Differential Expression of Two Basement Membrane Collagen Genes, COL4A6 and COL4A5, Demonstrated by Immunofluorescence Staining Using Peptide-specific Monoclonal Antibodies

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Abstract. Genes for the human α5(IV) and α6(IV) collagen chains have a unique arrangement in that they are colocalized on chromosome Xq22 in a head-to-head fashion and appear to share a common bidirectional promoter. In addition we reported a novel observation that the COL4A6 gene is transcribed from two alternative promoters in a tissue-specific manner (Sugimoto, M., T. Oohashi, and Y. Ninomiya. 1994. *Proc. Natl. Acad. Sci. USA.* 91:11679–11683). To know whether the translation products of both genes are colocalized in various tissues, we raised α5(IV) and α6(IV) chain-specific rat monoclonal antibodies against synthetic peptides reflecting sequences near the carboxy terminus of each noncollagenous (NC)1 domain. By Western blotting α6(IV) chain-specific antibody recognized 27-kD monomers and associated dimers of the human type IV collagen NC1 domain, which is the first demonstration of the presence in tissues of the α6(IV) polypeptide as predicted from its cDNA sequence. Immunofluorescence studies using anti-α6(IV) antibody demonstrated that in human adult kidney the α6(IV) chain was never detected in the glomerular basement membrane, whereas the basement membranes of the Bowman's capsules and distal tubules were positive. The staining pattern of the glomerular basement membrane was quite different from that obtained with the anti-α5(IV) peptide antibody. The α5(IV) and α6(IV) chains were colocalized in the basement membrane in the skin, smooth muscle cells, and adipocytes; however, little if any reaction was seen in basement membranes of cardiac muscles and hepatic sinusoidal endothelial cells. Thus, both genes are expressed in a tissue-specific manner, perhaps due to the unique function of the bidirectional promoter for both genes, which is presumably different from that for COL4A1 and COL4A2.

BASEMENT membranes are continuous sheets of specialized extracellular matrix composed of collagen IV, laminin, nidogen, heparan sulphate proteoglycan, and other glycoproteins that are found wherever cells meet extracellular matrix (Rohrbach and Timpl, 1993). This means that basement membranes are located outside of most of the cells: at the dermal-epithelial junction; at the base of all lining epithelia throughout the digestive, respiratory, reproductive, and urinary tracts; underlying endothelia of capillaries and venules; around Schwann cells, adipocytes, skeletal, cardiac, and smooth muscle cells; and at the base of parenchymatous exocrine and endocrine glands (Junqueira et al., 1992). Thus, they are the natural substrates on which most of the cells except blood cells grow and develop. They closely adjoin and are products of the overlying cells, and serve to compartmentalize the extracellular matrix and provide a barrier between cells and matrix.

Collagen type IV is a major structural component of basement membrane. The major form of this protein is a heterotrimer containing α1(IV) and α2(IV) chains, and this form appears to be ubiquitous in all basement membranes (Rohrbach and Timpl, 1993). Microsequencing of peptides made it possible to identify the human α3(IV) and α4(IV) chains (Wieslander et al., 1985; Butkowski et al., 1990). The primary structure of the human α3(IV) chain has been reported just recently (Mariyama et al., 1994) and cDNAs encoding the entire human α4(IV) chain have been isolated and characterized as well (Sugimoto et al., 1994; Leinonen et al., 1994). Lately, the nature of the α5(IV) chain has been identified by cDNA isolation

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(Hostikka et al., 1990; Zhou et al., 1992), and linkage between the α5(IV) gene (COL4A5) and Alport's Syndrome has been clarified (Barker et al., 1990). Zhou et al. (1993) and Oohashi et al. (1994) cloned genomic DNA and cDNAs coding for yet another α(IV) chain, α6(IV). These newly discovered α chains still contain the amino-terminal 7S domain, the central COL1 domain, and the carboxy-terminal noncollagenous (NC)1[1] domain (Oohashi et al., 1994) as found in the classical α1(IV) and α2(IV) chains; however we do not know the mechanism to explain which of the six α chains are selected, or how they come together, to form collagen type IV molecules.

Of interest is that the genes encoding the six individual α chains in humans are paired by two on three different chromosomes: the α1(IV) gene (COL4A1) and the α2(IV) gene (COL4A2) are located on chromosome 13 (Pihlajaniemi et al., 1985; Boyd et al., 1988; Griffin et al., 1987); COL4A3 and COL4A4 are on chromosome 2 (Mariyama et al., 1994; Kamagata et al., 1992); and COL4A5 and COL4A6 are on chromosome X (Hostikka et al., 1990; Zhou et al., 1993; Oohashi et al., 1994). Further, COL4A1 and COL4A2 are aligned in a head-to-head fashion sharing a common promoter between the two genes (Poschl et al., 1988; Soininen et al., 1988), whereas the precise upstream structure of COL4A3 and COL4A4 has not been reported yet. COL4A5 and COL4A6 have been found together on chromosome X at the segment q22 (Hostikka et al., 1990; Zhou et al., 1993; Oohashi et al., 1994) and have been reported to be also arranged in a head-to-head fashion and to share a bidirectional promoter (Zhou et al., 1993). Furthermore, we reported the novel observation that the COL4A6 gene is transcribed from two alternate promoters in a tissue-specific fashion (Sugimoto et al., 1994).

Presently we report for the first time the presence of the COL4A6 gene products as detected by Western blotting and immunofluorescence staining of various tissues by use of peptide-specific monoclonal antibodies. Our results demonstrate that the COL4A5 and COL4A6 genes are not necessarily coexpressed in several types of tissues.

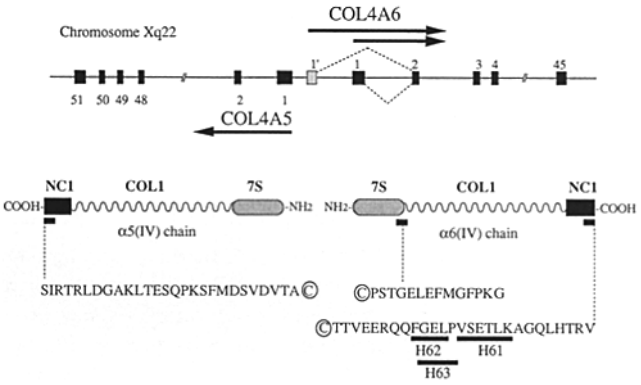


Figure 1. Schematic representation of the two genes COL4A5 and COL4A6 on chromosome Xq22. (*Top*) COL4A5 and COL4A6 are arranged head-to-head in close proximity on chromosome Xq22 (Sugimoto et al., 1994). Arrows indicate the direction of the transcripts for COL4A5 and COL4A6. There are two transcriptions for COL4A6. One transcription start site (from exon 1′) for COL4A6 is 442 bp away from the transcription start site of COL4A5, while an alternative transcription start site (from exon 1) is located 1050 bp apart from the first one and drives the expression of a second transcript that encodes an α6(IV) chain with a different signal peptide. (*Bottom*) Because of the direction of the two genes, the translation products, α5(IV) and α6(IV) chains, of the two genes are drawn accordingly. Chain-specific monoclonal antibodies were raised against synthetic peptides (*short bars*) of the NC1 domains close to the carboxyl termini of the two chains. The third peptide deduced from the third imperfection of the Gly-X-Y repeating sequence in the central COL1 domain was synthesized and used for monoclonal antibodies. The sequences of the three synthetic peptides are shown with cysteinyl residues at the amino termini. Using the Geysen's peptide scanning technique (Geysen et al., 1987) we determined the exact location of the epitopes for H61, H62, and H63. Relative location of the epitopes are shown by three thick bars under the α6(IV) peptide.

Materials and Methods

Synthetic Peptides

Earlier we determined the complete primary structure of the human α6(IV) chain (Oohashi et al., 1994; Zhou et al., 1994). When the amino acid sequences of all six α(IV) chains were compared, their noncollagenous (NC)1 domain portions showed the highest homology with each other. However, the amino acid sequences of certain subregions of NC1 were quite different from one another. We selected the peptide TTVEERQQFGELPVSETLKAGQLHTRV from the α6(IV) chain (Oohashi et al., 1994) and ATVDVSDMFSKPQSETLKAGDLRTRIS from the α5(IV) chain (Hostikka et al., 1990), both of which are close to the carboxyl ends, as shown in Fig. 1. We also synthesized a peptide, CPSTGELEFMGFPKG, derived from the third imperfection of the Gly-X-Y repeating sequence in the COL1 domain of the α6(IV) chain. A cysteinyl residue was added at the amino terminus to allow coupling of the peptide to the carrier protein. Oligopeptides were chemically synthesized by the solid-phase procedure of Merrifield (1963) with the aid of an automated peptide synthesizer (model 430; Applied Biosystems, Inc., Foster City, CA). The synthetic peptides were conjugated to a carrier protein, keyhole limpet hemocyanin, by the maleimide method (Ishikawa et al., 1983). In some experiments the peptides were directly used as antigen without conjugation to hemocyanin, and the antigenic efficiency was found not to differ much. Other peptides, ATIERSEMFKKPTPSTLKAGELRTHVS from the α1(IV) chain (Pihlajaniemi et al., 1985), TTIPEQSFQGSPSADTLKAGLIRTHIS from the α2(IV) chain (Hostikka and Tryggvason, 1988), ASLNPERMFRKPIPSTVKAGELEKIIS from the α3(IV) chain (Morrison et al., 1991), and TVKADFEFSSAPAPDTLKESQAQRQKI from the α4(IV) chain (Sugimoto et al., 1993), were used for raising chain-specific antibodies against the other human α chains.

Peptide-specific Monoclonal Antibodies

We introduced new steps to our previous method and developed a new efficient procedure, the rat lymph node method, for raising monoclonal antibodies (Kishiro et al., 1995). Briefly, WKY/NCrj rats (Charles River Japan, Yokohama, Japan) were immunized in the hind foodpads with 50 mg of hemocyanin-coupled synthetic peptide emulsified with Freund's complete adjuvant. Three or four weeks later the rats were killed, and lymphocytes obtained from the medial iliac lymph nodes of the rats were fused with mouse myeloma cells (SP2/0-Ag14). Supernatants from hybridoma cultures were screened by ELISA, using hemocyanin-free peptides from individual α(IV) chains.

The initial screening was performed by ELISA with the synthetic peptides themselves and/or native NC1 fractions isolated from the human kidney and other peptides of the same region from all α(IV) NC1 domains. 2,000 clones were screened and we picked 145 positive clones that reacted specifically with the α6(IV) peptide. The second screening was performed by indirect immunofluorescence using human tissue sections. Thus, three

1. *Abbreviations used in this paper*: COL, collagen; KLH, keyhole limpet hemocyanin; NC, noncollagenous; PVDF, polyvinylidene difluoride.

clones, H61, H62, and H63, out of the 12 positive clones for both ELISA and immunofluorescence were established.

Preparation of the NC1-Domain Fraction from Renal Basement Membrane

Renal basement membrane was prepared by the same method as that used for bovine renal basement membrane (Sado et al., 1991). In order to recover the NC1 domains from the collagen IV molecules we used bacterial collagenase and removed the central collagenous (COL) domains from the molecules. Namely, lyophilized basement membrane was solubilized with collagenase (Seikagaku Kogyo, Tokyo, Japan) at 47°C for 20 h by a method similar to that used for bovine nephritogenic antigen. After solubilization, the sample was centrifuged at 27,000 *g* for 10 min and insoluble material was removed. The supernatant was dialyzed against distilled water for 8 h, lyophilized, dissolved in PBS, and dialyzed against PBS overnight. The material was then centrifuged to remove insoluble materials formed during the dialysis. The supernatant was applied to a gel filtration column of UltrogelAcA 34(26 × 950 mm) at a flow rate of 30 ml/h. The main peak fraction was collected and concentrated.

Western Blotting

The NC1 fractions of collagen type IV thus prepared were analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions (Laemmli, 1970). SDS-containing 5% stacking and 11.5% homogeneous running polyacrylamide slab gels were used. Proteins separated on the SDS gels were blotted onto polyvinylidene difluoride (PVDF) membranes. The membrane was incubated with the primary antibodies first and then with the peroxidase-conjugated secondary antibodies (Sado et al., 1991).

Immunohistochemical Staining

Indirect immunofluorescence staining were performed as described previously (Sado et al., 1991). In the preliminary experiments we have tested different conditions varying the concentration of the urea, pH, incubation time, and temperature using the representative tissues including kidney, muscles, and skin. We set up to the standard acid-urea denaturation condition: incubating ethanol-fixed sections with 6 M urea-HCl buffer (pH 3.5) for 1 h at room temperature. 6 M urea treatment is necessary for most of the antibodies probably due to the linearized synthetic peptide antigens. Control sections for immunofluorescence staining were reacted with nonimmune rat sera or with the secondary antibody alone. These controls were negative.

The staining method for the kidney and skin. Normal portions of human kidneys were obtained from two patients with renal tumors (both males, 56 and 85 yr old). Renal biopsies were performed from two Alport's syndrome patients (males, 14 and 9 yr old). Skin specimens were obtained from the patients with Alport's syndrome and from patients with nonrenal diseases by surgical biopsy.

The tissues from the kidney and skin were snap-frozen in liquid nitrogen. 3-mm cryosections were fixed with acetone for 10 min. To reveal possible masked epitopes, we pretreated the sections with 6 M urea in 0.1 M glycine-HCl buffer (pH 3.5) for 10 min at room temperature. After having been washed with PBS, the sections were incubated with monoclonal antibodies for 1 h at room temperature. After another washing with PBS, they were incubated with FITC-labeled goat anti–rat IgG (Cappel Labs., Cochranville, PA) for 1 h, and then were examined under a fluorescence microscope (Axiophot, Zeiss, Germany). Control sections were treated by the same protocol except for the step of the incubation with the monoclonal antibody.

The staining for the extrarenal tissues. Various organs obtained from an autopsy case (64-yr-old female) were used as normal tissues. Acid-urea denaturation was performed by incubating acetone-fixed sections with 0.1 M glycine-HCl buffer (pH 3.5) containing 6 M urea for 2 to 30 min at room temperature. Positive control sections were stained with anti-α1(IV) or α2(IV) collagen antibody, as it is well known that both α1(IV) and α2(IV) chains are usually colocalized in basement membrane. Negative control sections were reacted with non-immune rat sera, with the secondary antibody alone, or with the primary antibody preabsorbed with the synthetic peptide.

Alport's Syndrome Patients

We examined two cases of Alport's syndrome diagnosed by clinical manifestations and histological examinations. One case, a 14-yr-old boy, had proteinuria, hearing loss, and eye lesions. Electron microscopic examination of the glomeruli showed basement membrane thickening with lamination and splitting. The second case, a 9-yr-old boy, had proteinuria (0.2–0.6 g/d) and hematuria since the age of three years. He displayed farsightedness of the eyes and hearing loss as well. Several relatives had renal diseases, and some of them had died. Histological and electron microscopic examination of the kidney demonstrated irregular thickening of the glomerular basement membrane, confirming the diagnosis.

Results

Production of α6(IV) Chain-specific Antibodies

We selected a relatively nonconserved region, close to the carboxy termini of the NC1 domains, as a pattern to prepare synthetic peptides for use as immunogens to elicit α(IV) chain-specific monoclonal antibodies. Keyhole limpet hemocyanin (KLH)-conjugated peptide emulsified with Freund's complete adjuvant was injected into hind footpads, and medial iliac lymph nodes of the immunized rat were used for fusion with myeloma cells. For α6(IV)-specific antibodies we repeated the cell fusion five times. In each experiment fused cells were plated on four 96-well plates. 145 positive wells from the five experiments were obtained by direct reaction with the synthetic peptides. We further screened by staining frozen sections of human kidney and selected 12 strongly positive clones, which were then tested with the synthetic peptides from the same region of the other α1 through α5 chains by ELISA. All of the 12 clones were specific for the α6(IV) chain. Three antibody clones out of these 12 clones for the α6(IV) peptide, H61, H62, and H63, were established after subcloning by limiting dilution. Monoclonal antibodies H11, H21, H31, H43, and H52 to the human α1(IV), α2(IV), α3(IV), α4(IV), and α5(IV) chains, respectively, were also obtained in the study. Significant similarity was not found between the peptide sequences used for antibodies and the known amino acid sequence in proteins by DDBJ or Swiss Plot sequence analysis. The specificity of the antibodies against the synthetic peptides was tested by ELISA. A typical specific reaction of each antibody to individual antigen peptides is demonstrated in Fig. 2. Using the Geysen peptide scanning technique (Geysen et al., 1987), we narrowed down the epitopes for H61, H62, and H63 to the amino acid sequences of VSETLK, FGEL, and GELP, respectively, within the synthetic peptide sequence. Relative location of the epitopes for H61, H62, and H63 is drawn in Fig. 1.

Immunoblot Analysis

The structure of the entire human α6(IV) collagen polypeptide was predicted by cDNA sequence analysis (Oohashi et al., 1994). The deduced collagen polypeptide contained 1690 amino acid residues, including a 21-residue signal peptide, a 24-residue amino-terminal NC domain, a central 1417-residue collagenous (COL1) domain, and a 228-residue carboxy-terminal NC1 domain. To detect the presumptive peptide at the protein level we utilized monoclonal antibody H63, specific for the cDNA-derived polypeptide that was mentioned above. Kidney was used as the starting material for Western blotting analysis. Since the predicted α6(IV) collagen polypeptide structure was simi-

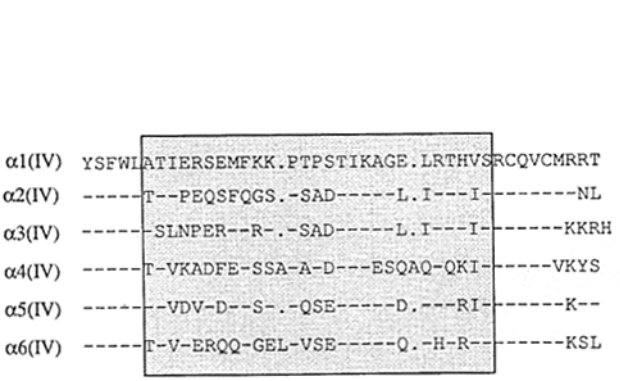


Figure 2. Specificity of the α(IV) chain-specific antibodies. (*A*) The carboxy-terminal ~40 amino acid residues of the α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), and α6(IV) chains are aligned to have the maximum homology. Dashes indicate the amino acid residues that are the same as for the α1(IV) chain. Gaps (-) are introduced to maintain alignment. The amino acid sequences of peptides used as immunogens for α chain-specific monoclonal antibodies are indicated by the shaded area. (*B*) The synthetic peptides used as immunogens for α chain-specific monoclonal antibodies were fixed onto the well bottoms of a 96-well plate. *Top row*: Control, only bovine serum albumin; *second row*: α1(IV) peptide; *third row*: α2(IV) peptide; *fourth row*: α3(IV) peptide; *fifth row*: α4(IV) peptide; *sixth row*: α5(IV) peptide; and *seventh row*: α6(IV) peptide. Nonimmune serum (7 wells in lane *1*), monoclonal antibodies of H11 (lane *2*), H21 (lane *3*), H31 (lane *4*), H43 (lane *5*), H52 (lane *6*), H63 (lane *7*), and dH2O (lane *8*) were added to the wells individually and incubated for 1 h. After the unbound antibodies had been washed out, the individual wells were blocked with bovine serum albumin. The peroxidase-conjugated secondary antibodies were added to all of the wells and reacted for 1 h. Note the specific reactions between the antigens and antibodies even though there is some similar sequence between peptide sequences used for raising the monoclonal antibodies.

lar to that of the other known α chains of collagen IV, we digested away the central collagenous domain by bacterial collagenase; and the materials were then electrophoresed and transferred to PVDF membranes. The membrane strips containing the blotted protein were incubated with antibody H63 made against the α6(IV)-derived peptide (see Materials and Methods). As shown in Fig. 3, the antiserum stained a band of ~27 kD in size, which is consistent with the NC1 size of the cDNA-derived α6(IV): 228 residues with a calculated molecular weight of 25,418 (Oohashi et al., 1994). This staining was blocked by preincubation of the antiserum with the antigen (data not shown). Preimmune serum was completely negative. When the other five antibodies against α1(IV) through α5(IV) peptides were reacted as controls with the same materials, several bands similar in size to those seen with α6(IV) sera and relatively distinct bands detected by α1 to α5 sera were obtained. The size of these bands was also reasonable for each NC1 domain. Each antibody also detected some bands at ~50 kDa, which we consider to be the dimeric forms (Johansson et al., 1992). Thus, α6(IV) antibody reacted indeed with the NC1 domain of the α6(IV) polypeptide, which is the first demonstration of the presence of the polypeptide in human kidney.

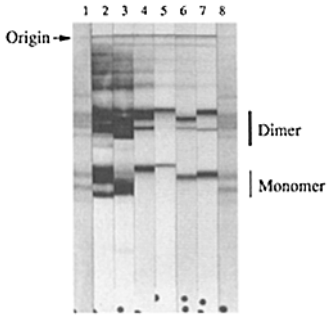


Figure 3. Western-blot analysis for the NC1-domain fraction from renal basement membrane. The NC1-domain fraction was prepared from renal basement membrane (see Materials and Methods), and SDS-PAGE was conducted on 11.5% polyacrylamide gels. After blotting of the proteins onto a PVDF membrane, the membrane was sliced into seven strips. Lanes *1* and *8* were stained with Coomasie-Brilliant Blue, and the rest of them were incubated with the individual α chain-specific monoclonal antibodies. Lanes *2–7* were incubated with H11, H21, H31, H43, H52, and H63, respectively. Note the sizes of the reactive peptides are different from strip to strip. The sizes of the peptides for lane *6* (for α5(IV)NC1) and *7* (for α6(IV)NC1) are estimated as ~26 and 27 kD, respectively. Several reactive peptides around the size of 50 kD probably represent dimerized forms.

Indirect Immunofluorescence Staining of Kidney Basement Membranes with Monoclonal Antibody against α6(IV)-derived Peptide

Immunofluorescence studies on frozen sections of human adult kidney with the α6(IV) peptide antiserum surprisingly gave a negative reaction in the glomerular basement membrane, whereas the basement membrane of the Bowman's capsules and of some tubules was positive, as shown in Fig. 4 *F*. The staining was completely blocked by preincubation of the antibody with the α6(IV) peptide used for raising antibody, H63 (Fig. 5 *B*). This staining pattern was quite in contrast with that obtained with the α5(IV) peptide antiserum (Fig. 4 *E*), in which basement membranes of glomerulus, Bowman's capsules, and some tubules were all positive. Thus, the two genes COL4A5 and COL4A6, controlled by a bidirectional promoter, are not always expressed at the same time; the cells that produce glomerular basement membrane express the α5(IV) gene but not the α6(IV) one at least in adult humans. Control experiments confirmed the specificity of these staining reactions. No reaction was obtained with the preimmune sera (data not shown). In contrast, antisera against α1(IV) and α2(IV) peptides gave a strong and similar staining pattern of all basement membranes of the glomeruli, tubules, and Bowman's capsules as well (Fig. 4, *A* and *B*). Intriguingly, the staining pattern for α3(IV) and α4(IV) antibodies was the same; both strongly stained the glomerular basement membrane and some distal tubules but mostly gave faintly positive reactions for basement membranes of Bowman's capsules (Fig. 4, *C* and *D*). The staining pattern for the kidney is summarized in Table I.

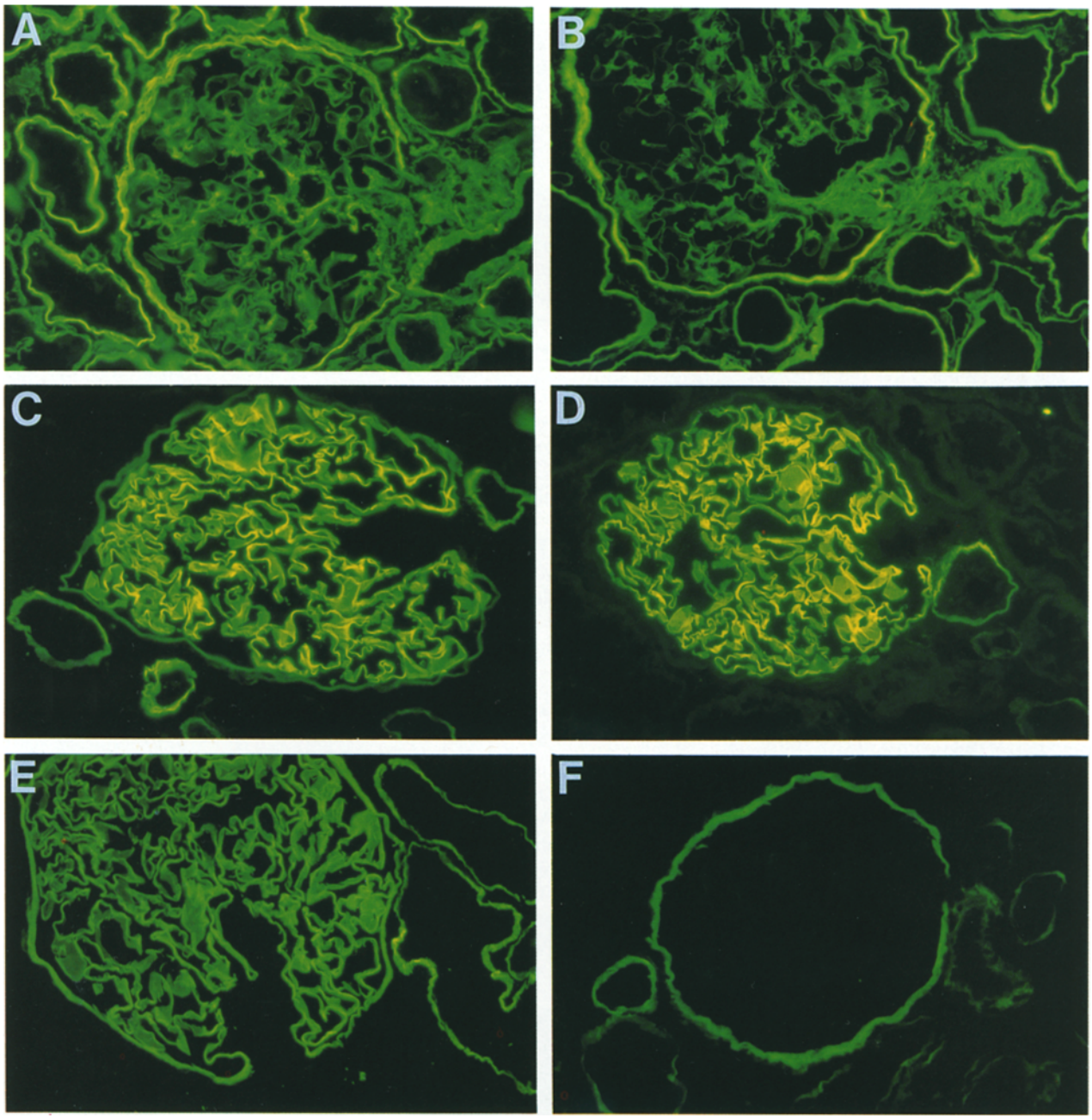


Figure 4. Differential staining of kidney basement membranes obtained with α(IV) chain-specific antibodies. α(IV) chain-specific antibodies were used to stain kidney basement membranes. Antibodies of H11, H21, H31, H43, H52, H63, and H64 were used for photos *A–F*, respectively. Note that α1(IV) and α2(IV) chains are coexpressed in all of the basement membranes in the kidney: glomeruli, Bowman's capsules, distal tubules, proximal tubules, and some capillaries. Antibodies specific for α3(IV) and α4(IV) chains demonstrated rather restricted staining pattern, i.e., glomerular, some tubular basement membranes; however these two chains are expressed together. Interestingly, α5(IV) and α6(IV) chains are not coexpressed; no staining for α6(IV) chain is seen in glomerular basement membrane (*F*), whereas α5(IV) chains are clearly expressed in the glomerulus (*E*).

To confirm that the monoclonal antibodies are recognizing the α6(IV) chain, we have done two experiments. One of them was to define the epitopes of the H61, H62, and H63 antibodies using Geysen's technique (Geysen et al., 1987). As mentioned above, the epitope sequences of VSETLK, FGEL, and GELP for H61, H62, and H63 antibodies, respectively, were all within the original peptide sequence but different from each other. However, the immunostaining pattern using the antibodies for the kidney sections were the same (data not shown). This suggested that the different antibodies recognizing different epitopes were reacting with the same molecule. The second experiment was to raise monoclonal antibodies to the peptide sequence within the COL1 domain of the α6(IV) chain. We selected CPSTGELEFMGFPKG in the third imperfection of the Gly-X-Y repeating sequence (Oohashi et al., 1994)

Table I. Distribution of Type IV Collagen α Chains in Kidney Basement Membranes

	α1(IV)	α2(IV)	α3(IV)	α4(IV)	α5((V)	α6(IV)
Glomerular BM	++	++	+++	+++	++	−
Mesangium	++	++	−	−	−	−
Bowman's BM	+++	+++	±	±	++	++
Tubular BM	++	++	++*	++*	++*	++*
Capillary BM	+	+	−	−	−	−

+, weakly positive; ++, moderately positive; +++, strongly positive; −, negative. *Not all the tubular basement membrances were positive for α3(IV), α4(IV), α5(IV), and α6(IV) antibodies.

within the COL1 domain. Among 11 positive clones by ELISA, the two clones, H64 and H65, were subcloned and purified. The immunostaining pattern for the kidney section using these two clones was the same as that of H63 shown in Fig. 5. These results indicate that the different antibodies derived from the different sequences still recognize the same material, the α6(IV) chain, in the tissues.

Differential Localization of the α5(IV) and α6(IV) Chains in Tissues Outside of the Kidney

To investigate whether the two genes are expressed in basement membranes in tissues other than the kidney, we stained several other organs and tissues, as shown in Fig. 6.

Skin. Epidermal basement membranes are thought to be produced by the keratinocytes, aligning at the base of the epithelial layers. Since the staining pattern for the α1(IV) and α2(IV) chains were almost the same, only the α2 staining pattern is shown in Fig. 6 *A*. As shown in Fig. 5, *B* and *C*, this basement membrane was stained by both antibodies against α5(IV) and α6(IV) chains, indicating that the keratinocytes express both genes together in human adult skin.

Muscle. Esophageal smooth muscle, cardiac muscle, and abdominal skeletal muscle were examined by using anti-α1(IV), -α2(IV), -α5(IV), and -α6(IV) antibodies. Both α5(IV) and α6(IV) chains colocalized in the basement membranes of the smooth muscle cells (Fig. 6, *E* and *F*). However, these two chains were never detected in cardiac muscle cells (Fig. 6, *H* and *I*), although both α1(IV) and α2(IV) chains were stained substantially (Fig. 6 *G*). Unexpectedly, in basement membranes surrounding the skeletal muscle cells, α5(IV) was negative and α6(IV) was weakly positive (Fig. 6 *L*) when the sections were stained with H-61 antibody. Further, this staining was blocked by incubating the antibody with the synthetic peptide of the α6(IV) chain. Relative to the α2(IV) staining, the α6 staining intensity was quite weak; therefore the level of the α6(IV) chain is presumed to be fairly low.

Adipocytes. Adipocytes expressed both α1(IV) and α2(IV) chains strongly (Fig. 6 *M*). Since α5(IV) and α6(IV) staining was quite low (Fig. 6, *N* and *O*), we had to expose the film longer to get a clear staining pattern. The level of α5(IV) and α6(IV) expression around adipocytes also thus appears to be quite low.

Liver. α2(IV) chain was present in the space of Disse (Fig. 6 *P*); however, neither α5(IV) nor α6(IV) chains were detected in this space underneath the sinusoidal endothelial cells (Fig. 5, *Q* and *R*).

Table II provides a summary of the above-mentioned staining patterns.

α5(IV) and α6(IV) Chains in Basement Membranes of Alport's Syndrome Patient

Immunohistochemical analysis was performed in kidney and skin from patients with X-linked Alport's syndrome. As shown in Fig. 7, antibodies against α1(IV) (photo not shown) and α2(IV) chains clearly stained basement membranes in glomeruli, Bowman's capsules, and tubules. However, neither of the antibodies against the α5(IV) and α6(IV) chains stained the basement membranes in the Alport kidney (Fig. 7, *B* and *C*). Antibodies against

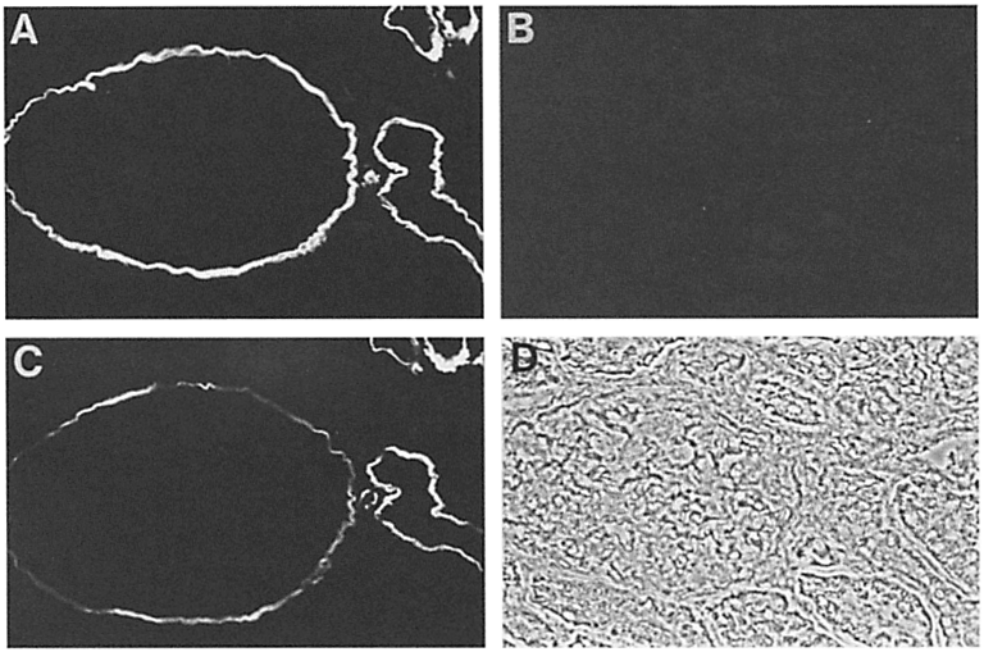


Figure 5. Staining of kidney basement membranes by α6(IV) antibodies. Serial sections of human kidney were stained by the two monoclonal antibodies to the different part of the α6(IV) chain: one (H63) is to the synthetic peptide from the NC1 domain (*A*) and the other (H65) is to the COL domain (*C*). Note the staining pattern using the two monoclonal antibodies is the same. Positive fluorescence staining using H63 antibody was completely blocked by the original synthetic peptide, TTVEERQQFGELPVSETLKAGQLHTRV(*B*). *D* shows nonstained phase contrast microscopic image of the neighboring section.

Table II. Distribution of Type IV Collagen α1(IV), α2(IV), α5(IV), α6(IV) Chains in Basement Membranes of Extrarenal Tissues

	α1(IV)	α2(IV)	α5(IV)	α6(IV)
Skin	+++	+++	+++	+++
Muscle				
Skeletal muscle	++	++	−	+
Cardiac muscle	++	++	−	−
Smooth muscle	++	++	++	++
Adipocyte	++	++	+	+
Liver	+	+	−	−

+, weakly positive; ++, moderately positive; +++, strongly positive; −, negative.

α3(IV) and α4(IV) chains also did not give positive staining in basement membranes in the kidney of the two patients examined (data not shown).

In Fig. 8, distribution of α(IV) chains in skin specimens from a normal individual and an Alport patient is shown. Antibodies against α1(IV) and α2(IV) chains demonstrated a clear linearized staining pattern in the epidermal basement membrane and basement membranes around capillaries and sweat glands in normal skin. α5(IV) and α6(IV) chains were detected only in the basement membrane of the epidermis but not in basement membranes around capillaries and sweat glands. In contrast to the normal skin, Alport skin showed negative staining with α5(IV) (Fig. 8 *H*) and α6(IV) (Fig. 8 *I*) antibodies in epidermal basement membranes, whereas α1(IV) (data not shown) and α2(IV) (Fig. 8 *G*) antibodies clearly stained the basement membranes.

Discussion

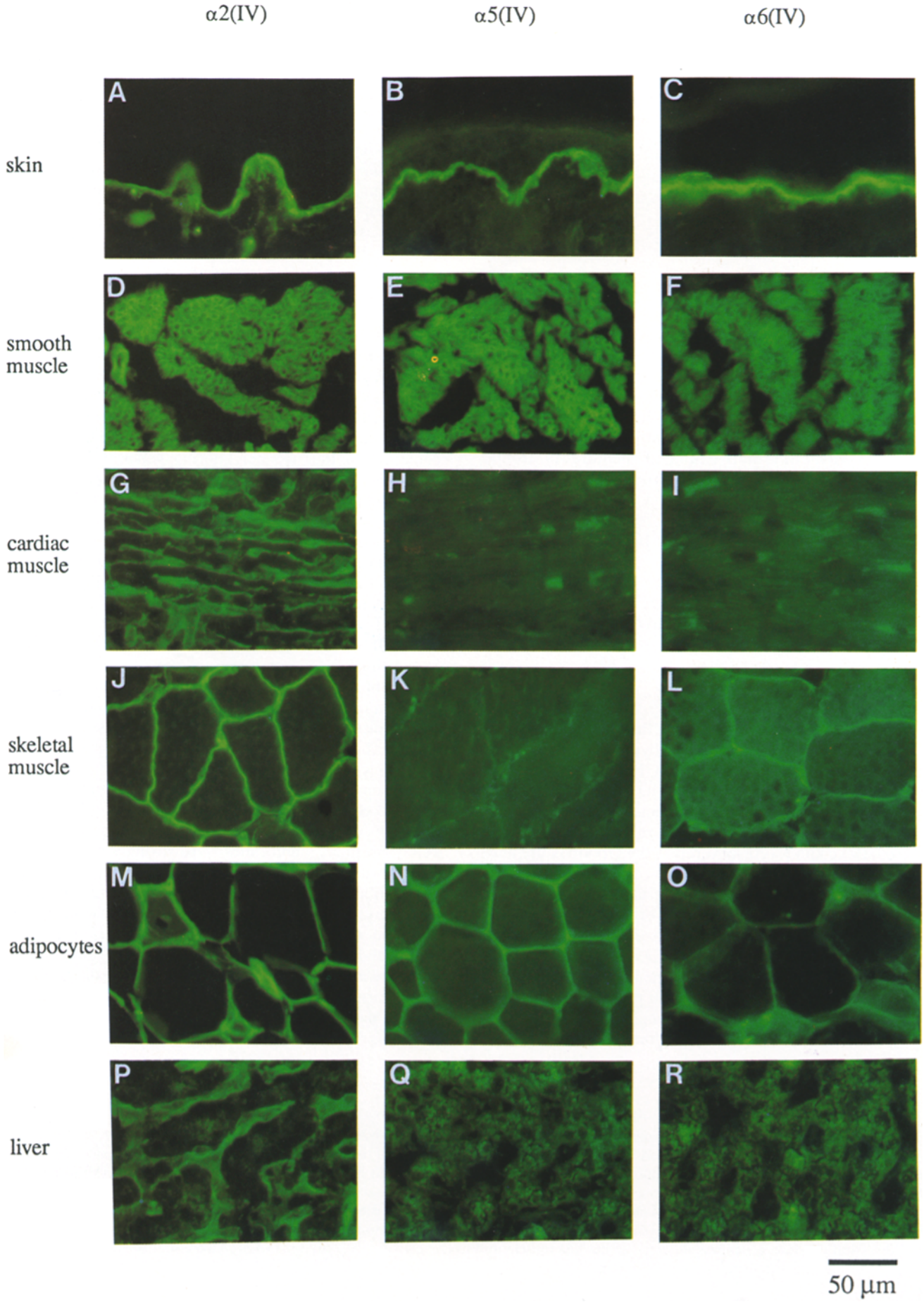
Diversity has been revealed among the subunits that form collagen IV molecules with the discovery of the α3, α4, α5, and α6 chains(Hudson et al., 1993). But chain assemblies that make up type IV molecules have been difficult to demonstrate since the α3-α6(IV) chains were studied primarily in the context of the disease-related human tissues. The α3(IV) chain was discovered as the Goodpasture antigen, which attacks primarily the alveolar basement membranes in the lung and the glomerular basement membranes in the kidney (Butkowski et al., 1987). Further, trials to purify the α3(IV) chain resulted in the discovery of another α-chain, α4(IV)(Gunwar et al., 1990). cDNA cloning of these two chains led to the identification of mutations in COL4A3 and COL4A4 in patients with autosomal recessive type Alport syndrome (Mochizuki et al., 1994; Lemmink et al., 1994). Most recently COL4A6 was shown to be located next to COL4A5 (Zhou et al., 1993; Sugimoto et al., 1994) and both genes were mutated in several X-linked cases of Alport syndrome combined with leiomyomatosis (Zhou et al., 1993).

Immunohistochemical analyses have shown that the α1(IV) and α2(IV) chains are abundant in all tubular basement membranes and in the glomerular mesangial matrix (Kleppel et al., 1989). However the α3-α5(IV) collagen chains have a rather limited distribution in human tissues; highly enriched in glomerular basement membranes but found only in a subset of tubular basement membranes (Hostikka et al., 1990; Sanes et al., 1990; Hudson et al., 1992; Yoshioka et al., 1994). Just recently, Miners and Sanes (1994) reported the distribution of α3, α4, and α5(IV) collagen chains in rodent basal laminae. They suggested from their immunohistochemical studies of various tissues that many combinations of α(IV) chains were possible, but α3 and α4(IV) chains were always coexpressed together, and appeared only in basal laminae that were α5(IV) chain positive. However, they did not examine the distribution of the newly discovered α6(IV) chain with respect to the other α(IV) chains. In the light of gene regulation of the two neighboring genes, COL4A5 and COL4A6, we herein examined and focused on the expression of the two genes at the protein level.

We first raised monoclonal antibodies specific for the human α6(IV) collagen chain and used them with antibodies produced by a previously isolated clone for α5(IV) chain to identify location of α5(IV) and α6(IV) chains in basement membranes of kidney, skin, muscle, liver, and fat cells. In addition, we used the monoclonal antibodies to identify the NC1 domains of α5(IV) and α6(IV) by Western blotting.

Our immunohistochemical survey with monoclonal antibodies to α5(IV) and α6(IV) chains indicated a very restricted distribution of these proteins in basement membranes. The basement membrane in the glomerulus is physiologically and clinically a carefully studied matrix. This basement membrane contains specialized structures that face cell layers of epithelial cells and endothelia or other cells on both surfaces. As shown in Fig. 4, the glomerular basement membranes were strongly positive when stained with α5(IV) chain-specific antibody, H51; however, antibody against the α6(IV) peptide did not show a fluorescence staining within the glomerular basement membrane. In contrast, basement membranes in Bowman's capsule and distal tubules were positive with both antibodies. There are at least two and possibly more different molecular forms in the glomerulus: $[\alpha1(IV)]_2\alpha2(IV)$ and one or more molecular forms of the combination of α3, α4, and α5 chains. In an Alport's nephritis case, neither of the

Figure 6. Staining of basement membranes in extrarenal tissues using α1(IV)/α2(IV), α5(IV), and α6(IV) antibodies. Expression of α5(IV), and α6(IV) chains was examined in basement membranes in extrarenal tissues; skin (*A–C*), smooth muscle cells (*D–F*), cardiac muscle cells (*G–I*), abdominal skeletal muscle cells (*J–L*), adipocytes (*M–O*), and liver (*P–R*). Since antibodies for α1(IV) and α2(IV) chains gave quite similar patterns, only the α2(IV) staining pattern using H21 is shown in *A, D, G, J, M,* and *P*. Expression of α5(IV) (*B, E, H, K, N,* and *Q*, using H52 antibody) and α6(IV) (*C, F, I, L, O,* and *R*, with H63 antibody) chains is compared in various basement membranes. Around the smooth muscle cells and at the epidermal/dermal junction, the α5(IV) and α6(IV) chains are coexpressed, but no expression of these genes was detected in cardiac muscle and liver. Intriguingly, only α6(IV) is weakly stained in skeletal muscle, whereas no staining is detected for α5(IV) chain.



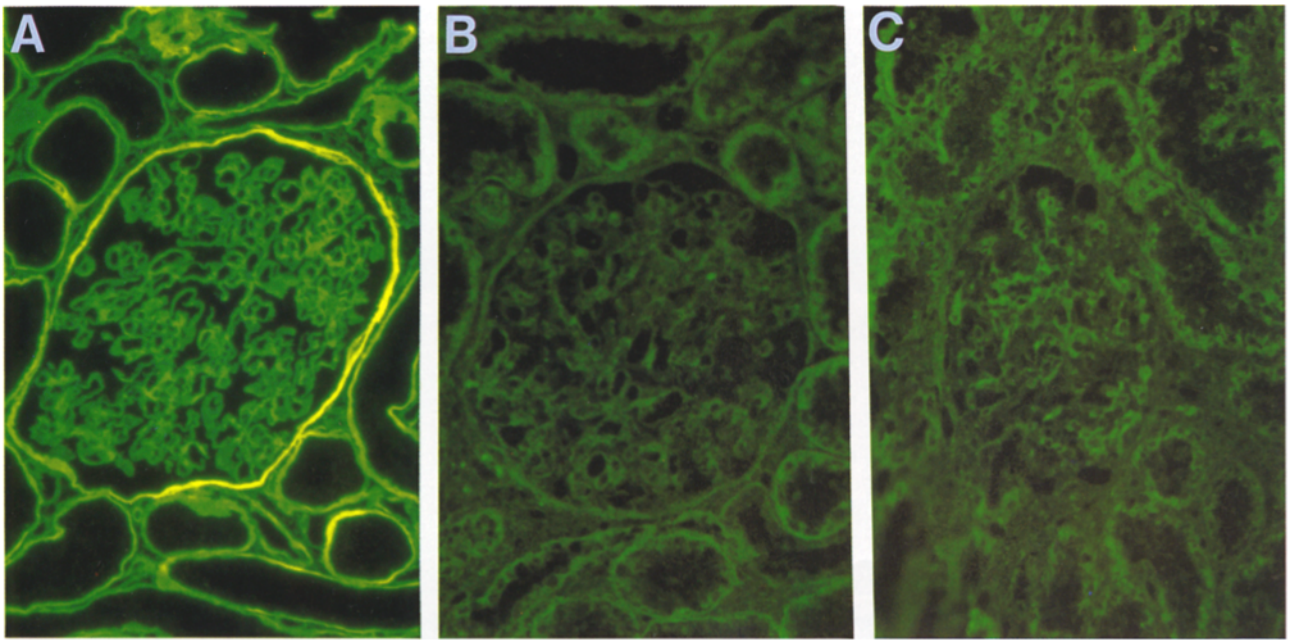


Figure 7. Distribution of α(IV) chains in kidney from Alport's syndrome patient. Similar staining pattern was obtained with α1(IV) (not shown) and α2(IV) (*A*) antibodies, but no clear staining is shown in any basement membranes in the kidney sections stained with monoclonal antibodies for α5(IV) (*B*, with H52) and α6(IV) (*C*, with H63) chains.

three chains, α3, α4, and α5, were found in the glomerulus (Fig. 6). We do not know the precise mutation of α(IV) gene in this case. However, since more than 50 different mutations have been identified in the COL4A5 gene but none in the COL4A6 gene, it is possible that a heterotrimeric molecule composed of the three chains, α3, α4, and α5, cannot be formed due to abnormal α5 chain synthesized from a mutated COL4A5 gene.

In addition to the human and murine α1(IV) and α2(IV) collagen genes (Killen et al., 1988; Soininen et al., 1988), there are several clustered genes in vertebrates that are organized in a head-to-head configuration and directed by bidirectional promoters. These include the histone H2a and H2b genes (Hentschel and Birnstiel, 1981), DHFR and mismatch repair protein 1 gene (Fujii et al., 1992), Wilms' tumor locus (Huang et al., 1990), a proliferation cell nuclear antigen gene (Rizzo et al., 1990), an SV40-like monkey genomic locus (Saffer et al., 1984), the GPAT/AIRC genes (Gavalas et al., 1993; Gavalas and Zalkin, 1995), and Drosophila ras2/rop genes (Lightfoot et al., 1994). Some of the promoters of these genes are bidirectional but the opposite genes are not identified. The promoter activity for one direction is quite different from that for the other direction, 10-fold different in some cases (Gavalas and Zalkin, 1995). Most of the paired genes are not homologous at all, however, the COL4A1/COL4A2 and COL4A5/COL4A6 genes are homologous, indicating that the clusters did evolve by gene duplication. Divergent transcription of the two genes from a bidirectional promoter can thus provide coexpression and coregulation of expression in some tissues. Proximal *cis*-acting elements as well as *trans*-acting factors such as CTC binding factor (Fischer et al., 1993) have the potential to modulate the expression of both genes simultaneously.

The genes for α5(IV) and α6(IV) chains are encoded on opposite DNA strands and have a common promoter region (Sugimoto et al., 1994). A question that arose immediately when we noticed the specialized relation of these genes was whether these genes always produce the two chains at a steady rate as COL4A1 and COL4A2 genes do. As we demonstrated in Fig. 6, keratinocytes, esophageal smooth muscle cells, fat cells, striated muscle cells and placental epithelial cells express α5(IV) and α6(IV) genes, whereas hepatocytes and cardiac muscle cells do not express either gene. However, of interest was that skeletal muscle cells only synthesized α6(IV) chain and that the glomerular basement membranes were positive with only with α5(IV) antibody in adult humans. These results are consistent with the analysis of transcripts found in a variety of tissues except for the heart (Zhou et al., 1993), although we have not analyzed tissues from human fetuses. The two genes are transcribed in the opposite direction in a tissue-specific manner, and regulation of expression for the two genes is controlled at the transcription level. One of the clues for the gene expression could be the presence of the alternative promoters on the COL4A6 gene (Sugimoto et al., 1994). The two different transcription forms for the α6(IV) gene driven by the alternative promoters are found in a tissue-specific manner. The tissue-specific distribution of the newly discovered α5(IV) and α6(IV) chains are probably related to the specialized function of individual basement membranes in different tissues.

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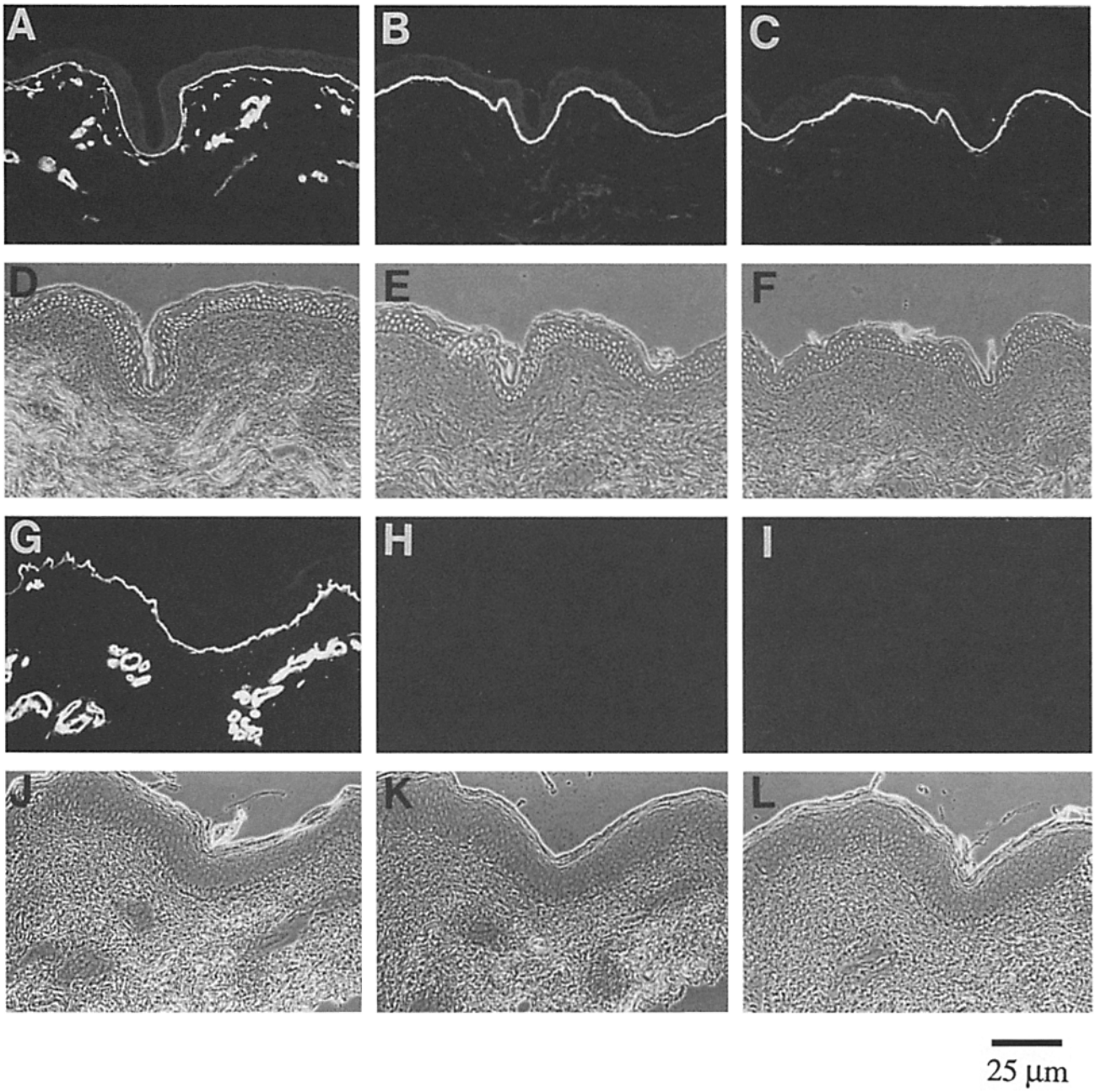

Figure 8. Distribution of α(IV) chains in dermal basement membrane from Alport syndrome patient. Since a similar staining pattern was obtained with α1(IV) and α2(IV) antibodies in basement membranes of dermal epidermal junction and capillaries, only the α2(IV) staining pattern (*A*, with H21) is shown in normal skin. Both α5(IV) (*B*, H52) and α6(IV) (*C*, H63) antibodies stain only the dermal basement membrane but not that of the capillaries. In the Alport patient case, a similar staining pattern was obtained for α1(IV) (not shown) and α2(IV) chains as in normal skin (*G*); however no clear staining was shown by monoclonal antibodies for α5(IV) (*H*) and α6(IV) (*I*) chains at all (*H* and *I*). Phase-contrast images are shown for individual samples in normal skin (*D–F*) and Alport skin (*J–L*).

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