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Recombinant Alpha 1(VIII) Collagen Chains Form Homotrimers *in Vitro*

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Type VIII collagen, a member of the short chain collagen family, is expressed in large blood vessels and in the subendothelium and mesangium of the glomerulus. While two genetically distinct type VIII collagen chains, alpha 1 and alpha 2, have been identified, it is not known whether type VIII collagen exists as homotrimers or heterotrimers. To determine whether alpha 1(VIII) collagen chains can associate to form homotrimers, a full length human alpha 1(VIII) collagen cDNA was generated by overlap extension PCR and used as a substrate for coupled *in vitro* transcription/translation. A translation product of 80kDa, the predicted size of alpha 1(VIII) collagen, was identified by autoradiography of SDS-gels containing radiolabelled translation products. Sensitivity of the *in vitro* translated protein to digestion with bacterial collagenase and trypsin and the size of the proteins generated by these digestions provide evidence that alpha 1(VIII) collagen can participate in the formation of homotrimers.

Type VIII collagen is a member of the short chain collagen family of proteins (reviewed in ref. 1 and 2). It is expressed in Descemet's membrane of the eye (3), the subendothelium of blood vessels (4), and in the glomerulus of the kidney (5). Two distinct type VIII collagen alpha chains, alpha 1 and alpha 2, have been described (2, 6, 7). The amino acid content of alpha 2(VIII) collagen is distinct from that of alpha 1(VIII) collagen (7). However, it is not known whether type VIII collagen trimers exist as heterotrimers of alpha 1(VIII) collagen and alpha 2(VIII) collagen chains and/or as alpha 1(VIII) collagen and alpha 2(VIII) collagen homotrimers. This distinction is important since the three dimensional structure of type VIII collagen polymers and binding interactions between type VIII collagen and other extracellular matrix molecules are predicted to vary according to the chain composition of mature type VIII collagen trimers. Studies of alpha 1(VIII) collagen and alpha 2(VIII) collagen chain assembly are hindered at present by the absence of a method to isolate pure preparations of the native forms of each of these chains. Therefore, another approach is needed to test the ability of these collagen chains to form homotrimers and heterotrimers. In the present study, the ability of alpha 1(VIII) collagen to form homotrimers has been tested by studying the protein products derived from cell-free transcription and translation of newly isolated full length human alpha 1(VIII) collagen cDNA. The results indicate that recombinant alpha 1(VIII) collagen protein chains can form homotrimers in this cell-free system.

MATERIALS AND METHODS

A 2.2 kb cDNA containing the complete human alpha 1(VIII) coding sequence was generated by overlap extension PCR (8) using a 16 kb genomic fragment encoding both exons of the human COL5A1 gene (6) (EMBL Databank, accession number X57527). In the first of a two step strategy, exons 1 and 2 were amplified, in separate reactions, using exact-match oligodeoxynucleotide primers. Primers were engineered to include: a Kozak sequence (9) in the exon 1 sense primer, 20 bp of exact-match sequence derived from the 5′ end of exon 2 at the 3′ end of the exon 1 antisense primer, and restriction enzyme sites in all primers. The primers sequences are: exon 1 sense 5′ - $(T)_5$GAATTC-CCACCATGGGGGTGATGGCTGTGCTGCCTGG - 3′, exon 1 antisense 5′ - TAAACTGGCTAATGGTATTTC-

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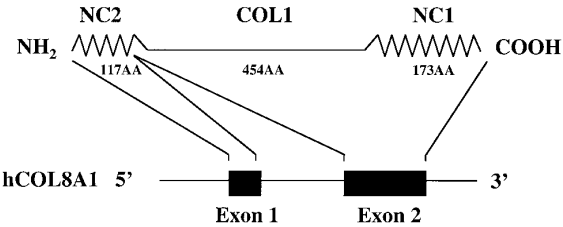

FIG. 1. Exon structure of human genomic DNA coding for the alpha 1(VIII) collagen chain (hCOL8A1) and domain structure of the translation product. Exons are indicated by filled boxes. The domain structure of the translation product is shown above the schematic of the gene. The central triple-helical domain (COL1) of 454 residues is flanked by an amino terminal nontriple helical domain (NC2, 117 amino acids) and a carboxy terminal nontriple helical domain (NC1, 173 amino acids).

TTTGCCTTTCTTGGGAACGG 3′, exon 2 sense 5′ - AAATACCATTAGCCAGTTTT - 3′, and exon 2 antisense 5′ - $(T)_5$AGATCTCATGGGATACAATAAATATC - 3′. PCR was preceded by denaturation of DNA at 94°C for 3 minutes followed by addition of Pfu DNA polymerase (Stratagene). PCR consisted of thirty-five cycles of 94°C for 1 minute, 58°C for 2 minutes and 72°C for 3 minutes performed in a programmable dri-block (Perkin-Elmer). In the second step, exon 1 and exon 2 DNA were used as substrates in a PCR using the exon 1 sense and the exon 2 antisense primers and the identical thermal cycling conditions used in the first step. A 2.2 kb PCR product was ligated to the mammalian expression vector, pSG5, (Stratagene). DNA sequencing was performed using the dideoxy chain-termination method and T4 DNA polymerase (Sequenase).

The recombinant pSG5 plasmid containing the full-length human alpha 1(VIII) collagen cDNA was used as a substrate for *in vitro* transcription/translation in the presence of ^{35}S-methionine, using a commercial kit (Novagen). Translation products were digested with 18U highly purified bacterial collagenase (Advance Biofactures) per 20 μl of reaction mixture for 1 hour at 37°C in a buffer consisting of 0.1M Tris-HCl, pH 7.5, and 10 mM CaCl. In separate reactions, translation products were digested with trypsin in 50 mM Tris-HCl, pH 8, and 100 mM NaCl for 2 hours at 22°C. After digestion proteins were precipitated with acetone in the presence of 0.1% SDS. Digested and undigested translation products were analyzed by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate under reducing conditions and then imaged by autoradiography. Molecular size was determined by comparison with globular molecular weight standards (NEN Research Products).

RESULTS

Cloning of a full length human alpha 1(VIII) collagen cDNA. The human alpha 1(VIII) collagen chain consists of a central triple helical domain flanked by an amino-terminal nontriple helical domain (NC2) and a carboxy-terminal nontriple helical domain (NC1) (6). This chain is encoded by the COL8A1 gene which consists of two exons separated by a large intron (Figure 1). Exon 1 encodes $109^1/_3$ codons of the NC2 domain including the signal domain. Exon 2 encodes $7^2/_3$ codons of the NC2 domain and the entire COL1 and NC1 domains.

Since a human alpha 1(VIII) collagen cDNA has not been previously isolated, overlap extension PCR (8) was used to generate a full length alpha 1(VIII) collagen cDNA. A two step strategy was used. First, exon 1 and exon 2 were amplified in separate PCR reactions using a human COL8A1 genomic fragment. This 16kb fragment (YMh801, kindly provided by Bjorn R. Olsen, Harvard Medical School) contains exon 1 and exon 2 and a large intervening intron. PCR using exon 1 and exon 2 primers generated DNA fragments approximately 330 bp and 1900 bp, the predicted sizes of exon 1 and exon 2, respectively (Figure 2A). Next, these amplified DNA fragments were pooled and used as substrates in a PCR using the exon 1 sense and exon 2 antisense primers. This reaction yielded three detectable bands, approximately 2.0 kb, 2.2 kb and 4.0 kb (Figure 2B). Since the 2.2 kb DNA band corresponded to the size expected for a band encoding exon 1 and exon 2, it was isolated and cloned. Recombinant clones containing this insert were then further analyzed to determine the sequence across the junction of exons 1 and 2. Since thermostable DNA polymerase has a propensity to add dA residues to the 3′ end of dsDNA, it was possible that sequence errors were present in the

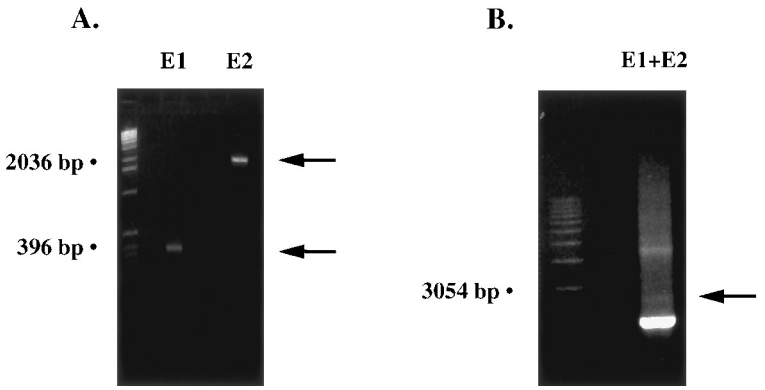

FIG. 2. PCR amplification of a full length alpha 1(VIII) collagen cDNA. Panel A. Ethidium bromide-stained agarose gel containing PCR products. Exact match primers were used to amplify exon 1 and exon 2 from genomic DNA in separate reactions. The DNA band corresponding to exon 1 (E1) is marked by the lower arrow. The DNA band corresponding to exon 2 (E2) is marked by the upper arrow. Panel B. Ethidium bromide-stained agarose gel containing PCR products. A full length alpha 1(VIII) collagen cDNA was amplified from exon 1 and exon 2 DNA by overlap extension PCR using exact-match exon 1 sense and exon 2 antisense primers (see methods). The arrow marks the position of an approximately 2.2 kb DNA band, the predicted size of the DNA construct containing the full length alpha 1(VIII) collagen coding sequence.

amplified alpha 1(VIII) DNA. Sequencing of twelve clones identified two clones, #58 and #63, with the correct sequence (Figure 3). The full length sequence of clone #58 was determined and shown to be identical to the known genomic sequence (data not shown).

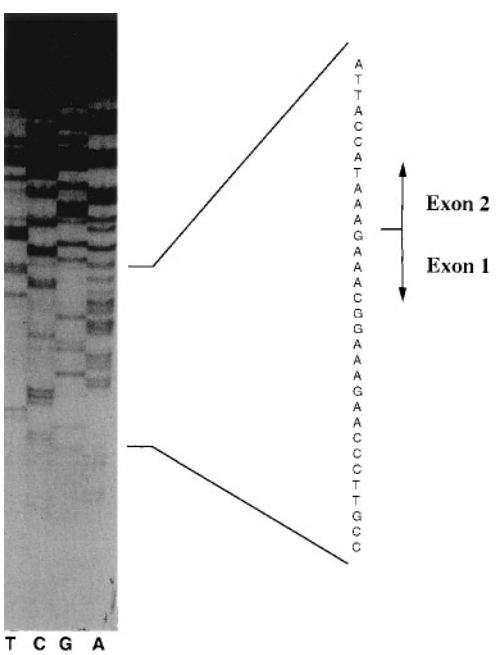

FIG. 3. DNA sequence obtained from clone #58, a full length alpha 1(VIII) collagen DNA construct. DNA sequencing was performed using an exact-match oligonucleotide primer encoding hCOL8A1 sequences upstream of the junction of exon 1 and exon 2. The sequence demonstrates that the spliced exon 1 and exon 2 sequence, encoded by clone #63, is identical to the genomic COL8A1 sequence.

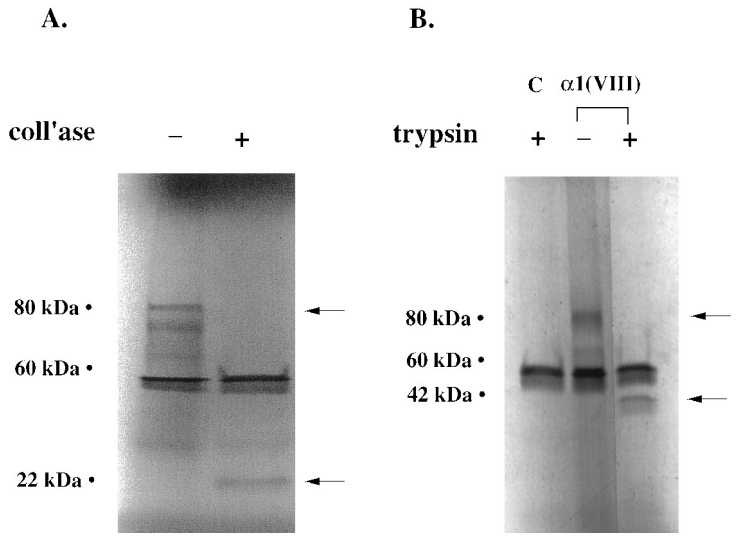

FIG. 4. Characterization of *in vitro* translated human alpha 1(VIII) collagen chains. Autoradiogram of SDS-gels containing ^{35}S-labelled products of a transcription/translation reaction before and after enzymatic digestions. Panel A. An aliquot of the translation products was treated with bacterial collagenase and the untreated and treated translation products were then electrophoretically separated. Translation yielded an 80 kDa product (upper arrow) and smaller truncated proteins. Digestion with collagenase resulted in degradation of these proteins and generation of an approximately 22 kDa collagenase resistant fragment (lower band). Panel B. An aliquot of the translation products was treated with trypsin and the untreated and treated translation products were then electrophoretically separated. Digestion with trypsin resulted in degradation of the 80 kDa translation product (upper arrow) and generation of an approximately 42 kDa trypsin resistant fragment (lower arrow). The lane marked 'C' refers to translation products generated in the absence of DNA substrate.

In vitro transcription/translation of human alpha 1(VIII) collagen. To determine whether alpha 1(VIII) collagen chains are capable of forming homotrimers, assays of trimer formation were used in *in vitro* translations of alpha 1(VIII) collagen mRNA. Two properties common to trimeric collagen molecules are important to this analysis: (1) the triple helical COL domains, assembled as trimers, are uniquely sensitive to digestion with bacterial collagenase and (2) the triple helical COL domains, assembled as trimers, are uniquely resistant to digestion with trypsin (10). In contrast, single collagen chains are resistant to collagenase and sensitive in all domains to digestion with trypsin. Therefore, digestion of translated collagen chains with bacterial collagenase and trypsin is a test of whether individual chains have participated in trimer formation. *In vitro* transcription/translation of recombinant human alpha 1(VIII) collagen DNA (clone #58) yielded a 80kDa band (Figure 4A), the size predicted for the complete alpha 1(VIII) collagen protein (5, 6). Lower molecular weight proteins, generated in this assay, represent incompletely translated proteins. Digestion of the translation products with highly purified bacterial collagenase resulted in partial degradation of the 80kDa protein and generation of a 21 kDa band, the predicted size of the collagenase resistant NC1 domain (Figure 4A). Digestion of the translation products with trypsin resulted in partial degradation of the 80kDa protein and generation of a 45 kDa trypsin resistant fragment, the size of the COL1 domain (Figure 4B). These results indicate that in a cell free system alpha 1(VIII) collagen chains are able to form homotrimers.

DISCUSSION

The manner in which proteoglycans and proteins are organized within the extracellular matrix to form stable three dimensional structures and how these structures interact with

adjacent cells are central issues in the study of the biology of extracellular matrices. We have sought to define novel matrix components expressed in the glomerular extracellular matrix which functions as a barrier to the filtration of macromolecules (11). We have demonstrated previously that the short chain collagenous protein, alpha 1(VIII) collagen, is expressed in the normal rat glomerulus in a subendothelial and mesangial distribution (5). The complete primary structure of alpha 1(VIII) collagen has been determined in the human (6), mouse (12), and rabbit (2). Comparison of the putative amino acid sequences across these species shows a remarkably high degree of identity ($>$ 94%) with amino acid differences being conservative in nature. The predicted alpha 1(VIII) collagen translation product consists of a 454 amino acid long triple helical domain bordered at the amino and carboxy terminal ends by a nontriple helical (globular) domain. The amino terminal (NC2) domain contains 117 amino acids and the carboxy terminal (NC1) domain contains 173 amino acids (Figure 1). In contrast to the fibrillar collagens (types I, II, III, V, XI) and like type X collagen (13), the alpha 1(VIII) collagen protein does not undergo proteolytic processing of its N- and C-terminal domains after secretion from the cell (5). In an identical fashion to all known collagenous proteins (14) alpha 1(VIII) collagen undergoes post-translational hydroxylation of prolyl and lysyl residues in the triple helical domain and promotes trimer formation by collagenous alpha chains (10). It is not known whether type VIII collagen exists as a homotrimer composed of three alpha 1(VIII) collagen chains or a heterotrimer composed of a mixture of alpha 1(VIII) collagen chains with one or two chains of another short chain collagen, alpha 2(VIII) collagen.

The experiments described here provide evidence that the size of the alpha 1(VIII) collagen chain is that predicted by its primary and secondary structure and that these chains are able to self-associate and form homotrimers. Recombinant human alpha 1(VIII) collagen chains containing the NC1, COL1 and NC2 domains were generated by synthesizing a full length alpha 1(VIII) cDNA and then using this DNA as a substrate in a coupled transcription/translation experiment. The translated chains are identical in size to alpha 1(VIII) collagen chains secreted by mesangial cells (5). The sensitivity of the translated proteins to digestion with bacterial collagenase and trypsin and the size of the proteins generated by these digestion provides evidence that alpha 1(VIII) collagen chains can participate in homotrimer formation. While hydroxylation of lysyl and prolyl residues is essential for trimer formation in intact cells, others have shown that a small amount of trimer is formed in the absence of this hydroxylation (10). This observation is consistent with the data here which show that proteins translated *in vitro* in the absence of enzymes which hydroxylate amino acids can form trimers.

The results described here provide a basis for determining the assembly of recombinant alpha 1(VIII) collagen chains expressed in intact cells. In turn, the expression of these chains will provide an opportunity to determine the binding of alpha 1(VIII) collagen trimers to other extracellular matrix components including heparan sulfate, predicted to be a binding partner for type VIII collagen containing alpha 1(VIII) collagen chains (2).

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