Cloning of α1(IV) and α2(IV) Collagen cDNAs from Rabbit Corneal Endothelial Cell RNA

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To understand the role of type IV collagen in embryogenesis, regeneration, and tissue repair of the cornea at the molecular level, the authors isolated clones coding for rabbit α1(IV) and α2(IV) chains from a cDNA library constructed with rabbit corneal endothelial cell RNA. The isolated α2(IV) cDNA clones encode a part of a 5' untranslated region, the signal peptide, the 7S domain, part of the triple-helical domain, and the entire carboxyl-terminal nontriple-helical (NC1) domain. By cross hybridization, using one of the α2(IV) cDNA inserts, a cDNA clone encoding the α1(IV) chain was isolated from a λgt10 library primed with oligo(dT). The clone covered a short portion of the triple-helical domain, the entire NC1 domain, and a short 3' untranslated region. The nucleotide-sequence analysis of these clones provides, for the first time to the authors' knowledge, the primary structure of the carboxyl-terminal portion of both rabbit α1(IV) and α2(IV) collagen chains. Between the α1(IV)NC1 and the α2(IV)NC1, 61% and 66% sequence similarities were observed at the amino acid and nucleotide levels, respectively. The locations of all the 12 cysteiny1 residues were conserved in the two NC1 sequences.

Materials and Methods

Endothelial Cell Cultures

Rabbit corneal endothelial cells were isolated from Descemet’s membrane as described. Briefly, the Descemet’s membrane–endothelial cell complex was peeled off 200 rabbit eyes, treated with 2.5 mg/ml trypsin (Cooper Biomedical, Malvern, PA), 2 mg/ml bacterial collagenase, and 0.5 mg/ml hyaluronidase (Worthington Biomedical Corp., Freehold, NJ). Cultures (10^4 cells/dish) were maintained in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY) supplemented with 20% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml of amphotericin B in a CO2 incubator. Trypsin was used for passage. Primary or secondary cells were used as sources of mRNA preparation.
cDNA Construction and Screening

Total cellular RNA was extracted from confluent cultures with guanidinium thiocyanate. Poly(A+)-RNA was obtained by oligo(dT)-cellulose chromatography. The rabbit cDNA libraries were generated with poly(A+)-RNA isolated from cultured corneal endothelial cells. The cDNA synthesis was done by primer extension using a kit from Amersham (Buckinghamshire, England) and based on an earlier method. As primer, we used a 14-mer oligonucleotide (5'-CCNGGNGGNCCNGG-3') to enrich for collagenous cDNAs. The size-fractionated cDNAs (> 400 base pairs) were C-tailed by terminal transferase and annealed to G-tailed pBR322 plasmid vector at the PstI site. The recombinants were transfected into competent cells (MC1061). Plasmid DNAs were isolated from 1 ml of overnight cultures of individual colonies and characterized by restriction-enzyme digestion and RNA transfer blot (northern blot) analysis.

Sau96I analysis was done to identify cDNA clones harboring collagenous sequences.

RNA Blotting and Nucleotide Sequencing

Poly(A+) or total RNA underwent electrophoresis in 0.8% agarose gels in the presence of formamide and formaldehyde, were blotted onto nitrocellulose filters, and probed with random-primed DNA as described. After hybridization in fivefold concentrated SSC (single-concentrated SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 1% N-lauroylsarcosine at 65°C, the filters were washed twice in threefold concentrated SSC and 0.5% N-lauroylsarcosine and twice in threefold concentrated SSC at 65°C. Nucleotide sequence was analyzed with the di-deoxy chain termination method using 35 S-deoxyadenosine triphosphate and T7 DNA polymerase. Some of the cDNA sequences were determined using specific oligonucleotides as sequencing primers on M13mp18 or M13mp19 templates containing the excised PstI cDNA inserts. The oligonucleotides were synthesized on a Milligen 7500 DNA synthesizer (Burlington, MA) according to the manufacturer's instructions.

Results

Construction and Screening of the cDNA Clones for \( \alpha_2(IV) \) Chain

A cDNA library constructed with a 14-mer primer (5'-CCNGGNGGNCCNGG-3') and poly(A)+RNA isolated from rabbit corneal endothelial cells was screened by Sau96I analysis to isolate cDNA clones harboring multiple GGNCC sites. The presence of multiple Sau96I sites in cDNAs is typical of collagenous clones because of multiple glycine–proline (Gly–Pro) repeats in collagens. We isolated 26 Sau96I-positive clones in 1000 randomly selected clones; 11 are shown in Figure 1. Inserts of these clones were used for northern blot analysis. Several clones showed positive hybridization to mRNAs, migrating slightly slower than the 28S rRNA marker in 0.8% agarose gels (Fig. 2). As shown in Figures 4 and 5, nucleotide-sequence analysis of the inserts of the three clones,
Northern-blot Analysis

![Fig. 2. Northern blot analysis using the clones encoding type IV collagen chains. Rabbit corneal endothelial cell poly(A) + RNA (0.2 µg) was applied on 0.8% agarose gel, electrophoresed for 5 hr at 50 V, and blotted onto nitrocellulose. Individual strips of the nitrocellulose filter were hybridized with inserts of cDNA clones coding for α1(IV) (NK11, lane 1) and α2(IV) (pCE1539, lane 2). As control type VIII collagen cDNA, pCE1230 was used on lane 3. Positions of 28s and 18s rRNA are indicated by arrows.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933382/)

pCE923, pCE1355, and pCE1539 showed that these three clones contain an open reading frame with a Gly-X-Y repetitive sequence, typical of collagen polypeptides. A characteristic feature of the repetitive sequence of these three clones is the presence of many imperfections in the Gly-X-Y repeat structure. It is well known that there are no imperfections in the Gly-X-Y repeats in the triple-helical domains of fibrillar types of collagen (types I, II, III, V, and XI), whereas fibril-associated collagen with interrupted triple-helix (FACIT) collagens (types IX and XII) and other, more recently characterized collagens (types VI, VIII, IX, and X) contain some imperfections. Because α1(IV) and α2(IV) collagen chains contain as many as 21 and 23 imperfections in their Gly-X-Y repeat domains, we examined the possibility that pCE923, pCE1344, and pCE1539 represented type IV collagen clones.

Two of the clones, pCE1539 and pCE1344, were overlapping clones. The composite translation product, containing 52 Gly-X-Y triplets and a 227 residue-long nontriple-helical domain, was compared with the primary structures of mouse and human α2(IV) collagen chains. This comparison identified the clones as coding for the rabbit α2(IV) chain. The locations and the sizes of the imperfections found in the triple-helical domain of the rabbit sequence are exactly the same as those present in the mouse and human α2(IV) chains.

The mobility of RNA that hybridized to the third clone pCE923 on northern blot analysis was the same as that of RNA detected by the other two clones, pCE1344 and pCE1539 (data not shown); however, there was no overlap between the nucleotide sequence of pCE923 and the composite sequence of pCE1344 and pCE1539 (Fig. 4). Computer analysis of the sequence of pCE923 showed a hydrophilic stretch (22 residues) between a methionyl residue (nucleotide number, 281–283) and an alanine residue (362–364), followed by a short nontriple-helical sequence with four cysteinyl residues. The putative signal sequence was conserved well (57% and 71% residues identical to the mouse and human signal sequences, respectively).

![Fig. 3. Schematic diagram of type IV collagen chain and relative locations of individual cDNA clones. Upper diagram represents the type IV collagen α chain. A central triple-helical domain indicated by a horizontal line is flanked by short amino and long carboxyl nontriple-helical (NC1) domains. A short horizontal bar at the amino end represents the position of the signal peptide. The relative locations of imperfections with Gly-X-Y repeat in α1(IV) and α2(IV) chains are indicated by short vertical bars above and below the line for the type IV collagen chain, respectively. A cDNA NK11, coding for α1(IV) covers part of the triple-helical domain and the whole NC1 domain, whereas the three α2(IV) cDNAs encode both 7S and NC1 domain as well as a portion of the triple-helical domain. Both α1(IV) NC1 and α2(IV) NC1 domains are covered by cDNAs. P and E indicate PstI and EcoRI cloning sites, respectively. Numbers in parentheses are nucleotide lengths of the inserts.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933382/)
Fig. 4. Nucleotide sequence and derived amino acid sequence of the α2(IV) clone, pCE923 covering the 7S domain. A box represents the signal peptide and the 7S domain. The putative signal peptidase cleavage site is indicated by an arrow head. Cysteinyl residues are encircled. Note that the first four cysteinyl residues are identical encode an open reading frame of 345 amino acids. Secondary structure of α2(IV) and α2(IV) C chains could be aligned with high homology. When the sequence of NK11 was compared with mouse and human α2(IV), the amino acid sequences of the two NC1 domains are identical (61% identity) in rabbit and human. Thus, the translation product deduced from the nucleotide sequence of the pCE923 insert was identified as the amino-terminal 25% of the α2(IV) chain.

Isolation and Characterization of cDNA Coding for the α1(IV) Chain

Overall sequence similarity between the primary structure of α1(IV)NCl and α2(IV)NCl in humans is 63%.23 This prompted us to use the α2(IV) cDNA sequence to isolate α1(IV) cDNA clones by cross hybridization. With standard hybridization and washing conditions, 14 positive clones were obtained from an λgt10 cDNA library constructed from rabbit corneal endothelial cell RNA. Two of these showed weaker hybridization than the rest of the clones. One of the weakly hybridized cDNAs, NK11, was purified further and characterized.

Northern blot analysis showed that the insert of the clone NK11 hybridized to RNA migrated slightly slower than α2(IV) mRNA (Fig. 2). Nucleotide sequencing (Fig. 3A) showed that the insert encoded a short (nine triplets) triple-helical domain and a nontriple-helical domain of 229 amino acid residues, followed by the termination codon (TGA). The amino acid sequence of this nontriple-helical domain was similar to that of both mouse and human α1(IV) chains (Table 1). We believe that the clone NK11 encodes the NC1 domain of the rabbit α1(IV) chain for several reasons. The length of the NC1 domain defined by NK11 is 229 amino acid residues, exactly the same as mouse and human α1(IV)NCl but different from α2(IV)NCl (227 residues). The locations of 12 cysteinyl residues were also the same as those in mouse and human α1(IV)NCl. Furthermore, when the sequence of NK11 was compared with mouse and human α1(IV) chains, 95% and 96% similarities were seen, respectively, whereas only 59% and 60% similarities were found with mouse and human α2(IV) chains. Although the length of the NC1 domain was different (229 amino acids in α1(IV) and 227 in α2(IV)) the amino acid sequences of the two NC1 domains could be aligned with high homology. When aligned with maximum homology, 138 amino acids were identical (61% identity) in rabbit α1 and α2 (Table 1).

Discussion

Primary Structure of α1(IV) and α2(IV) Chains

The DNAs coding for the rabbit α2(IV) chain we characterized encode an open reading frame of 345

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Discussion

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Fig. 5. Nucleotide sequence and derived amino acid sequence of cDNA encoding (A) α1(IV) (NK1 1) and (B) α2(IV) (pCE1344 and pCE1539) chains. Boxes indicate the NC1 domains of both α1 and α2 chains. Short bars at nucleotides 777–779 in (A) and 1289–1291 in (B) indicate the positions of termination codon, TGA. Other symbols are the same as in Figure 4. Note that the relative locations of all circled cysteinyl residues except for a residue at 695–697 in (B) are strictly conserved among α1 and α2 chains.
Table 1. Sequence similarities of the NC1 domain of rabbit, mouse and human α1(IV) and α2(IV) collagen chains

<table>
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<th>Comparison</th>
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<th>Identical nucleotides</th>
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<td>446/678</td>
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<td>95%</td>
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Reported cDNA sequences from mouse (m) α1(IV)20 and α2(IV)23 and human (h) α1(IV)20 and α2(IV)23 were compared with rabbit (r) cDNAs coding for α1(IV) and α2(IV).

dothingelium and result in corneal edema and opacity.1 Recently, in an attempt to investigate the mechanism of corneal fibrosis, others showed that polymorphonuclear leukocyte-conditioned medium modulated corneal endothelial cells into fibroblastic cells.28,29 This modulation corresponded to changes in the expression of different collagens in endothelial cells, ie, a change from type IV to type I collagen, after several cell passages.29 It was suggested that translational regulation might be involved in this event. The available rabbit α1(IV) and α2(IV) cDNAs should make it possible to study this phenotypic change in more detail. In addition, the probes can be used for investigations of morphogenesis, regeneration, and tissue repair to understand the role(s) that basement membranes play in these processes.

Key words: type IV collagen, Descemet's membrane, corneal endothelial cell, cDNA, cloning

Acknowledgments

The authors thank Dr, Bjorn R. Olsen for his support and suggestions on the manuscript and Ms. Masha Rodin for typing the manuscript.

References


