Maturation of Collagen Fibrils in the Corneal Stroma Results in Masking of Tyrosine-rich Region of Type V Procollagen

Donna M. Peters,* Randall L. Kintner,† Craig Steger,*, Krista Bultmann,* and Curtis R. Brandt†‡

Purpose. To determine the molecular form of type V procollagen in collagen fibrils in mammalian corneal stromas.

Methods. The presence of the tyrosine-rich region in the NH₂-propeptide of type V procollagen in collagen fibrils was examined in human, bovine, and mouse corneas and human corneal fibroblast cultures by immunofluorescence microscopy and immunoblot analysis using a polyclonal antibody specific for this region. The antibody was generated using a glutathione S-transferase-fusion peptide.

Results. The tyrosine-rich region was detected readily in frozen sections of 5- to 6-month-old mouse corneal stromas without the need for any unmasking techniques, indicating that this domain is exposed on the surface of striated collagen fibrils. In contrast, frozen sections of adult human and bovine corneas did not label with the polyclonal sera to the tyrosine-rich region. Immunoblot analysis of bacterial collagenase digests of human and bovine corneas, however, indicated that peptide fragments containing the tyrosine-rich region of type V procollagen and of the expected molecular weight of 70 to 85 kDa were present. Further immunofluorescence microscopic studies and immunoblot analysis of mouse corneas at different ages and of collagen fibrils formed in human corneal fibroblast cultures over time indicated that, initially, the tyrosine-rich region of type V procollagen could be detected in all these collagen fibrils; however, as the age of the mouse and the culture increased, the ability to detect this region decreased.

Conclusions. These results suggest that, in vivo, the tyrosine-rich region of type V procollagen is retained on type V procollagen molecules within mammalian collagen fibrils from corneal stromas and that this region becomes masked as collagen fibrils mature or the species ages. Invest Ophthalmol Vis Sci. 1996;37:2047-2059.

Type V procollagen, along with types I, II, and III procollagens, is a fiber-forming collagen involved in the assembly of striated collagen fibers found in most connective tissues. Sequence analyses have shown that structurally, type V procollagen is similar to the other fiber-forming collagens and that it consists of three pro α-chains. The chain composition of type V procollagen is heterogeneous and differs between tissues. In many tissues,1-3 the chain composition is mainly [α1(V)₂α2(V)], whereas in placenta and granulation tissue,4 the composition is [α1(V)α2(V)α3(V)]. Homotrimers of α1(V) chains also have been observed in Chinese hamster lung cultures and in cultures of chick embryo crop and blood vessels.2,5,6 Each pro α-chain contains three distinct domains—an amino terminal propeptide (NH₂-propeptide), a triple helical collagenous domain, and a carboxy terminal propeptide.7-9

The NH₂-propeptide of human type V procollagen consists of four specific regions. At the amino terminus of the NH₂-propeptide is a 208 amino acid cysteine-rich region. Next to this region is a 200 amino
acid, tyrosine-rich region, which is followed by a short, triple-helical collagenous domain of 95 amino acids (Col 2) and a short a-helical region of three amino acids. With the exception of the tyrosine-rich region, all the other regions of the NH2-propeptide appear to be highly conserved between species. The NH2-propeptide of the proα1(V) chain is related closely to the NH2-propeptide of the proα1(XI) chain. The major difference between these chains occurs within the tyrosine-rich (or variable) region.

Microscopic studies have shown that during assembly of type V procollagen into fibrils in the avian cornea, the carboxy terminus of the NH2-propeptide is retained on type V procollagen after it is assembled into a collagen fibril. This result is in the formation of a collagen fibril in which the tyrosine-rich region of the NH2-propeptide remains attached to the type V collagen molecule within the fibril. This region is exposed on the surface of the fibrils because it does not require the use of unmasking techniques to detect it in immunolabeling studies. In contrast, the helical domain of type V collagen is masked in the fibril in the corneal stroma and is not detected unless unmasking procedures are used. The presence of the NH2-propeptide of type V procollagen on the surface of fibrils has led to the suggestion that this region may serve as a regulatory domain during fibrillogenesis. Similar regulatory functions have been proposed for the NH2-terminal domains of types I and III procollagen.

During biosynthesis of type V procollagen, some portion of the NH2-propeptide is cleaved by an NH2-terminal procollagen propeptidase. The exact cleavage site is unknown, but, based on sequence homology with sites known to be cleaved by NH2-terminal procollagen propeptidase for types I and III procollagen, the cleavage site near the beginning of the triple helix. Studies by Moradi-Ameli et al, however, suggest that the putative cleavage site in humans must be closer to the carboxy terminus of the NH2-propeptide of type V procollagen because an antibody raised against a synthetic peptide that encoded for residues 284–293 in the human NH2-propeptide failed to recognize this region in the intact tissue. Thus, it is unclear how much of the NH2-propeptide is retained during fibrillogenesis and where the cleavage site for the NH2-propeptide is.

In this article, we attempt to address these questions. To do this, we constructed a fusion peptide containing the majority of the tyrosine-rich region from the amino terminus of human type V procollagen and used it to generate polyclonal antibodies. With immunofluorescence microscopy, we examined frozen sections of human and bovine corneas and mouse eyes for the presence of the tyrosine-rich region of type V procollagen. We also examined cultures of human cornea fibroblasts. Our results indicate that the tyrosine-rich region of type V procollagen is retained in fibrils in mammalian corneas. The organization of the type V procollagen in fibrils differs, depending on the age of the cornea or human corneal fibroblasts. In older corneas or cultures, the tyrosine-rich region can barely, if at all, be detected, suggesting that this region becomes masked as the collagen fibrils mature or the species ages.

MATERIALS AND METHODS

Fusion Vector Construction

A cDNA clone, pCW334 containing the NH2-propeptide of type V procollagen, was generously provided by Dr. Daniel Greenspan (University of Wisconsin–Madison) and was used to prepare the fusion protein. To clone the tyrosine-rich region of type V procollagen, pCW334 was digested with the restriction enzymes Bam HI and Nae I (Fig. 1). This resulted in the
formation of two 675 bp fragments, A and B. Fragment A coded for the last 34 residues of the cysteine-rich region and 192 residues of the tyrosine-rich region of the NH₂-propeptide of type V procollagen. Fragment B contained a portion of the 5' untranslated region, the signal peptide, and the amino terminus of the NH₂-propeptide. Both fragments were ligated into the pGEX-5X-2 glutathione S-transferase (GST) expression vector (Pharmacia, Piscataway, NJ) using the BamHI and Smal I sites in the polylinker. The ligation mix was then electroporated into Escherichia coli DH5α, and ampicillin-resistant clones were selected. Colonies containing fragment A were identified by digestion of plasmid minipreps (Wizard Miniprep; Promega Biotech, Madison, WI) with BsaI, which cuts only this fragment. A clone containing fragment A was isolated and screened by sequencing across the GST–procollagen junction to confirm that fragment A was in the appropriate reading frame. Sequencing of the first 28 amino acids was carried out with the Promega fmol system using the primer 5’GGGCTGGCAAGCCACGTTTGGTG3’. The 675 bp cloned fragment A was found to encode for a peptide that started with the sequence Ile-Leu-Glu-Glu-Val-Phe-Glu-Gly-Asp (data not shown). This sequence agrees with the expected sequence found at the BamHI site near the end of the cysteine-rich region in the NH₂-propeptide. The GST fusion peptide containing the tyrosine-rich region and 192 residues of the cysteine-rich region in the NH₂-propeptide was renamed GST-Col V.

Protein Expression and Induction

The GST-Col V fusion protein was expressed and purified using a modification of the procedure described by Smith and Johnson and by Sheibani and Allen–Hoffmann. Briefly, cultures of E. coli were grown overnight in Luria broth containing 0.05 mg/ml ampicillin until an OD₆₀₀ of 0.5 to 0.7 was reached. Cultures were incubated with 4 mM isopropanilthiogalactose for an additional 2 to 3 hours. Cells were pelleted, washed, and resuspended into 20 mM sodium phosphate buffer, pH 7.3, containing 150 mM NaCl (PBS). The following protease inhibitors were then added: 0.2 mg/ml N-tosylamide 2-phenylethyl ketone, 0.2 mg/ml soybean trypsin inhibitor, 4 μg/ml leupeptin, 4 mM phenylmethane sulfonlfluoride, 5 mM ethylenediaminetetraacetic acid (EDTA), 4 mM p-aminobenzoic acid, and 20 mM N-ethylmaleimide. Triton X-100 (final concentration, 1.5%) was added to the cells, and the cells were lysed by freeze-thawing in a dry ice–methanol bath followed by sonication on ice for 2 minutes. The cell lysate was centrifuged for 15 minutes at 12,000g. The supernatant was mixed with 200 μl of 50% glutathione–agarose beads (Sigma, St. Louis, MO) for 10 minutes at room temperature. The beads were centrifuged for 5 minutes in an IEC centrifuge (International Equipment Company, Needham Heights, MA) at 2700 rpm and washed with PBS containing 10 mM EDTA, and the GST–Col V fusion protein was eluted with 20 mM glutathione.

Production of Polyclonal Antibodies and Immunoblotting Procedure

Polyclonal antibodies to the Col V fusion peptide were made in New Zealand White rabbits by mixing GST–Col V fusion peptides still bound to glutathione–agarose with an equal volume of complete Freund’s adjuvant. The fusion peptide was retained on the agarose beads because this procedure was shown to increase the immune response. The emulsion was injected subcutaneously at multiple sites in the back of the rabbit. Rabbits were boosted every 2 weeks with the same amount of antigen mixed with an equal volume of incomplete Freund’s adjuvant. Immunoblots were made according to the procedure of Towbin et al to determine the specificity of the antibodies.

Preparations of Procollagens and Collagens

Human procollagens were isolated from human foreskin diploid fibroblasts, and mouse procollagens were isolated from a mouse fibroblast line (Mov IV) that overproduces type V procollagen. As shown previously, the ratio of α1(I) to α2(I) deviates from the normal 2:1 ratio observed in other cell lines. Both procollagens were isolated using a modification of the procedure described by Dzamba et al. Briefly, confluent cultures were incubated with either 25 μg/ml ³H-proline or ³H-amino acid mix (Dupont NEN, Boston, MA) in media containing Dulbecco’s modified Eagle’s medium, 0.2% bovine serum albumin, 50 μg/ml β-aminopropionitrilte, 50 μg/ml ascorbic acid, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml soybean trypsin inhibitor for 24 hours. Media were removed, and the protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 10 mM EDTA) were added. Procollagen was precipitated from the media using ammonium sulfate (176 mg/ml). Human type V collagen was purchased from Southern Biotechnology (Birmingham, AL) or was isolated from human placenta according to the procedure of Rhodes and Miller.

For some experiments, type V procollagen isolated from mouse fibroblast cultures was resuspended into 0.1 M Tris–HCl, pH 7.5, buffer containing 0.4 M NaCl, 5 mM N-ethylmaleimide, and 2.5 mM CaCl₂ and was digested with bacterial collagenase as described below.

Tissue Culture

Normal diploid corneal fibroblasts were prepared as described previously and were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine
serum. For immunofluorescent microscopy of nascent
collagen fibrils, cells were plated onto sterile glass cov-
erslips at a density of $4 \times 10^5$ cells/35 mm petri dish.
The next day, 50 $\mu$g/ml ascorbic acid was added, and
the cultures were grown for an additional 24 hours.
In other experiments, cells were plated onto glass cov-
erslips at a density of $1 \times 10^6$ cells/35 mm petri dish
and were grown to confluency. Cultures were labeled
with anti-Col V sera at 4°C for 30 minutes using the
procedure described by Dzamba et al. Labeled cells
were then incubated with donkey anti-rabbit conjugated
to fluorescein for 45 minutes at 4°C (Jackson
ImmunoResearch Laboratories, West Grove, PA).

Immunofluorescent Microscopy of Frozen
Sections
Normal human corneas were obtained 24 to 48 hours
after death from the Madison Eye Bank. Mouse cor-
neas were obtained from the University of Wisconsin
Animal Care Facility. Bovine corneas were obtained
from the Muscle Biopsy Laboratory (University of Wis-
consin-Madison). Corneas were washed with 0.1 M so-
dium phosphate, pH 7.4, and were incubated over-
night with 7% sucrose in PBS, 100 U/ml penicillin,
and 100 $\mu$g/ml streptomycin sulfate at 4°C. The cor-
neas were embedded in Tissue Tek II OCT compound
at $-20^\circ$C, and 8 to 10 $\mu$m sections were cut. Sections
were air dried onto poly L-lysine-coated slides at room
temperature.

Sections were treated with 0.5 mg/ml sodium bor-
ohydride for 1 hour and then were preblocked with
1% bovine serum albumin for 2 hours. Sections were
labeled with either rabbit anti-Col V sera or rabbit
nonimmune sera overnight at 4°C, washed for 1 hour
with PBS, and labeled with a goat anti-rabbit immuno-
globulin conjugated to either rhodamine or indodi-
carbocyanine (Cy5) for 3 hours at room temperature.
All antibodies were diluted 1:100 in PBS containing
1% bovine serum albumin and 20 mM sodium azide.
Frozen sections were mounted in PBS containing 1
mg/ml phenylendiamine and viewed with a Nikon
Microphot (Nippon Kogaku K.K., Tokyo, Japan).

All experimental procedures adhered to the
ARVO Statement for the Use of Animals in Ophthalmic
and Vision Research. Investigations using human tis-
sue followed the tenets of the Declaration of Helsinki,
and the appropriate institutional approval was ob-
tained.

Collagenase Digestion
Eyes from mice at various ages were washed twice with
0.1 M Tris-HCl, pH 7.5, buffer containing 0.4 M NaCl,
5 mM Nethylmaleimide, and 2.5 mM CaCl$_2$, and their
wet weights were determined. The wet weights of 2-
5-, 9-, and 10-month-old mouse eyes were 19 mg, 22
mg, 43, and 38 mg, respectively. Eyes were homoge-
nized on ice, and the homogenate was incubated with
25 U/ml of purified Bacterial Collagenase (Advance
Biofactures, Lynbrook, NY) for 3 hours at 37°C as pre-
viously described. The digestion was stopped with 5
mM EDTA (final concentration), and the sample was
prepared for sodium dodecyl sulfate (SDS)—polyacryl-
amide gel electrophoresis. Corneas from adult bovines
and humans were removed from the eyes and were
trimmed to remove any sclera that may have re-
ained. Each human cornea was cut in half, and one
half of a cornea was digested with bacterial collagenase
as described above. Bovine corneas were quartered
and $1/4$ of the cornea was digested with bacterial colla-
genase as described above. The wet weight of average
human and bovine corneas were 130 mg and 340 mg,
respectively.

Results
Expression of GST—Col V Fusion Peptide
Polyacrylamide gel analysis of the affinity purified
GST—Col V fusion peptide revealed the presence of
three major bands with apparent molecular weights
of 63 kDa, 31 kDa, and 29 kDa (Fig. 2, lane 2). The
63 kDa band that was the most prominent expressed
by the GST-Col V fusion vector was not expressed in
cells transformed with GST vector only (Fig. 2, lane
1). This suggests that this band was the fusion peptide
containing the tyrosine-rich region of type V procolla-
gen. The 63 kDa fusion protein containing the tyro-
sine-rich region migrated at a higher molecular weight
than expected because the calculated molecular
weight for the fusion peptide is 51.5 kDa (29 kDa for
The anti-Col V serum was specific for the propeptide region of type V procollagen because it did not recognize pepsiomized human type V collagen (Fig. 3A, lane 2), which contains only an intact triple helical region and lacks the propeptide regions. To establish that the anti-Col V serum recognized the amino terminal propeptide region of type V procollagen and not the carboxy terminal propeptide, mouse type V procollagen was digested with bacterial collagenase, and the digest was analyzed on an SDS gradient polyacrylamide gel. As shown in Figure 4, lane 2, the anti-Col V serum recognized a bacterial collagenase-resistant fragment that migrated as a broad band between 78 and 85 kDa (arrow). Because the molecular weights expected for both the amino terminal bacterial collagenase-resistant fragments from the proal (V) chains on an SDS polyacrylamide gel were between 74 and 85 kDa, this band more than likely contained both bacterial collagenase-resistant fragments from the NH₂ propeptide of type V procollagen. Thus, the anti-Col V serum appeared to recognize the amino terminal of type V procollagen. Failure to separate the two bacterial collagenase-resistant fragments from the proal (V) chains was attributable to the fact that we ran a gradient gel.

The anti-Col V serum also recognized a 60 kDa band in both the undigested procollagen (Fig. 4, lane 1) and the bacterial collagenase-digested procollagen (Fig. 4, lane 2). We assumed that this band is a nonspecific proteolytic degradation product of the amino terminal of type V procollagen that arose during our tissue culture procedures because it was not observed in our procollagen preparations before we started the bacterial collagenase digest.

Localizatlon of Type V Procollagen in Cornea

Immunofluorescent microscopy studies using the anti-Col V serum indicated that the tyrosine-rich region of

The abnormal behavior of the tyrosine-rich region, however, was not entirely unexpected because the amino terminal bacterial collagenase-resistant fragment containing this region also migrates with a higher molecular weight than expected.

In both insert-containing and vector-only cell lysates, a smaller band at 29 kDa was observed. This 29 kDa fragment was presumable the glutathione-S-transferase fragment. The other band migrating at 31 kDa and the faint bands observed in lane 2 were probably proteolytic degradation products of the 65 kDa fusion protein because they were not observed in the cell lysates from cells transformed with vector only, and the intensity of these bands increased if protease inhibitors were omitted during the purification procedure (data not shown).

Immunoblot Analysis of Anti-Col V Serum

Tritiated-labeled procollagen preparations containing both type V and type I procollagens were electrophoresed on 5% SDS polyacrylamide gels and analyzed by immunoblot analysis to determine the specificity of the anti-Col V serum. As shown in Figure 3A, lane 3, the mouse procollagen preparations contained the proal, proal', and proal₂ chains from type V procollagen, as well as the proal₁ and proal₂ chains from type I procollagen. Immunoblot analysis of the same preparation showed that serum generated against the GST-Col V fragment recognized only the proal₁ and proal₂ chains from mouse type V procollagen (Fig. 3A, lane 1). The serum did not recognize the proal₂ chain from type V procollagen and did not cross-react with any of the proα-chains of type I procollagen (Fig. 3A; compare lanes 1 and 3) even though they were present in the mouse procollagen preparation. The serum also recognized the proal₁ chains from human type V procollagen (Fig. 3B). The faint band recognized in the mouse procollagen preparation presumably represented a proal₁ (V) chain that had been processed partially because it migrated between the proal₂ chain of type V procollagen and the proal₁ chain of type I procollagen.

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Localizatlon of Type V Procollagen in Cornea

Immunofluorescent microscopy studies using the anti-Col V serum indicated that the tyrosine-rich region of
type V procollagen was retained in fibrils in the mouse corneal stroma (Fig. 5A). Staining was specific because labeling was not detected using a nonimmune serum (Fig. 5B). Detection of the tyrosine-rich region in mouse corneal stroma could be achieved without the need to unmask the epitope, indicating that this region was readily accessible. Neither human corneal stroma (Fig. 5C) nor bovine corneal stroma (Fig. 5D), however, stained positive with the anti-Col V serum, suggesting that the tyrosine-rich region of type V procollagen either was not retained during the assembly of type V collagen into collagen fibrils in the corneal stroma or that it was masked. Panels E and F represent the nonimmune controls for the human and bovine cornea. The fluorescence observed in the epithelium in all the panels was the result of autofluorescence and could be observed in unstained tissues (data not shown), as well as in tissues labeled with nonimmune serum, indicating that this was nonspecific labeling. The labeling observed in the stroma of the nonimmune controls was caused by nonspecific binding of the anti-Col V sera to the fibroblasts in the stroma.

**Bacterial Collagenase Digestion**

Attempts to unmask the tyrosine-rich region of type V procollagen in fibrils using acetic acid10 to swell the fibrils, 4 M urea or 6 M guanidine to denature the fibrils,32 or specific lyases such as chondroitin ABCase or heparinase to remove proteoglycans were unsuccessful (data not shown). Thus, to determine whether the tyrosine-rich region was masked or cleaved in the bovine and human corneal stroma, bovine and human corneas and mouse eyes were homogenized and digested with bacterial collagenase to remove the triple helical gly-pro-pro regions of collagens in the stroma and to release the tyrosine-rich region into the surrounding buffer.

As shown in Figure 6, lane 1, when 3-week-old mouse eyes were digested with bacterial collagenase and analyzed on an immunoblot, several major bands were detected with the anti-Col V serum, including bands at 85 and 74 kDa (asterisks), which are the expected sizes for amino terminal bacterial collagenase-resistant fragments containing the tyrosine-rich region from proα1(V) and proα1'(V) chains, respectively.29,31 Thus, this procedure apparently can detect peptides containing the tyrosine-rich region of type V procollagen and having the correct molecular weight in tissues in vivo. The two major bands observed above the 85 kDa bands presumably represented incomplete digestion of the amino terminal propeptide and were expected because we only partially digested the tissue homogenate. The partial digestion of collagenase-sensitive collagens was done to avoid extensive proteolytic degradation of the amino propeptide by the various proteolytic enzymes that were released from the lysed cells in the tissue homogenate. As shown in Figure 2, the tyrosine-rich region was sensitive to proteolytic degradation. The lower molecular weight bands at 47 kDa and 69 kDa presumably were the result of some proteolytic degradation. Lane 2 was the nonimmune control.

A similar result was obtained when adult human corneas were digested with bacterial collagenase (Fig. 6, lanes 5 and 6). These digests showed that an 85 kDa band specifically labeled with the anti-Col V se-
FIGURE 5. Immunofluorescent micrographs of mouse, human, and bovine corneas. (A,B) Frozen sections of a 5- to 6-month-old mouse cornea. (C) Frozen section of human cornea, and (D) is a frozen section of bovine cornea. (A,C,D) stained with the rabbit anti-coll V serum as described in Materials and Methods. Sections of mouse cornea were then labeled with goat anti-rabbit immunoglobulin G (IgG) conjugated to Cy5. Sections of human and bovine cornea were labeled with goat anti-rabbit IgG conjugated to rhodamine. B, E, and F were labeled with a rabbit nonimmune serum followed by goat anti-rabbit IgG conjugated to Cy5 or rhodamine. Bars = 62.5 μm (A,B,C,E); = 125 μm (D,F).
FIGURE 6. Immunoblot analysis of mouse eyes and human bovine corneas digested with bacterial collagenase. Four 2-week-old mouse eyes (lanes 1 and 2), one fourth of a bovine cornea (lanes 3 and 4), and one half a human cornea (lanes 5 and 6) were digested with bacterial collagenase, as described in Materials and Methods. The amount of material digested and loaded on the gel were adjusted so that equal amounts of tissue (wet weight) were used. Lanes 1, 3, and 5 were incubated with rabbit anti-Col V serum (1:200 dilution), followed by goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (1:1000 dilution). Lanes 2, 4, and 6 were stained with rabbit nonimmune serum (1:200 dilution), followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (1:1000 dilution). All antibodies were diluted in 1% bovine serum albumin in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl. Molecular weights of standards are indicated in kDa for each blot. Asterisks indicate 74 to 85 kDa bands that specifically labeled with the anti-Col V serum. Bovine and mouse digests were electrophoresed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. Human digests were analyzed on an 8% SDS polyacrylamide gel. Human digests were analyzed on an 8% SDS polyacrylamide gel. All samples were reduced with 5% β-mercaptoethanol.

rum could be detected, indicating that the tyrosine-rich region of type V procollagen was also present in human cornea (Fig. 6, lane 5, asterisks). Two other bands with apparent molecular weights of 45 kDa and 40 kDa also were observed (Fig. 6, lane 5). These lower bands are presumably degradation products of the 85 kDa band. The 55 kDa band appears to represent nonspecific staining because it is also observed in the nonimmune control (compare lanes 5 and 6, Fig. 6).

Fragments containing the tyrosine-rich region of type V procollagen were detected in bacterial collagenase digests of adult bovine cornea. Immunoblot analyses of the supernatant from these digests indicated that two bands with apparent molecular weights of 106 kDa and 70 kDa could be detected specifically with the anti-Col V serum (Fig. 6, lane 3, asterisks). Because the 70 kDa band migrated with the molecular weight expected for the proα1(V) amino terminal bacterial collagenase-resistant fragment, it indicated that the tyrosine-rich region of type V procollagen was present in collagen fibrils in the bovine cornea. The molecular weight of the 106 kDa band was slightly higher than expected based on the observed molecular weight of these fragments from chicken type V procollagen. It is not known if the larger size of this fragment resulted from differences between species or from incomplete digestion. The remainder of the bands detected on the blot appeared to result from nonspecific staining because they also were observed in the nonimmune control (Fig. 6, lane 4).

Interestingly, neither the human bacterial collagenase digest nor the bovine cornea digest contained the second lower molecular weight collagenase-resistant fragment. Because it had been assumed that this fragment was from the proα1(V) chain (Fig. 6, compare lane 1 to lanes 3 and 5), the absence of this chain in those digests suggested that the proα1(V) chain may not be found within either human or bovine cornea and that production of this chain in cornea may depend on the species.

Localization of Type V Procollagen in Corneal Fibroblast Cultures

Cultures of human corneal fibroblasts also were examined to see whether the tyrosine-rich region of type V procollagen was present in these matrix fibrils. Immunofluorescent studies using the anti-Col V serum indicated that the tyrosine-rich region of type V procollagen could be observed in the extracellular matrix of fibroblast cultures plated for only 48 hours (Fig. 7A). When 10-day-old cultures were observed, however, the staining pattern was much fainter (Fig. 7C) and barely
Localization of Type V Procollagen in Collagen Fibrils

FIGURE 7. Immunofluorescent micrographs of human corneal fibroblast cultures. Unfixed cells were incubated at 4°C for 30 minutes with either the rabbit anti-col V serum (A,C) or a rabbit nonimmune serum (B,D), followed by a goat anti-rabbit immunoglobulin G conjugated to rhodamine. All antibodies were used at a 1:100 dilution. Fibrils containing the tyrosine-rich region of type V procollagen (arrowheads) are observed readily in cultures plated for 24 hours (A). In contrast, fibrils containing this site (arrowheads) are barely detected in cultures grown for 10 days (D). B is the nonimmune control for A, and D is the nonimmune control for C. The exposure time was 6 seconds for the freshly seeded cultures and 41 seconds for the 10-day-old cultures. Bar = 19.8 μm.

Immunoblot and Immunofluorescent Analysis of 10-Month-Old Mouse Eyes

As with the human corneal fibroblast cultures, the age of the mice analyzed determined how well we could detect the tyrosine-rich region of type V procollagen. As shown in Figure 8, the tyrosine-rich region of type V procollagen could be detected by immunofluorescent microscopy studies in frozen sections of the stroma from a 2-month-old mouse. The 74 and 85 kDa frag-
FIGURE 8. Immunofluorescent micrographs of corneas from 2- and 10-month-old mice. Frozen sections of corneas from 2-month old (A) and 10-month-old mice (B,C) were labeled with rabbit anti-col V serum (A,B) and nonimmune serum (C) as described in Materials and Methods, followed by a goat anti-rabbit immunoglobulin G conjugated to rhodamine. The tyrosine-rich region is detected in the 2-month-old mouse eye but not in the 10-month-old mouse eye. Bar in = 62.5 μm.

ments containing this region of type V procollagen, as well as some partially digested material, and the 55 kDa proteolytic degradation fragment could be observed in immunoblots of bacterial collagenase digests of 2-month-old mice (Fig. 9). In contrast, this region

FIGURE 9. Bacterial collagenase digest of eyes from 2-, 5-, 9-, and 10-month-old mice. The 74 and 85 kDa tyrosine-rich fragments are found in the digests of 2- and 5-month-old mouse eyes but is not found in the digests of eyes from the older mice. Eyes from 2-, 5-, 9-, and 10-month-old mice were digested with bacterial collagenase and analyzed on immunoblots as previously described. All lanes were stained with the rabbit anti-Col V serum, followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase as described in Figure 9. Numbers on the right indicate the molecular weight of standards. All samples were run on a 8% sodium dodecyl sulfate polyacrylamide and were reduced with 5% β-mercaptoethanol.
Localization of Type V Procollagen in Collagen Fibrils

barely was detected in frozen sections of the stroma from a 10-month-old mouse by immunofluorescence microscopy (Figs. 8B, 8C), and the 74 kDa and 85 kDa fragments containing the tyrosine-rich region could no longer be isolated from the digested tissue (Fig. 9). The only detectable bands on the immunoblot of the older mouse eyes were several faint higher molecular bands observed in the 10-month-old eye. Thus, the age of the mouse tissue made a difference in how well the tyrosine-rich region of type V procollagen could be detected in the tissue.

DISCUSSION

In this article, we have described the preparation of polyclonal antibodies to the tyrosine-rich region of human type V procollagen with a GST-fusion peptide, and we used this antiserum to detect the tyrosine-rich region in mammalian corneas and human corneal fibroblast cultures. By immunoblot analysis, the antibody, anti-Col V, was specific for the propeptide region of human and mouse type V procollagen. The cross-reactivity with rodent type V procollagen is not surprising given that the homology between the human and hamster protein sequences within that region is 80%.

Immunofluorescent labeling studies using the anti-Col V serum showed that collagen fibrils in corneal stromas of young mice contained the tyrosine-rich region of type V procollagen, indicating that mouse type V procollagen, like avian type V procollagen, retains its tyrosine-rich region as an exposed structure on the surface of fibrils in the corneal stroma. In contrast, the tyrosine-rich region of type V procollagen could not be detected in either adult human or bovine corneas, suggesting that the tyrosine-rich region of type V procollagen either is not readily accessible on the surface of collagen fibrils or is cleaved. Digestion of human and bovine corneas with bacterial collagenase, however, detected fragments having the expected molecular weight of the amino terminal bacterial collagenase-resistant fragment containing the tyrosine-rich region. This indicates that the tyrosine-rich region of type V procollagen is present in adult human and bovine corneas and suggests that the fragment is masked rather than cleaved off.

Attempts to unmask the tyrosine-rich region by swelling the fibrils with acetic acid, denaturing the fibrils with guanidine or urea, or digesting the stroma with lyases failed to reveal the presence of this fragment in the stroma. Our inability to unmask the fibrils is not necessarily in conflict with the work of Linsenmayer and Birk. Their studies used embryonic avian corneas that had been pretreated with β-amino-propionitrile to prevent cross-linking. In contrast, our adult tissues have not been pretreated with β-amino-propionitrile. Therefore, it is possible that cross-linking in the tissue prevented our ability to expose the tyrosine-rich region of type V procollagen. Nevertheless, it is unclear what masks the tyrosine-rich region of type V procollagen in fibrils and how, if at all, this masking affects the proposed regulatory role of this propeptide in fibrillogenesis.

The process of masking the tyrosine-rich region, however, appeared to be age dependent because immunofluorescence microscopy studies showed that the tyrosine-rich region of type V procollagen could be detected readily in fibrils from freshly seeded human cornea fibroblast cultures and young mice corneas, but not in older cultures or mice corneas. Thus, our data suggested that the tyrosine-rich region is retained in the matrix, presumably within fibrils, but that this region of type V procollagen becomes masked with increasing age.

Bacterial collagenase studies with the older mice further suggested that the inability to detect this region may result from an increased cross-linking of the collagen in the stroma as the mice age because the tyrosine-rich region of type V procollagen appeared to be cross-linked into a higher molecular weight complex and could not be isolated readily as the 85 kDa tyrosine rich fragment. Although the cause of this cross-linking is unknown, additional cross-linking of collagen during aging has been reported to occur as a result of nonenzymatic glycosylation of the collagen. It is possible, therefore, that this same process may mask the tyrosine-rich region of type V procollagen in the stroma. Alternatively, some of the NH2-propeptide may be removed from the stroma as the species ages, and we were unable to detect it is because the amounts remaining in the stroma were below the detection limits of the immunofluorescence procedure.

Finally, these studies also predicted that in mouse, bovine, and human corneas, the cleavage site used by the NH2-terminal propeptidase is located between the prolyl-glutamyl residues at positions 252–253 near the beginning of the tyrosine-rich region. The other possible cleavage sites at the beginning of the α-helix would remove completely the tyrosine-rich region from type V procollagen in the fibril. A similar conclusion was obtained in studies on the avian corneal stroma, which also predicted that the cleavage site used by the NH2-terminal propeptidase in cornea stroma is located before the start of the tyrosine-rich region. This, of course, does not rule out the possibility that cleavage sites besides those mentioned may exist.

Others may have failed to detect the tyrosine-rich region of type V procollagen in vivo because of the way type V collagen was extracted from these tissues. We found that this region is susceptible to
proteolytic degradation (data not shown). Alternatively, failure may have resulted from variation in the types of tissues used. Both our study and Linsenmayer et al. used corneal tissue, whereas Niyibizi and Eyre used fetal calf skin and Moradi-Ameli et al. used bone extracts or collagen from umbilical cord. Clearly, additional studies on the organization of this region in fibrils are needed if we are to understand fully the functional role of the tyrosine-rich region of type V procollagen in collagen fibrillogenesis.

Key Words

aging, collagen fibrils, corneal stroma, extracellular matrix, type V procollagen

Acknowledgments

The authors thank Mr. Terry Timm and the staff of the Muscle Biopsy Program at the University of Wisconsin for generously providing the bovine corneas, and they thank Dr. David Pauza and Ms. Brenda Preuninger (Department of Pathology and Laboratory Medicine, University of Wisconsin) for helping with the sequence analysis. They also thank Donald Cha and Afsar Sokhansanj for technical support and Drs. Hermann Bultmann and Tim Kern for insightful discussions.

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