
collagen VIII expressed with or without co-transfection with prolyl 4-hydroxylase, dialysed into
column buffer containing 2M urea, was also chromatographed on the Superose 6 10/30 column
pre-equilibrated in 2M urea in order to determine if the high-Mᵣ assemblies were dissociated in
these conditions.

**Pepsin digestions**

Medium containing collagen VIII was digested for 2 hours with 100 µg/ml pepsin in 0.05M
acetic acid at temperatures of 4°C and 37°C. Samples were then resolved on SDS PAGE gels and
visualized by western blotting using a monoclonal anti-collagen VIII antibody that recognizes epitopes within α1(VIII) and α2(VIII) collagenous regions. Medium containing collagen VIII were also treated with a mixture of trypsin and chymotrypsin after incubation at a range of temperatures from 30°C to 90°C, as previously described (17).

Atomic force microscopy

Metal support stubs (15 mm diameter) were obtained from Veeco Instruments Inc. (Santa Barbara, California, USA), muscovite mica sheets were obtained from Agar Scientific (Stansted, Essex, UK). Trimmed mica sheets were adhered to the sample stubs using clear nail varnish. Cleaved mica surfaces were prepared by peeling off the top layer of mica with adhesive tape immediately prior to sample deposition. Aliquots of collagen VIII, from Superose 6 10/30 column fractions were diluted 1:2 with distilled H2O and incubated on the mica surface for 1 minute. Excess liquid was removed by capillary action and the surface was washed three times with 300 µl distilled H2O. Prepared samples were allowed to air dry for at least 2 hours prior to microscopical analysis.

All samples were imaged by intermittent tapping mode in air using a Multimode atomic force microscope with a Nanoscope IIIa controller and an E scanner. Olympus high aspect ratio etched silicon probes with a spring constant of 42 N/m were obtained from Veeco Instruments Inc. (Santa Barbara, California, USA). Cantilever oscillation varied between 200 – 300 kHz, the drive amplitude was determined by the Nanoscope software and images were captured at a scan rate of 1.2 Hz. The setpoint was adjusted to just below the point at which tip-sample interaction was lost. All measurements were conducted on height data, which was flattened using the Nanoscope...
software and exported to routines written in Microsoft Visual Basic 6.0 for averaging. Gaussian curve fitting to α2(VIII) angle and distance data was performed in SigmaPlot 8.0 (SPSS Science, Chicago, IL, U.S.A.).

**Rotary shadowing electron microscopy**

Recombinant collagen VIII from Superose 6 10/30 column runs (void volume, 700 kDa fractions) were diluted with distilled H$_2$O then adsorbed onto mica and shadowed with tungsten/platinum, using a modification of the mica sandwich technique, as previously described (29). Samples were visualised using a JEOL 1200EX electron microscope.
RESULTS

Expression of recombinant collagen VIII

cDNA fragments encoding α1(VIII) and α2(VIII) were inserted into pCEP4 episomal vectors then transfected into 293-EBNA cells for recombinant collagen VIII expression (Fig. 1). In this system, 293-EBNA cells maintain cell plasmid population and efficient episomal expression. 293-EBNA cells transfected with pCEP4 alone were generated as an empty vector control. Primary SMC were isolated and cultured for production of endogenous collagen VIII.

Collagen VIII secreted into transfected 293-EBNA or SMC culture medium was analysed by SDS-PAGE in reducing and non-reducing conditions, and western blotting using a monoclonal anti-collagen VIII antibody (Fig. 1A). Immunoreactive bands at 180 kDa were detected that corresponded to α1(VIII)3 and α2(VIII)3 homotrimers and to SMC collagen VIII trimers. Collagen VIII was not detected in the empty vector control cells (not shown), and 293-EBNA cells did not produce detectable endogenous collagen VIII. No collagen VIII α-chains (~60 kDa) were detected. More recombinant α2(VIII) was consistently produced than α1(VIII). The pREP8αP4H expression construct, which contains the full-length sequence of the α-subunit of prolyl 4-hydroxylase, was transfected into 293-EBNA cells already stably transfected with α1(VIII) or α2(VIII) in order to ensure efficient prolyl hydroxylation and stable triple helix formation. The empty vector pREP8 was transfected into α1(VIII) and α2(VIII) expressing cell lines as a control. Recombinant α1(VIII)3, α2(VIII)3 and α1(VIII)3 / α2(VIII)3 expressed in these co-transfected cells also migrated as 180 kDa homotrimers on SDS-PAGE gels under reducing and non-reducing (Fig. 1B).
Recombinant secreted collagen VIII, with and without co-expression with prolyl 4-hydroxylase, was subjected to pepsin digestion (100µg/ml) for 2 hours at 4°C or 37°C then compared for helical stability with SMC collagen VIII (Fig. 2A, B). Pepsin-resistant fragments corresponding to those observed in SMC preparations were detected following digestion of recombinant α1(VIII)₃ and α2(VIII)₃ that had been expressed by the co-transfected cells. Thus, co-expression of prolyl 4-hydroxylase with recombinant collagen VIII had ensured the formation of stable triple helical collagen VIII molecules. However, digestion experiments using chymotrypsin and trypsin digestion, after incubation at a range of temperatures from 30°C to >90°C, showed no differences in banding patterns (not shown). In all subsequent experiments, recombinant collagen VIII was co-expressed with prolyl 4-hydroxylase in order to ensure that stable helical collagen VIII assemblies were analysed.

Size exclusion chromatography of native collagen VIII assemblies

Size exclusion chromatography of recombinant collagen VIII [(α1(VIII)₃, α2(VIII)₃ and α1/α2(VIII)₃] in native conditions was conducted in order to determine whether collagen VIII formed higher-order assemblies in vitro, as previously shown for collagen X (24). Recombinant collagen VIII and SMC collagen VIII were eluted over a Sephacryl 200 16/60 column with a volume of 120 ml, exclusion limit of 2.5 × 10⁵ kDa, and a void volume of 32.7 ml. Eluted fractions were then analysed by SDS-PAGE and western blotting. Both recombinant and SMC collagen VIII eluted in the column void volume but resolved on SDS-PAGE as 180 kDa bands (not shown). Thus, recombinant collagen VIII formed higher-order supramolecular assemblies in native conditions, although the detection of a single 180 kDa band in these high-Mr, fractions by SDS-PAGE showed that trimers were the fundamental subunit of these assemblies.
Recombinant collagen VIII samples, expressed without (Fig. 3i) or with (Fig. 3ii) co-expression of prolyl 4-hydroxylase, were then eluted over a Superose 6 HR 10/30 column which had a volume of 24 ml, a void volume of 7.7 ml, and an exclusion limit of $4 \times 10^7$ kDa, in order to investigate the presence of intermediate assemblies. The molecular weight markers laminin (900 kDa), fibronectin (500 kDa), thrombospondin (450 kDa) and IgG (167 kDa) were first eluted over the column in native non-reducing conditions to calibrate the column. Collagen VIII was then fractionated and identified in column fractions by western slot blotting using an anti-collagen VIII monoclonal antibody (Fig. 3). All recombinant native collagen VIII samples expressed without or with prolyl 4-hydroxylase were detected as high-$M_r$ assemblies >900 kDa (Fig. 3i and ii, A and C).

When recombinant $\alpha_1$(VIII)$_3$ and $\alpha_2$(VIII)$_3$ expressed by cells, without or with prolyl 4-hydroxylase, were dialysed into buffer containing 2M urea then chromatographed on a Superose 6 HR 10/30 column pre-equilibrated in 2M urea, they eluted with an $M_r$ of 700 kDa (Fig. 3i and ii, B and D). Thus, native high-$M_r$ assemblies were dissociated by urea but the stable 700 kDa assemblies were urea-resistant. When the recombinant $\alpha_1$(VIII)$_3$ and $\alpha_2$(VIII)$_3$ that had been expressed without or with prolyl 4-hydroxylase and equilibrated in 2M urea, were then dialysed to remove the urea then re-chromatographed, the elution profiles obtained were comparable to those obtained prior to urea treatment (not shown). Thus, the 2M urea disruption of the high-$M_r$ collagen VIII assemblies was reversible in these conditions, which will dissociate non-covalent molecular complexes but will not denature molecules. The elution profiles of co-expressed $\alpha_1/\alpha_2$(VIII)$_3$ also paralleled those observed for $\alpha_1$(VIII)$_3$ and $\alpha_2$(VIII)$_3$ assemblies.
**Immunofluorescence analysis of collagen VIII deposited in cell layers**

Immunofluorescence of collagen VIII in the transfected 293-EBNA cells and empty vector control cells was conducted to determine whether secreted collagen VIII was deposited in the extracellular matrix (Fig. 4) after days 4 and 6 in culture. Cells were cultured on glass slides were stained for nuclei using DAPI and collagen VIII using a monoclonal anti-collagen VIII antibody and a polyclonal anti-α2(VIII) antibody (Fig. 4B, 4D). Medium from these cell cultures was also analysed by SDS-PAGE and western blotting using the anti-collagen VIII monoclonal antibody (Fig. 4A) or the anti-collagen α2(VIII) polyclonal antibody (Fig. 4C).

Using the monoclonal anti-collagen VIII antibody, 180 kDa SDS-PAGE bands were detected (Fig. 4A). Untransfected 293-EBNA cells and empty vector control cells showed no collagen VIII staining (not shown). α1(VIII) was observed as prominent mainly cell-associated and pericellular immunostaining (Fig. 4B, days 4-6), whereas only cell-associated immunostaining was detected for α2(VIII)-transfected cells (Fig 4B, days 4-6). Immunostaining of co-expressed α1(VIII)₃/α2(VIII)₃ showed cell-associated and pericellular immunostaining (Fig 4B, days 4-6). Similar experiments were conducted using the anti-collagen α2(VIII) polyclonal antibody (8) (Fig. 4D). This antibody strongly recognised the 180 kDa α2(VIII) homotrimer, but also detected a 50kDa band in the α1(VIII) preparation and trimers in the co-expression preparations. α1(VIII) recombinant collagen was detected as weak diffuse staining surrounding the cells (Fig. 4D, day 6). In contrast, prominent α2(VIII) extracellular filaments (labelled with white arrows) were observed (Fig. 4D, day 4), with more present by day 6 (Fig 4D day 6). Immunostaining of α1(VIII)₃/α2(VIII)₃ revealed both pericellular and filamentous immunoreactivity, together with cell-associated structures with central nodes (marked with red arrows) and arrays of...
interconnecting filaments between them (Fig. 4D, day 6). Mouse IgG primary and mouse conjugated FITC antibody controls showed only DAPI fluorescence (not shown).

**Microscopy of recombinant collagen VIII assemblies**

In order to determine the molecular organisation of collagen VIII assemblies, recombinant collagen VIII was examined by tapping mode atomic force microscopy and rotary shadowing electron microscopy (Figs. 5,6). For both α-chains, individual homotrimeric were detected that exhibited a molecular length of 135 nm (Fig. 5Ai-iv), which correlates well with previous observations of recombinant collagen X (24). Arrays of α2(VIII)3 collagenous molecular assemblies were detected with commonly observed branching angles of ~114º (Fig. 5Bi-iii), and some partial hexagons were apparent. Rotary shadowing of urea-treated and native collagen VIII from the Superose 6 HR 10/30 column fractions revealed small assemblies with rod-like arms and globular centres, and intermediate angular assemblies and large networks with helical associations, respectively (Fig. 6).
DISCUSSION

Collagen VIII is an essential structural component of vascular, ocular, renal and other extracellular matrices, yet its molecular composition and mechanism of assembly into polygonal lattices remain poorly defined. We have expressed stable triple helical recombinant \( \alpha_1(\text{VIII})_3 \) and \( \alpha_2(\text{VIII})_3 \) homotrimers in mammalian cells, and shown that the secreted form is highly stable triple-helical trimers, and that these trimers form urea-resistant intermediate assemblies of \( \sim \)700 kDa. These data suggest a tetrahedron model of higher-order collagen VIII assembly into 3D hexagonal lattices.

The physiological molecular composition of collagen VIII has proved difficult to resolve. Early studies of collagen VIII extracted from Descemet’s membrane suggested heterotrimers based on a ratio of two \( \alpha_1(\text{VIII}) \) chains to one \( \alpha_2(\text{VIII}) \) (2,5). Subsequent studies indicated both collagen VIII chains were present in equimolar ratios in Descemet’s membrane, whilst immuno-localisation studies using chain-antibodies showed distinct localisation of \( \alpha_1(\text{VIII}) \) and \( \alpha_2(\text{VIII}) \) in cornea, optic nerve, aorta and kidney mesangium (8). We previously used an \textit{in vitro} translation system supplemented with semi-permeabilised HT1080 cells to generate stable triple-helical \( \alpha_1(\text{VIII})_3 \) and \( \alpha_2(\text{VIII})_3 \) homotrimers (17). These intracellular molecules resolved on SDS-PAGE, after denaturation at 100°C, as \( \alpha \)-chains with traces of trimers comprising three triple-helical molecules. In the present study, secreted recombinant collagen VIII and endogenous SMC collagen VIII was in the form of trimers that were thermally stable and resistant to SDS and reducing conditions. A previous study of homologous collagen X expressed in SaOS-2 cells showed that generally more trimer was present in medium than cell lysates (28, see Figs. 8, 9).
These studies highlight stability differences in intracellular and secreted trimers, possibly due to altered conformation in the extracellular environment.

Co-transfection of collagen VIII expressing cells with prolyl 4-hydroxylase enhanced the triple-helical stability of homotrimers, as judged by increased resistance to pepsin digestion. Interestingly, chymotrypsin/trypsin digestions failed to show stability differences between collagen VIII expressed with or without prolyl 4-hydroxylase showing that, for collagen VIII, harsher acidic conditions are required to expose potentially susceptible sequences. The unusually stable NC1 trimers are likely to contribute to this effect and to the complex digestion patterns observed.

Immunofluorescence analysis of transfected cell layers confirmed the deposition of recombinant collagen VIII within the extracellular matrix (Fig. 4). An α2(VIII)-specific polyclonal antibody showed that α2(VIII)₃ forms distinctive higher-order rod-like structures. Interestingly, a monoclonal antibody that recognizes a collagenous sequence close to the NC1 domain of both α-chains, showed only cell-surface associated staining, so these epitope(s) are not accessible in the higher-order assemblies. It is likely that α1(VIII)₃ forms similar higher-order structures to α2(VIII)₃. Co-expressed α-chains exhibited similar patterns as single α-chains, as predicted since they are structurally homologous and form similar intermediate assemblies. There was also evidence for short filaments emanating from central nodes. It is not known why more α2(VIII)₃ than α1(VIII)₃ was consistently expressed. α1(VIII) may be transcribed or translated more slowly than α2(VIII), or the α1(VIII) transcript may be less stable than the α2(VIII) transcript, or it may be post-translationally modified and secreted more slowly than α2(VIII).
The crystal structure of collagen VIII trimeric NC1 domains has revealed an intimate trimeric assembly strengthened by a buried cluster of calcium ions (26). Both collagen VIII chains possess the conserved aspartic acid residues that, in collagen X, ligate calcium. A previously proposed explanation for the high thermal stability of collagen X NC1 trimers, based on hydrophobic interactions between NC1 domains, is unlikely as the residues considered responsible for this effect do not form a hydrophobic plug at the base of the trimer (25,26). Another possible contribution to collagen X and VIII trimer stability may be interactions between the non-collagenous sequences preceding the NC1 domains of collagens VIII and X.

Both collagen VIII and X undergo supramolecular assembly into hexagonal lattices in vitro and in vivo. Immuno-microscopy showed that collagen VIII is an integral component of a hexagonal lattice structure within Descemet’s membrane of the eye, and similar collagen VIII-containing assemblies were observed in cell layers (7,27). Purified chick collagen X formed hexagonal lattices in thermal aggregation studies in vitro (24), and recombinant mammalian α1(X) NC-1 domains also aggregated (25). In this study, microscopy has shown that recombinant α1(VIII)3 and α2(VIII)3 homotrimers form large supramolecular assemblies in native conditions. Microscopy of urea-treated and native collagen VIII from the Superose 6 HR 10/30 column fractions revealed small assemblies with rod-like arms and globular centres, and numerous arrays of rod-like molecules aligned at angles of ~114° and large networks with helical associations, respectively. The ability of recombinant collagen VIII to assemble readily was also shown using size exclusion chromatography. Using a Superose 6 HR 10/30 column, virtually all recombinant native collagen VIII homotrimers were present as high-M, aggregates in the void volume. Addition of 2M urea led to disruption of these large native aggregates into intermediate
assemblies of 700 kDa, which corresponds closely to the $M_r$ of four homotrimers (720 kDa). SDS-PAGE gels of the column fractions confirmed that the high-$M_r$ and intermediate assemblies were all formed from non-covalently associated 180 kDa trimers.

The novel urea-resistant collagen VIII intermediates and networks with helical associations identified in this study provide important clues to collagen VIII assembly (Fig. 7). It may be formed by the association of four NC1 trimers, each making three hydrophobic contacts via its three hydrophobic patches, one on each NC1 domain within a trimer, similar to those identified in the collagen X trimer crystal structure (25). Such an arrangement would generate angular tetrahedron-shaped subunits that could then readily assemble into a 3-dimensional hexagonal lattice as a result of secondary interactions involving N-terminal sequences with each other (Fig. 7Bi) or with both terminal and helical sequences (Fig. 7Bii). The latter arrangement would involve anti-parallel aligned helices and therefore thicker inter-node ‘struts’. Since many examples of helical associations are apparent in our microscopy preparations, and have previously been shown for collagen X (24), we predict that the anti-parallel overlap model (Fig. 7Bii) is how three-dimensional collagen VIII lattices assemble.

In summary, we have expressed stable recombinant collagen VIII homotrimers, shown that they assemble and are deposited in the extracellular matrix, and have identified an intermediate of assembly that forms the basis for three-dimensional collagen VIII assembly.
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FIGURE LEGENDS

FIGURE 1

Western blots of collagen VIII secreted by α1(VIII) and α2(VIII) transfected 293-EBNA cells and by SMC

A, B. Collagen VIII secreted into culture medium by transfected 293-EBNA cells, without (A) or with (B) co-transfection with prolyl 4-hydroxylase, was electrophoresed in reducing or non-reducing conditions then probed in western blots using an anti-collagen VIII monoclonal antibody that recognises α1(VIII)₃ and α2(VIII)₃ (10,27). Collagen VIII immunoreactive bands of 180 kDa were detected in all samples.
Western blots of collagen VIII pepsin digests secreted by α1(VIII) and α2(VIII) transfected 293-EBNA cells and by SMC

A, B. Collagen VIII secreted into culture medium by transfected 293-EBNA cells, without (A) or with (B) co-transfection with prolyl 4-hydroxylase, was digested with pepsin at 4°C (lanes 2,5,8) or 37°C (lanes 3,6,9-12) for 1 hour prior to electrophoresis in reducing conditions, then western blotting using the anti-collagen VIII monoclonal antibody. Both α1(VIII) and α2(VIII) without co-transfection with prolyl 4-hydroxylase (A) were partially digested at 4°C with no pepsin-resistant fragments, and completely digested at 37°C. Homotrimers generated in 293-EBNA cells that had been co-transfected with prolyl 4-hydroxylase (B), and SMC collagen VIII, were partially digested at 37°C but pepsin-resistant fragments were present.
FIGURE 3

Size fractionation of recombinant collagen VIII on a Superose 6 HR 10/30 column.

Collagen α1(VIII)3 (A,B) and α2(VIII)3 (C,D) were expressed without (i) or with (ii) co-transfection with prolyl 4-hydroxylase, then chromatographed on a Superose 6 HR 10/30 gel filtration column in native conditions (A,C) or equilibrated in 2M urea (B,D).

Slot immunoblot analysis of column fractions, using an anti-collagen VIII monoclonal antibody (10,17), identified the fractions containing recombinant collagen VIII. For both homotrimers, with or without co-transfection with prolyl 4-hydroxylase, urea treatment resulted in the loss of high-M₉ material but the appearance of a prominent peak corresponding to M₉ ~700 kDa.

Chromatography markers used to calibrate the column were laminin (900 kDa), fibronectin (500 kDa), thrombospondin (450 kDa) and IgG (167 kDa).
Recombinant collagen VIII is deposited in 293-EBNA cell layers

Western blotting was conducted on collagen VIII secreted by 293-EBNA cells transfected with \( \alpha_1(\text{VIII})_3, \alpha_2(\text{VIII})_3 \) and co-expressed \( \alpha_1(\text{VIII})_3/\alpha_2(\text{VIII})_3 \) into culture medium, using the anti-collagen VIII monoclonal antibody (A) or a polyclonal \( \alpha_2(\text{VIII}) \) chain specific polyclonal antibody (B). Collagen VIII deposition by 293-EBNA cells transfected with \( \alpha_1(\text{VIII}), \alpha_2(\text{VIII}) \) and co-expressed \( \alpha_1(\text{VIII})_3/\alpha_2(\text{VIII})_3 \) was visualised by immunofluorescence using an anti-collagen VIII monoclonal primary antibody (C) (10,27) or a polyclonal antibody specific for \( \alpha_2(\text{VIII}) \) (D) (8), and appropriate FITC-conjugated second antibodies. DAPI was used for nuclear staining. Cells were visualised at x 400 magnification. Filamentous recombinant collagen VIII is labelled with white arrows in (D). Immunostaining of \( \alpha_1(\text{VIII})_3/\alpha_2(\text{VIII})_3 \) cell-associated structures with central nodes is labelled with red arrows. Control lanes contain medium from untransfected 293-EBNA cells.
FIGURE 5

Ultrastructural organisation of $\alpha_1$(VIII)$_3$ and $\alpha_2$(VIII)$_3$ visualised by intermittent contact mode AFM.

(Ai) Isolated $\alpha_1$(VIII)$_3$ monomers appeared as two globular regions connected by a rod-like triple helical region (extracted region from a 2$\mu$M x 2$\mu$M scan).

(Aii) Mean axial height profile for $\alpha_1$(VIII)$_3$ monomers. Mean height of the triple helical region was 0.8 nm (SEM = 0.1, n = 4) and the mean monomer was 134.4 nm (SEM = 4.3, n = 4).

(Aiii and Aiv) Mean height maps of $\alpha_1$(VIII)$_3$ (iii) and $\alpha_2$(VIII)$_3$ (v) monomers.

(Bi-iii) Atomic force micrographs showing $\alpha_2$(VIII)$_3$ monomers forming complex branched networks (1 x 0.4 $\mu$m scan (i), 200 nm x 150 nm (ii and iii), scale bar = 200 nm). Within these networks the mean node-node distance was 88.4 nm (SEM = 4.4, n = 40) and the frequency distribution of the branching angle fitted a uni-modal Gaussian peaked at 114° (table Biv).

Scale bars represent 200 nm.
FIGURE 6

Ultrastructural organisation of α2(VIII)₃ visualised by rotary shadowing.

Rotary shadowing electron micrographs of recombinant α2(VIII)₃ from Superose 6 column fractions (high-Mᵣ and intermediate assemblies).

(i-iii) Intermediate assemblies had rod-like triple helical arms and a central globule. Images (i-iii) are 100nm x 100nm.

(iv-x) Large assemblies appeared as angular arrays, and extensive networks were also present. Images (iv-ix) are 200nm x 200nm. Image (x) is 600nm x 400nm.
Tetrahedron model of collagen VIII assembly

(A) Four triple-helical collagen VIII molecules may associate to form a tetrahedron (720 kDa). Each molecule has a hydrophobic patch on each of its three C-terminal NC1 domains (26), which could interact with corresponding hydrophobic patches on the C-termini of the other three molecules within the tetrahedron. *En face* images (i,ii) and offset images (iii, iv) of a tetrahedron highlight how the four molecules may be arranged.

(B) Models showing how collagen VIII tetrahedrons could interact to form 3-dimensional hexagonal lattices. In (i), tetrahedrons undergo higher-order N- to N- terminal interactions. In (ii), tetrahedrons undergo higher-order N- to C-terminal interactions. The inter-node struts in (ii) would be formed from two anti-parallel triple helices, and would therefore be predicted to be wider than struts in (i).
Figure 1

**A: COL VIII +/- DTT**

| DTT | + | + | + | - | - | - | kDa |
|-----|---|---|---|---|---|---|-----|
| α1(VIII) | 188 |
| α2(VIII) | SMC |
| α1(VIII) | 188 |
| α2(VIII) | SMC |

**B: COL VIII + P4H**

| DTT | + | - | + | - | + | - | kDa |
|-----|---|---|---|---|---|---|-----|
| α1(VIII) | 188 |
| α2(VIII) | P4H |
| α1(VIII) | 188 |
| α2(VIII) | P4H |
| α1(VIII)/α2(VIII) | P4H |
**Figure 2**

A: COL VIII + PEPSIN

| lane no. | pepsin (+/-) | temp(°C) |
|----------|--------------|----------|
| 1        | +            | 37       |
| 2        | +            | 37       |
| 3        | +            | 37       |
| 4        | +            | 37       |
| 5        | +            | 37       |
| 6        | +            | 37       |
| 7        | +            | 37       |
| 8        | +            | 37       |
| 9        | +            | 37       |

B: COL VIII + P4H + PEPSIN

| lane no. | pepsin (+/-) | temp(°C) |
|----------|--------------|----------|
| 10       | +            | 37       |
| 11       | +            | 37       |
| 12       | +            | 37       |

- α1(VIII) | α2(VIII)     | SMC (VIII) |
- 188 kDa  | 98 kDa       | 62 kDa     |

- α1(VIII) P4H | α2(VIII) P4H | SMC (VIII) |
- 188 kDa      | 98 kDa       | 62 kDa     |
Figure 3

i) COLLAGEN VIII

Fibronectin (500KDa)
Laminin (900KDa)
Thrombospondin (450KDa)
IgG (167KDa)

α1(VIII)
α1(VIII) + 2M urea
α2(VIII)
α2(VIII) + 2M urea

FRACTION

A
B
C
D

11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

ii) COLLAGEN VIII + P4H

Fibronectin (500KDa)
Laminin (900KDa)
Thrombospondin (450KDa)
IgG (167KDa)

α1(VIII)
α1(VIII) + 2M urea
α2(VIII)
α2(VIII) + 2M urea

FRACTION

A
B
C
D

11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Figure 4

A: Cell media blot with monoclonal antibody

B: Monoclonal antibody Immunofluorescence

| Control | α1(VIII) | α2(VIII) | α1(VIII)/α2(VIII) |
|---------|----------|----------|-------------------|
| day 4   | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| day 6   | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |

D: Polyclonal anti α2(VIII) antibody Immunofluorescence

| Control | α1(VIII) | α2(VIII) | α1(VIII)/α2(VIII) |
|---------|----------|----------|-------------------|
| day 4   | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| day 6   | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |

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Figure 5

**A**

- Image i: Close-up of a specific area.
- Image ii: Graph showing the distribution of distances with a peak at 134 nm and a variation of 0.8 nm.
- Image iii: Another close-up with a different perspective.
- Image iv: A detailed view highlighting specific features.

**B**

- Image i: A section with 112 nm highlighted.
- Image ii: An area with a node-node distance of 0.8 nm.
- Image iii: A close-up showing pixel values.

| Angle (°) | Node-node distance (nm) |
|----------|-------------------------|
| Mean     | 113.8                   | 88.4          |
| SD       | 21.8                    | 27.9          |
| N        | 40                      | 40            |
A) Tetrahedrons comprising four collagen VIII triple-helical molecules

- **i**
- **ii**
- **iii**
- **iv**

B) Tetrahedron interactions that generate 3D hexagonal lattices

1. (i) N- to N- terminal interactions
2. (ii) N- to C- terminal interactions
Expression and supramolecular assembly of recombinant a1(VIII) and a2(VIII) collagen homotrimers
Simon Stephan, Michael J. Sherratt, Nigel Hodson, C. Adrian Shuttleworth and Cay M. Kielty

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