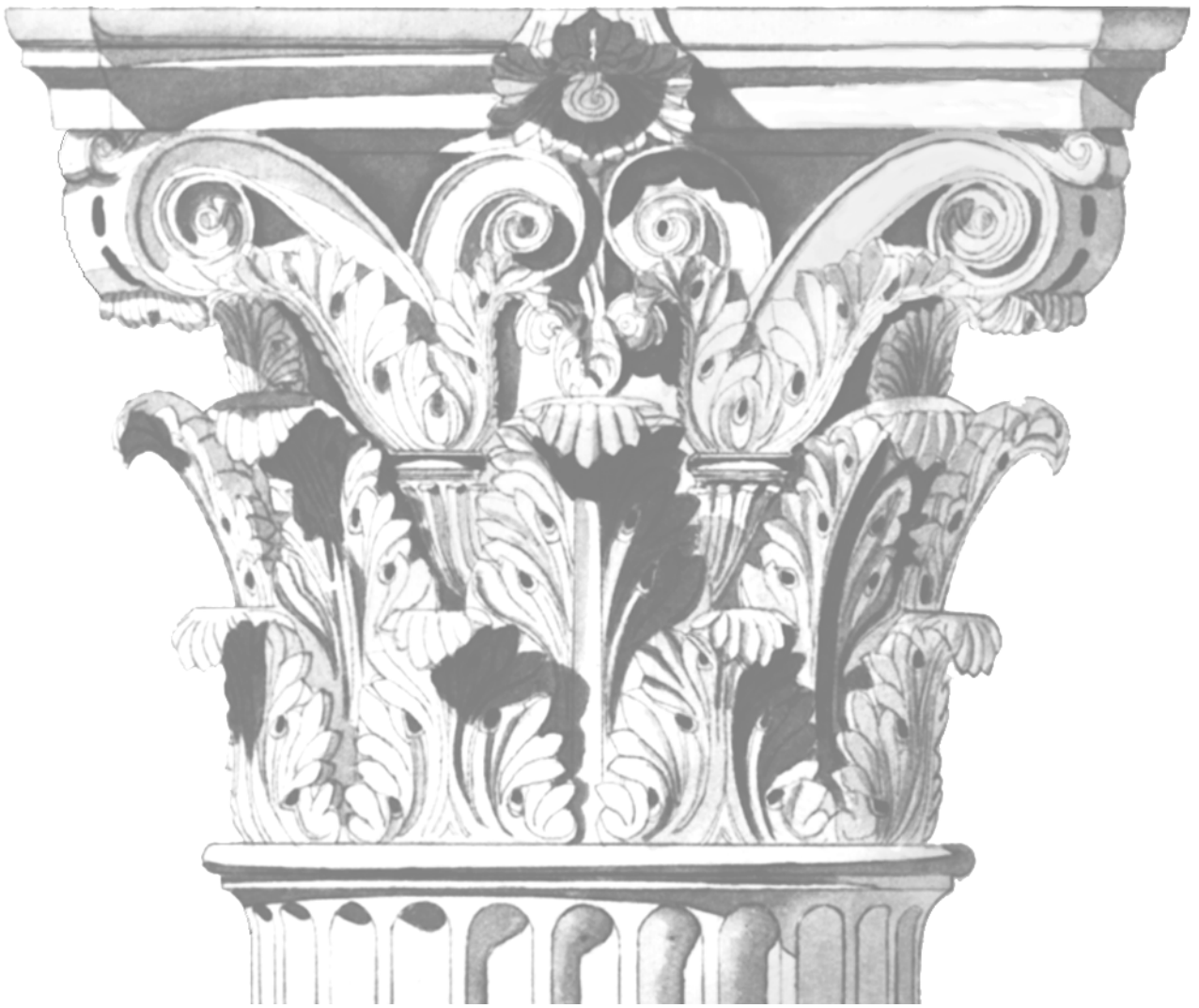
TYPE IV COLLAGEN

Characterization of the COL4A5 gene, mutations in Alport syndrome, and autoantibodies in Alport and Goodpasture syndromes

PAULA MARTIN

Biocenter Oulu and Department of Biochemistry

OULU 2000



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Abstract

Type IV collagen is only found in basement membranes, where it is the major structural component, providing a framework for the binding of other basement membrane components and a substratum for cells. The type IV collagen molecule is triple-helical and composed of three α chains which exist as six distinct forms (α1 – α6). Abnormalities in this basement membrane collagen structure and function are connected to both inherited and acquired diseases.

Alport syndrome is a hereditary kidney disease associated with extrarenal complications, such as sensorineural deafness and eye abnormalities. The disease is caused by mutations in the COL4A3, COL4A4 and COL4A5 genes, coding for the type IV collagen α3, α4 and α5 chain genes, respectively. About 85% of the Alport syndrome cases are X-linked dominant, caused by mutations in the COL4A5 gene. In order to develop a basis for automated mutation analysis of the COL4A5 gene, previously unknown intron sequences flanking exons 2 and 37 were determined. Intron sequences flanking the other 49 exons were expanded from 35 to 190, and additionally, two novel 9 bp exons (exons 41A and 41B) were characterized in the large intron 41. In addition to optimization of the PCR amplification and sequencing conditions for all 51 exons and exon flanking sequences, optimization for the 820 bp promoter region and for the two novel exons was performed as well. Mutations were found in 79 unrelated patients of the 107 studied. This gives a high mutation detection rate of almost 75% in comparison with 50%, at its best, in other extensive mutation analyses of the COL4A5 gene using SSCP analysis. None of the mutations involved the promoter region or exons 41A and 41B.

Circulating antibodies against basement membrane components have been recognized in some autoimmune diseases. Goodpasture syndrome is a rare autoimmune disease characterized by progressive glomerulonephritis and pulmonary hemorrhage. The target of the antibodies in this disease has been shown to be the noncollagenous NC1 domain of type IV collagen α3 chain. For unknown reasons, a minority of Alport syndrome patients also develops antibodies against α3 and α5 chains after renal transplantation with manifestation of severe anti-GBM disease. In order to investigate the antibodies both in Goodpasture and Alport syndrome, the NC1 domains of all six type IV collagen chains were produced as recombinant proteins in bacterial and mammalian expression systems, and an ELISA method was developed for antibody detection. Antibodies were found in both syndromes, interestingly also in Alport syndrome patients without the anti-GBM disease.

The results of this work have a significant clinical value by providing for the first time complete, effective DNA-based analysis of all exon/intron and promoter regions of the COL4A5 gene in Alport syndrome.

Keywords: alternative splicing , basement membrane, hereditary nephritis

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Oulu, June 2000 Paula Martin

Abbreviations

α1(IV)	type IV collagen α1 chain, and other collagen polypeptide chains accordingly
AS	Alport syndrome
BFH	benign familial hematuria
BM	basement membrane
COL4A1	type IV collagen α1 chain gene, and other genes accordingly
dNTP	deoxynucleotide triphosphate
EBM	epidermal basement membrane
ESRD	end stage renal disease
GBM	glomerular basement membrane
HSPG	heparan sulfate proteoglycan
NC1	noncollagenous domain 1
Ni-NTA	nickel-nitrilo-tri-acetic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SSCP	single-strand conformation polymorphism
STBM	seminiferous tubule basement membrane
WB	washing and binding
X	any amino acid
Xq	long arm of the chromosome X
Y	any amino acid

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Martin P, Heiskari N, Zhou J, Leinonen A, Tumelius T, Hertz JM, Barker D, Gregory M, Atkin C, Styrkarsdottir U, Neumann H, Springate J, Shows T, Pettersson E & Tryggvason K (1998) High mutation detection rate in the COL4A5 collagen gene in suspected Alport syndrome using PCR and direct DNA sequencing. J Am Soc Nephrol 9:2291-2301.

II Martin P, Heiskari N, Pajari H, Grönhagen-Riska C, Kääriäinen H, Koskimies O & Tryggvason K (2000) Spectrum of COL4A5 mutations in Finnish Alport syndrome patients. Hum Mutat 15: 579.

III Martin P & Tryggvason K: Two novel alternatively spliced 9 bp exons in the COL4A5 gene. Pediatr Nephrol, in press.

IV Martin P, Ruotsalainen V, Vuolteenaho R, Tryggvason K & Höyhtyä M: Use of recombinant proteins to detect antibodies against type IV collagen in serum. Manuscript

Contents

1 Introduction

Type IV collagen is a triple helical molecule, composed of three polypeptide chains, termed α chains. Six genetically distinct α chains, α1(IV) to α6(IV), have been characterized. Type IV collagen is ubiquitously found in basement membranes, where it forms a supportive network for adhesion of cells and for the binding of other basement membrane components. Abnormalities in type IV collagen are involved in some genetic and acquired diseases.

Alport syndrome (AS) is a hereditary kidney disease with typical extrarenal symptoms, such as sensorineural hearing loss and eye abnormalities. The most common form of the disease is inherited dominantly on the X chromosome. In this form, mutations have been found in the COL4A5 gene encoding the type IV collagen α5 chain. Rarer autosomal forms, which represent about 15% of the cases, also exist. Autosomal AS is caused by mutations in the COL4A3 and COL4A4 genes, coding for the α3(IV) and α4(IV) chains, respectively. Type IV collagen mutations can be considered responsible for abnormalities in the structural framework of the glomerular basement membrane (GBM), with consequent kidney manifestations. As in many collagen diseases, mutations in Alport syndrome are dispersed all over the huge type IV collagen genes. Extensive studies of COL4A5 have been carried out, but difficulties have arisen, in that usually about 50% of the mutations have remained unidentified.

Goodpasture syndrome, an autoimmune disorder, is caused by circulating anti-basement membrane antibodies, the targets of which have been shown to be the noncollagenous domain (NC1) of the α3 chain of type IV collagen. Some Alport syndrome patients produce antibodies against the transplanted allograft, developing a very rare and dramatic anti-GBM nephritis. Targets of these antibodies have been shown to be the NC1 domains of both the α3(IV) and α5(IV) chains.

In order to increase the possibilities for identification of mutations in the COL4A5 gene in Alport syndrome, further characterization of the gene and optimization of the conditions for mutation analysis were performed. Again, COL4A5 mutations were detected from foreign Alport syndrome patients as well as from Finnish AS families. To study if AS patients without anti-GBM nephritis have antibodies against type IV collagen,

the NC1 domains were produced by two different expression systems and an ELISA method was developed for antibody detection.

2 Review of the literature

2.1 Type IV collagen

Type IV collagen is a member of the collagen superfamily that contains at least nineteen different proteins. Collagens, the primary structural proteins of the body, either form extracellular fibrils or network structures providing the main support for tissues (Vuorio & de Crombrugghe 1990, Prockop & Kivirikko 1995). Type IV collagen is ubiquitously present in a specific type of extracellular matrix, the basement membranes. As their major structural component, type IV collagen forms a tightly cross-linked network connected through entactin (nidogen) to a less dense laminin network (Aumailley & Gayraud 1998).

2.1.1 α(IV) chains

Type IV collagen protein is a triple helical molecule composed of three α chains. To date, six genetically distinct type IV collagen α chains, α1(IV)-α6(IV), have been characterized. Each chain contains a ~1400-residue collagenous domain with Gly-Xaa-Yaa repeats, which is interrupted at several sites by short noncollagenous sequences. At the amino terminus, there is a ~15-residue noncollagenous domain and at the carboxyl terminal end, a ~230-residue noncollagenous (NC1) domain (Hudson *et al*. 1993). The amino terminal noncollagenous segment and the short cysteine-rich collagenous sequence next to it is called the 7S domain (Timpl 1989). The α chains are highly glycosylated, with numerous hydroxylysine-linked disaccharide units and an asparagine-linked oligosaccharide unit in the 7S domain (Langeveld *et al*. 1991, Nayak & Spiro 1991). The presence of a glycine residue as every third amino acid in the collagenous domain is essential, as it is the only amino acid small enough to fit into the center of the collagen

triple helix. The interruptions in the Gly-X-Y repeat sequences are thought to provide flexibility for the triple helical molecules (Hudson *et al*. 1993).

The most common isoform of type IV collagen, present in all basement membranes, is a trimer with two α1(IV) chains and one α2(IV) chain ($\alpha1_2\alpha2$). The complete sequence of both chains has been determined for different species including human (Soininen *et al.* 1987, Hostikka & Tryggvason 1988), mouse (Muthukumaran *et al*. 1989, Saus *et al*. 1989) and *Caenorhabditis elegans* (Guo *et al.* 1991, Sibley *et al*. 1993). Additionally, the α1(IV) sequence is known for *Drosophila* (Blumberg *et al*. 1988) and sea urchin (Exposito *et al*. 1993), and the α2(IV) sequence for *Ascaris suum* (Pettit & Kingston 1991). The primary sequences of the more tissue-specific α3(IV) (Mariyama *et al*. 1994), α4(IV) (Leinonen *et al*. 1994), α5(IV) (Zhou *et al*. 1992a, 1994b) and α6(IV) (Zhou *et al*. 1994a, Oohashi *et al*. 1995) chains have been characterized for man.

The collagen IV chains are very homologous and can be divided into two classes. The α1(IV), α3(IV) and α5(IV) chains belong to an α1-like class, and the α2(IV), α4(IV) and α6(IV) to an α2-like class. The α-chains can be also classified in a way that the abundant α1(IV) and α2(IV) chains are termed ”classical” chains to distinguish them from less abundant, tissue-specific ”novel” α3(IV) to α6(IV) chains (Hudson *et al*. 1993).

2.1.1.1 Special features

The six type IV collagen α chains all share some distinct, highly conserved features which presumably are related to the common and unique functions of this network forming collagen type. However, there are also certain differences between the primary structures of some chains, probably giving some special biological functions to each individual chain (Zhou & Reeders 1996).

The noncollagenous carboxyl terminal NC1 domains have been proposed to serve two major functions, which are common among the type IV collagen α chains in all species. They are essential for correct chain association that is followed by formation of the triple helix (Dölz *et al*. 1988). Additionally, they are important for type IV collagen assembly into the network structure, during which covalent disulfide bonds are formed between individual NC1 domains of two neighboring molecules (Timpl *et al*. 1981). Thus, it is not surprising that the amino acid sequences of the NC1 domains are highly conserved between species and chain types. The NC1 domains contain about 230 residues and the sequence identity varies between 52% to 83% in man (Leinonen *et al*. 1994). Twelve cysteines are completely conserved between *Drosophila* and man, six cysteines being located in each half, when NC1 domains are divided into two homologous repeating units (Siebold *et al*. 1988, Pihlajaniemi *et al*. 1985). These cysteines first form intradomain bonds, but during the extracellular molecular assembly, rearrangement of these bonds occurs, so that six cysteines form disulfide bonds with cysteine residues of the NC1 domain of a neighboring molecule (Siebold *et al*. 1988). The role of other individual conserved amino acids is not understood, but obviously the maintenance of a conserved

three-dimensional configuration, required for the normal function of the NC1 domain, demands a high degree of sequence similarity.

The collagenous domains of the six human type IV collagen α chains contain 1,410 to 1,449 residues (Table 1). The main reason for the size difference between α chains is the presence of 21-26 noncollagenous interruptions of varying length in the Gly-Xaa-Yaa-repeat sequence. It is, however, possible to make an alignment of the Gly-Xaa-Yaa-repeat sequences of all the α chains when interruptions are ignored (Leinonen *et al*. 1994). The interruptions are thought to give flexibility both to the type IV collagen molecules and the network itself (Hofmann *et al*. 1984, Dölz *et al*. 1988).

A characteristic feature of the collagenous domain is the conservation of four cysteine residues in the 7S domain at the amino terminus. These cysteines are essential for the cross-linking of four triple helical molecules through disulfide bonds (Timpl *et al*. 1981, Siebold *et al*. 1987). The α3(IV), and particularly the α4(IV) chain, are different from the other α chains in that they contain more unconserved cysteines. The α3(IV) contains five and the α4(IV) chain thirteen unconserved cysteines, as compared to 2-3 in the other chains. This indicates that isoforms containing the α3(IV) and α4(IV) are more cross-linked and, thus, might be required in basement membranes that are subject to more stress than others. This hypothesis is supported by the observation of a developmental switch in the GBM, where the α1:α2 network is abundant in embryo and the α3:α4:α5 network is predominant after birth (see section 2.5.5.1.).

The α3(IV) and α5(IV) chains have been reported to undergo alternative splicing events in certain tissues. Several differentially spliced α3(IV) products have been found in human kidney samples (Bernal *et al*. 1993, Feng *et al*. 1994, Penades *et al*. 1995). All these transcripts lack one or more of the exons from the NC1 domain. However, it has not been demonstrated if these alternatively spliced products are translated. In the case that they were, they might have a role in controlling the amount of functional α3(IV) chains. The α5(IV) chain has also been shown to have differentially spliced transcripts. Saito *et al*. (1994) found human kidney and skin transcripts lacking exon 50 sequences. Additionally, a splice product with an extra 18 bp sequence between exons 41 and 42 has been reported in human kidney (Guo *et al*. 1993). The significance of such alternative splice products has remained unknown.

Table 1. Main features of the six human type IV collagen α chains.

α chain	Amino acid residues					Number of iterruptions in collagenous domain
	Complete translation product	Mature α chain	Signal peptide	Collagenous domain	NC1 domain	
α1(IV)	1 669	1 642	27	1 413	229	21
α2(IV)	1 712	1 676	36	1 449	227	23
α3(IV)	1 670	1 642	28	1 410	232	23
α4(IV)	1 690	1 652	38	1 421	231	26
α5(IV)	1 685	1 659	26	1 430	229	22
α6(IV)	1 691	1 670	21	1 417	228	25

References: Hostikka & Tryggvason 1988, Leinonen *et al*. 1994, Mariyama *et al*. 1994, Soininen *et al*. 1987, Zhou *et al*. 1992a, 1994b.

2.1.2 Biosynthesis and molecular assembly

The biosynthesis of type IV collagen includes many complex co- and posttranslational modifications similar to those of collagenous proteins in general (Kivirikko & Myllylä 1987). During translation, the nascent α chains undergo several enzymatic modifications. These include cleavage of the signal peptide, as well as hydroxylation of prolyl and lysyl residues. Again, in type IV collagen α chains, almost all hydroxylated lysyl residues are glycosylated to form galactosyl-hydroxylysine or glucosyl-galactosyl-hydroxylysine. The presence of hydroxyproline is required for the maintenance of a stable triple helix through the formation of hydrogen bonds between the individual α chains. The hydroxylysyl residues are believed to stabilize cross-links between molecules, but the function of the carbohydrates is still unknown. Structure of the carboxyterminal noncollagenous domains allows their association, and both intrachain and interchain disulfide bonds are formed. Once the triple helix has been formed, the molecules are secreted into the extracellular space for assembly.

The triple helical type IV collagen molecules, monomers, self-assemble into a complex network (Fig 1). Several modes of interactions are known. Linear dimers are formed through association between the NC1 domains of the two molecules. The resulting hexameric NC1 complex is mostly stable via interchain disulfide bond formation (Timpl *et al*. 1981). The 7S domain at the amino terminus contains four cysteine residues that participate in both intra- and intermolecular disulfide bonds (Siebold *et al*. 1988). Tetrameric structures are formed through noncovalent interactions, parallel and antiparallel associations of the aminoterminal ends of four monomers. The

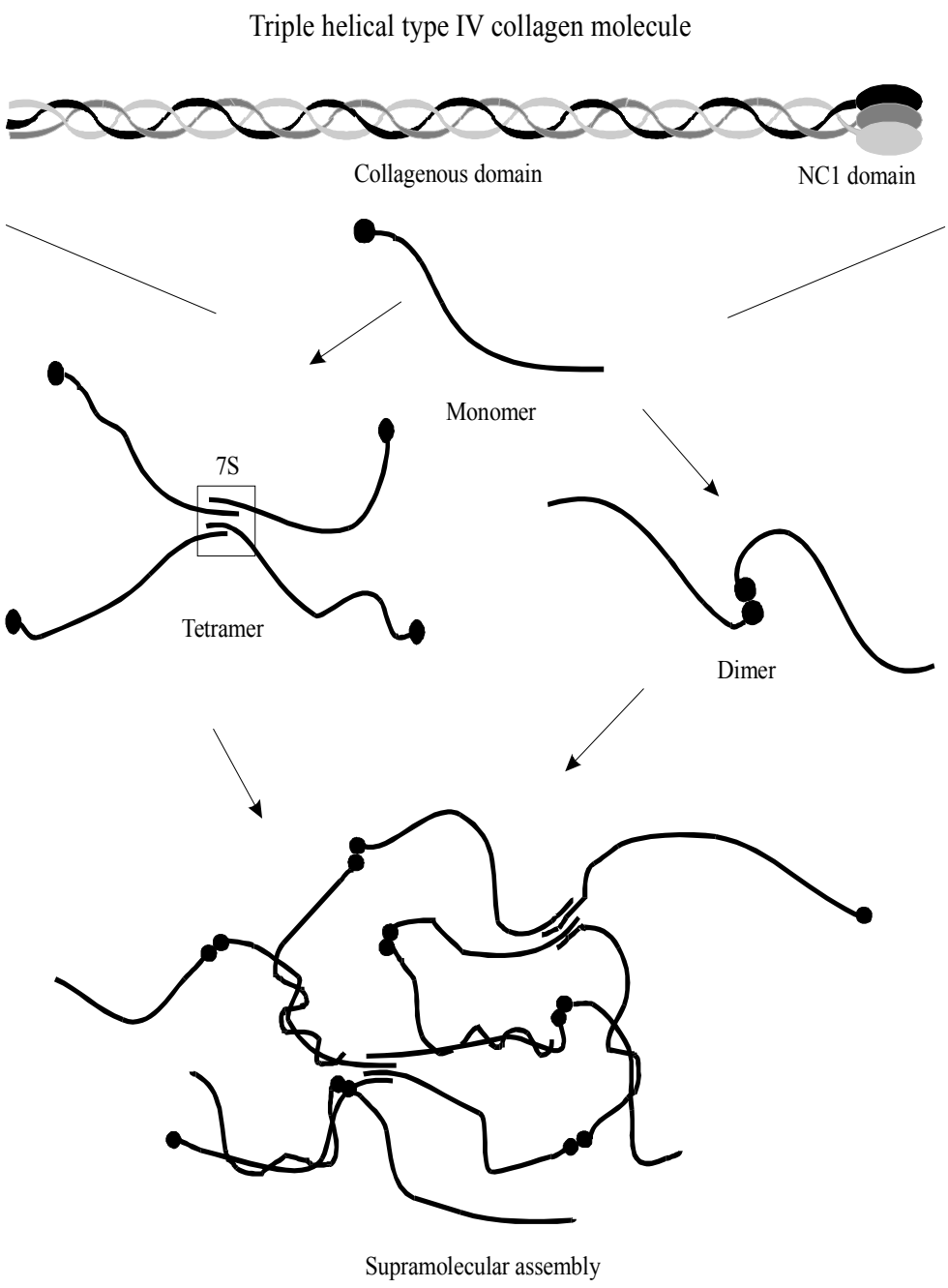

Fig 1. Schematic illustration of the type IV collagen molecules assembling into a supramolecular network structure. See text for details.

dimers and tetramers further assemble forming a network of highly branched filaments that include laterally aligned molecules and molecules twisting around each other (Siebold *et al*. 1988, Yurchenko & O'Rear 1993).

Theoretically, the existence of six different α(IV) chains allows formation of 56 different kinds (isoforms) of triple helical molecules (Hudson *et al*. 1993). As mentioned earlier, the most common form of type IV collagen, present in most basement membranes, is a heterotrimer with two α1(IV) chains and one α2(IV) chain (Hudson *et al*. 1993). Homotrimers of α1(IV) chains have also been reported (Haralson *et al*. 1985, Saus *et al*. 1988). Possible combinations of the other four novel α chains (α3-α6) have been intensively studied. Whether the novel chains are in the same molecule or not is still a question of controversy, but evidence for different networks has been presented. Different populations of NC1 domains, one with a heterotrimeric composition of two α3(IV) chains and one α4(IV) chain (Johansson *et al*. 1992), and another with a homotrimeric composition of α3(IV) chains (Saus *et al*. 1988), have been shown to exist in the glomerular basement membrane. Immunohistochemical stainings of kidney sections from Alport patients usually show the absence of α3(IV)-α5(IV) chains which are normally present in the GBM. Recently, Gunwar and co-workers (1998) identified a novel disulfide-cross-linked α3:α4:α5 network of type IV collagen in the kidney. This finding explains several features of the GBM abnormalities in Alport syndrome (see also section 2.5.5.1.). Seminiferous tubule basement membrane (STBM) has an important role in spermatogenesis, in that ultrastructural abnormalities of the STBM are thought to lead to infertility. STBM has been shown to contain two different networks: one with α1(IV) to α6(IV) chains and the other with α3(IV) to α6(IV) chains (Kahsai *et al*. 1997).

2.2 Basement membranes

Type IV collagen forms tight and stable scaffolding for basement membranes. It is covalently crosslinked due to disulfide bonds and nonreducible lysyl oxidase-derived bonds formed both at the carboxy- and aminoterminal ends of the molecule (Yurchenko & O'Rear 1993). Laminin molecules, capable of self-association, also form a network in basement membranes. Again, nidogen has a critical role in connecting type IV collagen and laminin networks, and in anchoring other components, such as perlecan and fibulins (Aumailley & Gayraud 1998) (Fig 2).

In general, basement membranes (BMs), also called basal laminae, are extracellular matrices which form thin, sheet-like structures, surrounding cells and tissues. The BMs have highly specialized mechanical and biological functions. They provide physical support for tissues and an anatomical barrier for cells of different origins. The biological role of basement membranes is diverse. They are involved in development, cell attachment and migration, tissue regeneration and repair and maintenance of cell polarization. They also act as reservoirs of growth factors, enzymes and plasma proteins. In kidney glomeruli, the basement membrane has an additional important role, in that the GBM serves as a molecular sieve for the selective removal of small molecules from blood.

The thickness of basement membranes can vary between 50-350 nm, being usually 60-80 nm. By electron microscopy, two morphologically distinct layers, the *lamina lucida (rara)* and *lamina densa*, can be observed. Some basement membranes, such as the GBM, appear to have three layers: a subendothelial electron–translucent layer, named *lamina rara interna,* an electron-dense central layer, *lamina densa,* and a subepithelial electron-translucent layer, *lamina rara externa* (Fig 3). This three-layered structure may be a result of synthesis of the laminae individually by glomerular epithelial and endothelial cells, following the fusion of these layers at an early embryonic stage (Kasinath & Kanwar 1993). However, this structure may also be an artifact of the routine fixing techniques, as studies of tissue samples made by the quick-freeze method have shown the basement membranes not to contain any distinct layers (Goldberg & Escaig-Haye 1986).

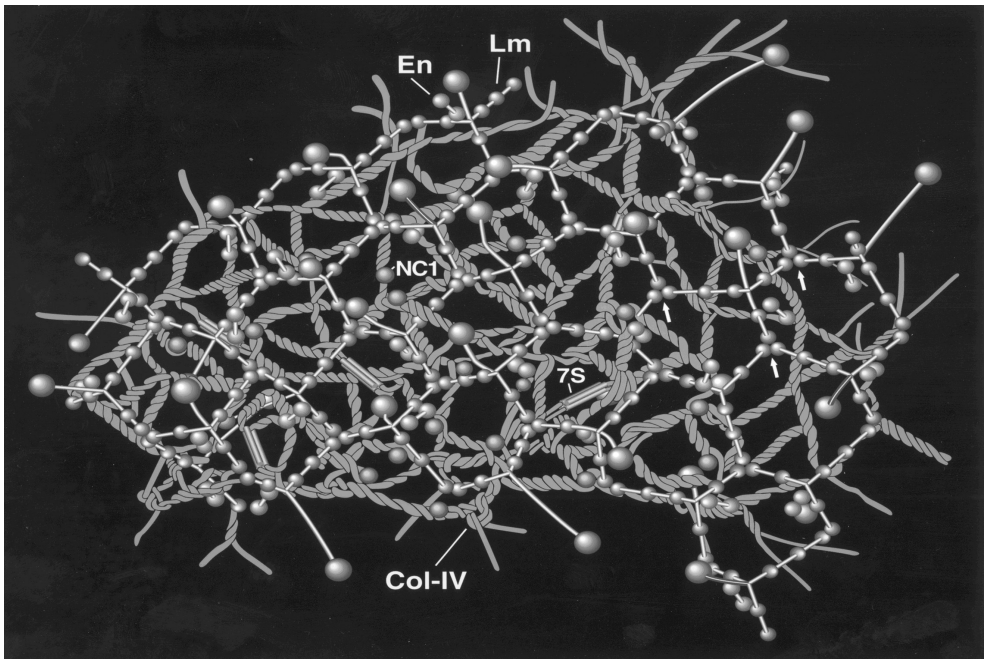

Fig 2. Hypothetical architecture of the basement membrane. Type IV collagen (Col-IV) and laminin (Lm) form double and interwoven polymer networks. The collagen molecules bind to each other via N-terminal tetrameric (7S), C-terminal globular dimeric (NC1), and lateral bonds. The laminin scaffold contains end-to-end interactions of short arms (white arrows) and apparently long arms as well. Entactin/nidogen (En) functions as a bridge between collagen and laminin networks. Proteoglycans are furthermore connected to the network in a yet unknown manner. Courtesy, Dr. Peter Yurchenco, Department of Pathology, Robert-Wood-Johnson Medical School, Piscataway, New Jersey.

2.2.1 Basement membrane components

In addition to type IV collagen, laminin is present in basement membranes as a major noncollagenous protein. Laminin, a glycoprotein, is a structurally important component with many biological functions, including cell attachment, proliferation and differentiation. Laminin was initially isolated and characterized from a murine Engelbreth-Holm-Swarm (EHS) tumor forming basal lamina matrix (Timpl *et al*. 1979). The laminin molecule is a heterotrimer having a characteristic cross-shaped structure with three short arms and one long arm. This structure is formed by the assembly of three chains, an α chain, which is 300-400 kD in size, and a β and a γ chain, both 180-200 kD in size. Each chain forms one short arm, and all chains together the long arm linked by disulfide bonds. All three short arms have a central and terminal globular domain separated by rod-like segments. The long arm of laminin is a flexible rod with a large terminal globule composed of five subdomains. Like type IV collagen, laminin molecules interact with each other via their terminal globular domains forming dimers, trimers and oligomers (Timpl 1989). In 1994, a new nomenclature for the laminins was suggested because of the continuous discovery of new isoforms and the complexity in old designations (Burgeson *et al*.). To date, several different laminin chains and isoforms with variable tissue distributions and biological functions have been characterized (Tryggvason 1993, Miner 1998).

Proteoglycans consist of different types of core proteins with covalently attached glycosaminoglycan side chains. They are found in all extracellular matrices, but those found in basement membranes differ from many others. Polyanionic heparan sulfate proteoglycans (HSPGs) are thought to have an essential role in glomerular filtration by creating a charge-selective filtration barrier to the GBM (Kanwar *et al*. 1980, Kanwar 1984). The best-studied proteoglycan is perlecan. The human perlecan core protein is one of the largest polypeptides in the human body, with a core protein of 467 kDa in size (Kallunki & Tryggvason 1992). Chondroitin sulfate and hyaluronic acid have also been found in basement membranes. Bamacan is a chondroitin sulfate proteoglycan with a core protein of 138 kDa (Wu & Couchman 1997). It has been found in the basement membranes of Bowman's capsule and within the mesangial matrix, but not in the capillary GBM (McCarthy *et al*. 1994). Recently, a novel proteoglycan, also bearing chondroitin sulfate glycosaminoglycans, was characterized and termed leprecan. It has a core protein of about 100 kDa (Wassenhove-McCarthy & McCarthy 1999). Leprecan is present in the GBM and mesangial matrix.

Nidogen (Timpl *et al*. 1983) /entactin (Carlin *et al*. 1981) is an abundant glycoprotein found in all BMs. It is a single 148-kD polypeptide with many carbohydrate moieties (Paulsson *et al*. 1986). Electron microscopy has revealed nidogen to be a dumbbell-shaped protein with a large and a small globular domain connected by a rod-like segment. As mentioned earlier, due to the multiple binding activities, nidogen is an important link in the structural organization of various BM components (Aumailley & Gayraud 1998).

Other minor components include BM40 (Dziadek *et al*. 1986)/osteonectin (Termine *et al*. 1981)/SPARC (soluble protein, acidic and rich in cysteine; Sage *et al*. 1984), agrin and fibulins, which are present in some specialized types of basement membranes (Aumailley & Gayraud 1998, Yurchenko & O'Rear 1993).

2.2.2 Glomerular filtration barrier

The molecular structure of the glomerular basement membrane is very much the same as that of other basement membranes, with type IV collagen being its major component. Mutations in three type IV collagen genes, COL4A3, COL4A4 and COL4A5, are causative for the formation of ultrastructurally abnormal GBM, which is the main molecular sieve in the filtration barrier. These structural alterations and the consequent malfunction of the glomerular filtration barrier cause the major complications of Alport syndrome, hematuria and proteinuria.

Primary urine is formed by ultrafiltration of plasma in the kidney glomeruli (Fig 3). The glomerular capillary is divided into several loops (glomerular tuft). The inner part of the capillary faces the mesangium, while the outer part forms the filter. The filtration barrier is composed of three layers: the fenestrated endothelium, the glomerular basement membrane, and the tight podocyte layer of interdigitating epithelial cells, named foot processes. The adjacent processes are separated by 20-30 nm wide slit pores, or filtration slits, which contain thin diaphragms (Rodewald & Karnovsky 1974). Under normal conditions, proteins with the size of albumin (67 kDa) or larger do not penetrate the filtration barrier. In contrast, there is an extremely high permeability to water and small solutes. Filtration across the barrier depends on the size of the molecules; *i.e.* it is a size-dependent permeability barrier (Caulfield & Farquhar 1978). Additionally, the filtration barrier shows selectivity according to the electrical charge of the molecules, in that, cationic and neutral molecules traverse the membrane more easily than anionic molecules of the same size (Brenner *et al*. 1978). As mentioned earlier, HSPGs are thought to be important in this action. A loss of charge as well as size selectivity in various disease stages has been found in association with proteinuria and loss of HSPGs (Rohrbach & Murrah 1993). Very recently, Ruotsalainen *et al*. (1999) showed that the novel protein nephrin is highly specific for the slit diaphragm between the glomerular foot processes of podocytes. Additionally, the NPHS1 gene encoding the nephrin is mutated in the congenital nephrotic syndrome (Kestilä *et al*. 1998). This indicates that nephrin also has a direct role in filtration.

2.3 Tissue distribution of the type IV collagen α chains

The six α(IV) chains differ considerably with respect to tissue distribution. Immunohistological studies with human tissues have shown that the α1(IV) and α2(IV) chains are ubiquitously present in all BMs, whereas the α3(IV)-α6(IV) chains have a more restricted tissue distribution (Hudson *et al*. 1993). In the kidney, the α3, α4 and α5 chains of type IV collagen are the major chains in the glomerular basement membrane. However, they are found only in a subset of tubular BMs, *i.e*., they are expressed in Bowman's capsule and distal tubules, and the α5(IV) chain is furthermore present in collecting ducts (Zhou & Reeders 1996). The α6(IV) chain expression is almost identical to that of α5(IV)

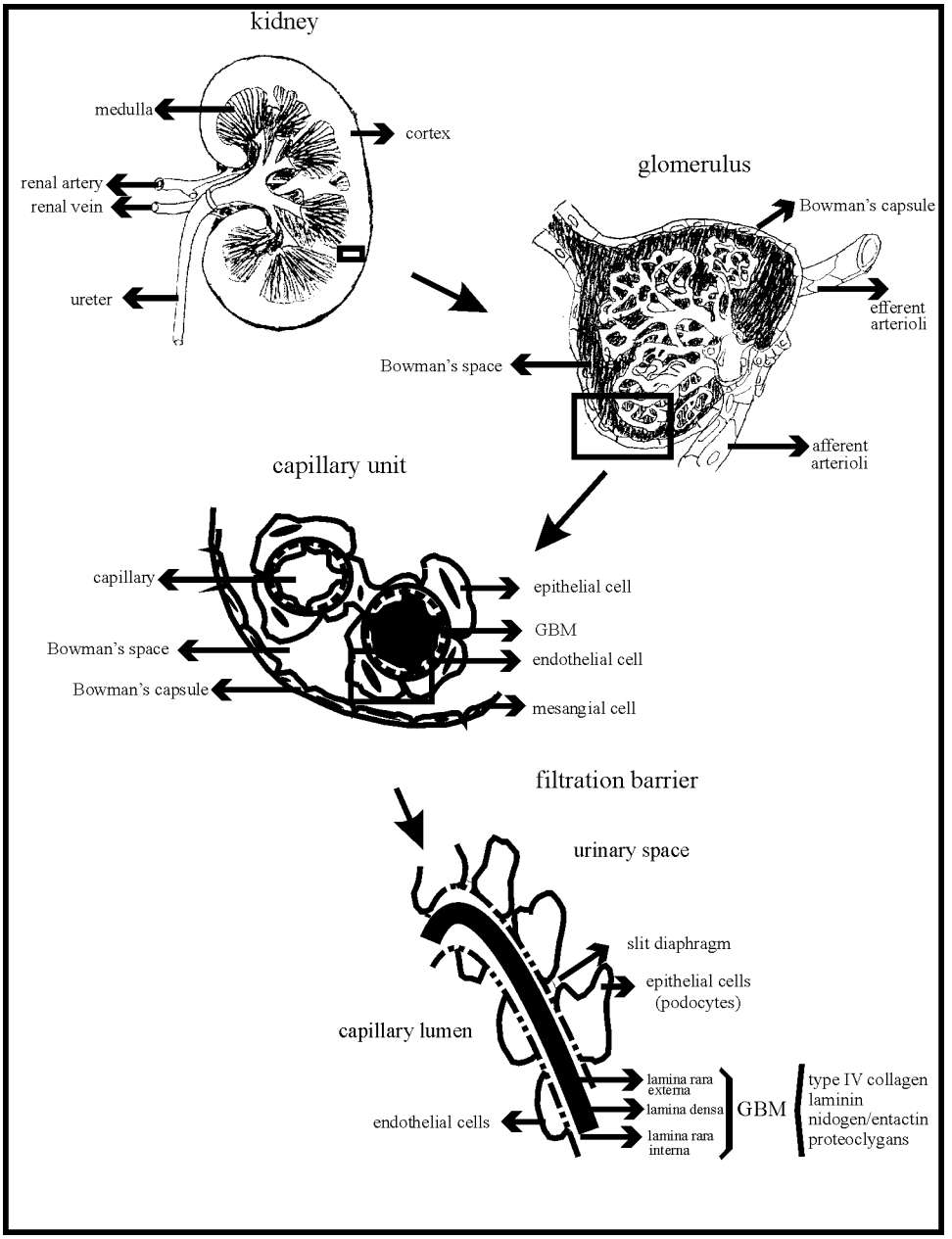

Fig 3. Schematic illustration of the units participating in kidney filtration.

throughout the kidney, but it differs in that it is totally absent from the GBM (Peissel *et al*. 1995). In the adult glomerulus, the α1(IV) and α2(IV) chains are present in the mesangium, but no expression of chains α3(IV) to α6(IV) can be seen (Ninomiya *et al*. 1995, Kashtan *et al*. 1996). There is a developmental switch in expression of type IV collagen α1:α2 chains and α3:α4:α5 chains in the GBM (Miner 1998), which will be discussed in more detail in section 2.5.5.1.

Tissue distribution of different α(IV) chains in Alport syndrome patients is described in section 2.5.5. (see Table 3). The basement membranes that are affected in Alport syndrome normally express the α3(IV), α4(IV) and α5(IV) chains as their major chains. In addition to the GBM, these chains are also present in the lens capsule, internal limiting membrane of the retina, Bruch's membrane and Descemet's membrane in the eye, and several basement membranes of the cochlea (Kashtan & Michael 1996). The type IV collagen chains are also known to be present in extrarenal non-Alport target tissues; the α3(IV) and α4(IV) chains are expressed in the lung, brain and synaptic fibers of muscle, and the α5(IV) and α6(IV) chains in the lung, esophagus and skin (Zhou & Reeders 1996).

2.4 Type IV collagen genes

The six mammalian type IV collagen genes share a unique feature in that they are located pairwise in a head-to-head fashion on three different chromosomes. The COL4A1/COL4A2 gene pair encoding the α1(IV) and α2(IV) chains, respectively, is located on chromosome 13 (Boyd *et al*. 1988). The α3 and α4 chains of type IV collagen are encoded by the gene pair COL4A3/COL4A4, respectively, and these genes have been co-localized to chromosome 2 (Mariyama *et al*. 1992). Again, the gene pair COL4A5/COL4A6, respectively coding for the chains α5(IV) and α6(IV), has been mapped to chromosome X (Zhou *et al*. 1993). The 5' ends of each gene pair are adjacent to each other, separated by sequences, which contain motifs involved in the regulation of transcriptional activity (Kashtan & Michael 1996).

The 5' ends of the COL4A1 and COL4A2 genes overlap each other and the region between transcription start sites is only about 130 bp. The genes are transcribed from opposite DNA strands and they share a common bi-directional promoter (Soininen *et al*. 1988). The arrangement of the COL4A5 and COL4A6 genes is similar, but the distance between transcription start sites is longer, 442 base pairs. In addition, the COL4A6 gene has two alternative promoters, indicating usage of different regulatory elements for the expression of the gene, and resulting in two distinct transcripts in a tissue-specific manner (Sugimoto *et al*. 1994). Studies of the murine genes have shown the bi-directional operation model for the promoter elements of the α3(IV) and α4(IV) genes (Hlaing *et al*. 1994). Recently, Momota and coworkers (1998) showed that the human COL4A3 and COL4A4 genes are also transcribed in opposite directions and share a common promoter region. Furthermore, the COL4A4 gene has two alternative transcripts, the expression of which appears to be tissue-specific. One transcription start site of COL4A4 is only 5 bp

away from the transcription start site of COL4A3 and the other starts 373 nucleotides downstream from the first. Two kinds of transcripts are generated that differ in the 5' untranslated region, thus resulting in the same deduced amino acid sequences.

All the genes encoding the human type IV collagen α chains are very large because of the large sizes of introns. It has been estimated by macrorestriction mapping that the sizes of the α1(IV) and α2(IV) genes are 100-160 kb each (Cutting *et al*. 1988). The α5(IV) gene is 230-250 kb, and the α6(IV) gene has been estimated to be at least 425 kb (Srivastava *et al*. 1995). There is, however, a discrepancy in the reported sizes of the second large intron of COL4A6. According to Zhang *et al*. (1996), the size of the intron 2 should be as large as 340 kb based on a published YAC contig (Srivastava *et al*. 1995). In contrast, a detailed restriction map (Heidet *et al*. 1995) as well as a complete YAC contig map (Kendall *et al*. 1997) has limited the size of this intron to between 65 and 180 kb, thus leading to a variation in the size of the whole COL4A6 gene (Table 2). The combined size of the COL4A3/COL4A4 gene pair has been estimated to be about 500 kb (Mariyama *et al*. 1992), and recently, Boye *et al*. (1998) estimated the size of the COL4A4 gene to be at least 113 kb. The α1(IV) gene contains 52 exons and the sizes of exons vary from 27 to 213 base pairs (Soininen *et al*. 1989). The number of exons of the COL4A2 gene is 47, the smallest exon being 36 bp and the largest one 287 bp (Heikkilä & Soininen 1996). Previously, the exons encoding the most 3' end of the COL4A3 and COL4A4 genes had been characterized (Quinones *et al.* 1992, Sugimoto *et al*. 1993), but Boye *et al*. (1998) showed the COL4A4 gene to have 48 exons, of which the smallest is 9 bp and the largest 287 bp. The type IV collagen α5 chain gene has been shown to contain 51 exons and they are almost of the same size as those in the α1(IV) gene, the smallest one is 27 bp and the largest one 213 bp (Zhou *et al*. 1994b). However, the size of the exon(s) coding for the extra 18 bp sequence found in kidney mRNA (Guo *et al*. 1993) has not been reported. The COL4A6 gene contains 46 exons including exon 1', which is alternatively transcribed with exon 1 from alternative promoters. The sizes of exons vary from 36 to 222 bp (Oohashi *et al*. 1995).

Table 2. Properties of the six type IV collagen genes.

	COL4A1	COL4A2	COL4A3	COL4A4	COL4A5	COL4A6
Chromosome locus	13q34	13q34	2q35	2q35	Xq22	Xq22
Size of the gene (kb)	< 100	< 100	≈ 250	> 113	250	425 (?)
Number of exons	52	47	?	48	51[1]	46
Size of the shortest exon (bp)	27	36	?	9	27	36
Size of the longest exon (bp)	213	287	?	287	213	222

[1]This gene may contain an additional exon(s) encoding an alternatively spliced 18 bp sequence (Guo *et al*. 1993).
References: Boyd *et al*. 1988, Boye *et al*. 1998, Cutting *et al*. 1988, Heidet *et al*. 1995, Heikkilä & Soininen 1996, Kendall *et al*. 1997, Mariyama *et al*. 1992, Oohashi *et al*. 1995, Soininen *et al*. 1989, Srivastava *et al*. 1995, Zhou *et al*. 1993, 1994b.

2.5 Role of type IV collagen in Alport syndrome

The most dramatic symptom of Alport syndrome is a dysfunction of the kidneys. The filtration of plasma is impaired and macromolecules, larger than albumin, leak into urine. Furthermore, red blood cells can escape into the urine causing hematuria. The kidney filtration occurs in the filtration units, the glomeruli, which in man amount to about 0,8-1,2 million per kidney. The glomerular basement membrane and the slit diaphragm located between the podocyte foot processes are the main molecular sieves responsible for normal plasma filtration. Mutations in the type IV collagen are responsible for abnormalities in the structural framework of the GBM.

2.5.1 Alport syndrome

Alport syndrome, also termed hereditary nephritis, was the first basement membrane disease whose pathogenesis was clarified at the gene level (Hostikka *et al*. 1990, Barker *et al*. 1990). Professor Arthur Cecil Alport initially described the disease in 1927 as an inherited kidney disease characterized by hematuria and sensorineural deafness. Later, association of eye abnormalities was noted. Irregularities and disruptions in the GBM,

seen by electron microscopy, are typical for this disorder as well (Atkin *et al*. 1988a). The disease is progressive, usually leading to renal failure during adolescence or before middle age. Based on the age of onset of end-stage renal disease (ESRD), Alport syndrome is divided into two clinical subgroups. In the juvenile type, the mean age of ESRD in men is about 18 years, while in the adult type it is about 37. A good dividing line between the two types is thirty-one years of age (Gregory *et al*. 1996).

The most common form of Alport syndrome is inherited as an X-linked dominant trait, but lately both autosomal recessive and autosomal dominant forms have been described (Knebelmann *et al*. 1993, Mochizuki *et al*. 1994, Jefferson *et al*. 1997). The gene defective in the X-linked form was localized by several investigators to the long arm of the X chromosome during 1988 and 1989 (Atkin *et al*. 1988b, Brunner *et al*. 1988, Flinter *et al*. 1989). In 1990, the COL4A5 gene encoding the type IV collagen α5 chain was discovered and localized on chromosome X to the Alport gene region (Hostikka *et al*.). This was soon followed by the identification of mutations in the COL4A5 gene in AS patients (Barker *et al*. 1990, Zhou *et al*. 1991). More recently, mutations have also been reported in the genes for the α3(IV) and α4(IV) chains in the rarer autosomal forms of Alport syndrome (Mochizuki *et al*. 1994, Lemmink *et al*. 1994, Ding *et al*. 1995, Boye *et al*. 1998). Presently, about 300 different mutations have been identified in type IV collagen genes in Alport patients, almost all of them in the COL4A5 gene (Knebelmann *et al*. 1996, Netzer *et al*. 1996, Lemmink *et al*. 1997, Plant *et al*. 1999).

2.5.2 Phenotypic classification and criteria for the diagnosis of AS

Phenotypic expression of Alport syndrome is complex, and Atkin *et al.* (1988a) have divided AS into six classes according to the mode of inheritance, age of onset of ESRD and occurrence of nonrenal abnormalities. This classification has been widely adopted and is clinically sufficient if the following aspects are considered: (a) the mode of inheritance is heterogeneous, but in most cases X-linked; (b) in the X-linked types males are more severely affected than females; (c) the age of onset of both renal failure and hearing defects varies a lot; (d) there may be defects of other organs, and (e) a generally similar phenotype can be expected in each member within a family.

Due to the heterogeneous nature of the disease, it has been difficult for physicians to draw the lines in defining Alport syndrome. In 1987, Flinter and co-workers defined four criteria, *i.e.* (1) familial history, (2) hearing loss, (3) ocular lesions, and (4) GBM abnormalities, of which three have to be fulfilled by a patient with hematuria or/and chronic renal failure for the diagnosis of Alport syndrome. However, there are numerous patients with progressive renal disease and mutations in type IV collagen genes who do not fulfill these criteria. Again, inheritance cannot always be demonstrated, as *de novo* mutations may account for as many as 15 % of cases (Lemmink *et al*. 1997). Also, there is quite a large proportion of patients with renal failure and mutations in type IV collagen genes who do not have hearing loss or eye manifestations. Because of new knowledge of the complexity of the clinical definition of Alport syndrome, the above described criteria

were expanded by Gregory *et al*. (1996). They have listed ten criteria which must be satisfied differentially depending on the each case. These criteria are:

1) family history of nephritis or unexplained hematuria in a relative of first-degree or in a male relative linked through any number of females;
2) persistent hematuria without evidence of another possibly inherited nephropathy (*i.e*. polycystic kidney disease, IgA nephropathy or thin GBM disease);
3) bilateral sensorineural hearing loss in the range of 2,000-8,000 Hz, the hearing loss develops gradually and is not present in early infancy but commonly at the age of thirty at latest;
4) a mutation in the gene encoding the α3, α4 or α5 chain of type IV collagen;
5) immunohistochemical evidence of complete or partial lack of the Alport epitope in the GBM, epidermal BM or both;
6) widespread ultrastructural abnormalities of GBM;
7) ocular abnormalities (anterior lenticonus, posterior subcapsular cataract, posterior polymorphous dystrophy and retinal flecks);
8) gradual progression to ESRD in the index case or at least in two family members;
9) macrothrombocytopenia or granulocytic inclusions, and
10) diffuse leiomyomatosis of esophagus, female genitalia or both.

To diagnose Alport syndrome in a family for clinical or research purposes, at least four of these criteria must be fulfilled in closely related family members. In the case of an individual, AS must be present in the family and the patient has to be on the line of descent for the postulated mode of transmission. For a diagnosis of probable AS, one of the criteria 2 to 10 must be satisfied and two of the criteria for a diagnosis of definite AS. Without a positive family history, at least four of the criteria must be present to establish the diagnosis of AS in an individual. Some criteria are of greater value than others, but the definition satisfactorily discriminates the currently known forms of Alport syndrome. However, any child who presents visible hematuria under the age of six, regardless of the family history, should have a renal sonogram to exclude the possibility of Wilm's tumor. (Gregory *et al*. 1996).

2.5.3 Different forms of Alport syndrome

About 85% of the Alport syndrome cases are X-linked dominant, affecting 1:5000 males in the Intermountain West of the United States (Hastedt & Atkin 1983). The gene frequency in Rhode Island was estimated to be 1:10 000 (Shaw & Kallen 1976) and in Finland it is even lower, about 1:53 000 (Pajari *et al*. 1996). In the X-linked form, males are severely affected and the disease progresses to end-stage renal failure during adolescence or before middle age. About 70% of the patients have sensorineural hearing loss affecting primarily high tones. Eye abnormalities, such as anterior lenticonus, premature cataracts or perimacular retinal flecks are observed in about 25% of patients.

Autosomal recessive disease should be suspected if an individual is symptomatic, without positive family history of the disease, especially in cases where females are as severely affected as males (Kashtan & Michael 1996). In the rare autosomal dominant form of the disease, males and females are equally severely affected. However, individuals show a milder phenotype than is usually found in the X-linked form, *i.e.*, an older age of onset of ESRD, minimal deafness, and an absence of eye signs (Jefferson *et al*. 1997).

For an unknown reason, a minority of Alport patients develops antibodies against renal allografts, and about one third of those patients reject the transplant. This anti-GBM nephritis is a rare but dramatic manifestation of Alport syndrome. The onset of anti-GBM nephritis usually occurs within the first year following transplantation, with eventual loss of the allograft. Unfortunately, anti-GBM nephritis recurs in most patients after retransplantation (Kashtan *et al*. 1996).

2.5.4 Alport syndrome associated disorders

Association of Alport syndrome with diffuse leiomyomatosis (DL-AS) was first described by Garcia-Torres and Guarner in 1983. DL is a rare disorder characterized by benign proliferation of smooth muscle cells in the esophageal wall (Heald *et al*. 1986, Antignac & Heidet 1996). Both sporadic and hereditary cases have been described (Bourque *et al*. 1989, Marshall *et al*. 1990, Antignac & Heidet 1996). In addition to the esophagus, other organs such as the female genital tract and tracheobronchial tree may be affected.

In some cases, Alport syndrome has been shown to be associated with different hematological abnormalities, such as macrothrombocytopenia and granulocyte inclusions (Epstein *et al*. 1972, Brivet *et al*. 1981, Peterson *et al*. 1985). Macrothrombocytopenia is characterized by the presence of abnormally few, but exceptionally large blood platelets. In many published cases, it is a possibility that AS and the hematologic disease are transmitted independently within the same kindred (Gregory *et al*. 1996). Nevertheless, the association has been noted frequently enough to assume that there might be an autosomal dominant form of AS associated with macrothrombocytopenia and, in some families, also granulocyte inclusions.

Recently, Jonsson *et al*. (1998) described a family with X-linked AS with additional features including mental retardation, dysmorphic facies with marked midface hypoplasia, and elliptocytosis. The disorder was later named AMME contiguous gene deletion syndrome (Vitelli *et al*. 1999). AS has also previously been described in conjunction with mental retardation and macrocephaly in males (Robson *et al*. 1994).

2.5.5 Immunohistology of basement membranes

Immunohistochemical studies have shown that in most cases the type IV collagen α3, α4 and α5 chains are absent in the GBM of male patients with X-linked Alport syndrome (Gubler *et al*. 1998), whereas expression of the α1(IV) and α2(IV) chains is increased (Peissel *et al*. 1995, Naito *et al*. 1996). Expression of the α6(IV) chain is normally seen in Bowman's capsules and tubules, but not in these patients. This is also the case with the α3(IV), α4(IV) and α5(IV) chains (Ninomiya *et al*. 1995) (Table 3). Women who are heterozygous for X-linked AS exhibit segmental expression of the α3(IV), α4(IV) and α5(IV) chains in the GBM while expression of the α1(IV) and α2(IV) chains is preserved (Nakanishi *et al*. 1994). Immunohistochemical analyses of skin tissues from males with X-linked Alport syndrome have shown a complete absence of the α5(IV) and α6(IV) chain expression. A discontinuous or mosaic staining of these two chains is seen in the epidermal basement membrane (EBM) of heterozygous females (Ninomiya *et al*. 1995, Hino *et al*. 1996). Cheong *et al*. (1994) have studied the expression of type IV collagen α chains in another Alport target tissue, anterior lens capsule, in X-linked cases and demonstrated the same feature as with the GBM, *ie*. the lack of α3(IV) to α5(IV) chains.

The distribution of α(IV) chains in autosomal recessive AS cases has been studied by Gubler *et al*. (1995). Usually, no expression of the α3(IV), α4(IV) and α5(IV) chains can be seen in the GBM, but deviating from the X-linked expression pattern, the α5(IV) chain is normal in Bowman's capsules and distal tubular basement membrane (Table 3). This similar expression pattern can be seen in both sexes. In addition, the α5(IV) chain shows normal staining in the EBM while the α3(IV), α4(IV) and α5(IV) chains are absent in the GBM. This combination cannot be seen in X-linked AS, as it is well known that only the α5(IV) chain of these three is present in the EBM (Gubler *et al*. 1995).

The possibility to use the above-mentioned differential pattern of staining as an instrument for distinguishing between X-linked and autosomal recessive forms or even diagnosis of Alport syndrome, has been demonstrated by Kashtan (1999). If immunohistochemical analysis of α5(IV) expression from skin biopsy shows absence of this expression in a male, or a clearly mosaic expression in a female, diagnosis is X-linked Alport syndrome caused by a COL4A5 mutation. In contrast, normal expression of α5(IV) in the skin of a patient suspected of having Alport syndrome gives several explanations. The patient may still have a COL4A5 mutation that allows skin expression of α5(IV) (van der Loop *et al*. 1999), or the patient has autosomal recessive Alport syndrome (Gubler *et al*. 1995). Yet another possibility is that the patient does not have Alport syndrome. Thus, if skin expression of α5(IV) was normal, kidney biopsy or molecular genetic analysis would be the next step.

Table 3. Distribution of the six type IV collagen α chains in normal tissues and in tissues of Alport patients.

Tissue	α1(IV)	α2(IV)	α3(IV)	α4(IV)	α5(IV)	α6(IV)
Normal distribution						
Kidney						
GBM	+	+	+	+	+	-
Bowman's capsules	+	+	(+)	(+)	+	+
Distal tubules	+	+	(+)	(+)	+	+
Collecting ducts	+	+	-	-	+	+
Mesangium	+	+	-	-	-	-
Eye						
Anterior lens capsule	+	+	+	+	+	?
Internal limiting membrane	+	+	+	+	+	?
Bruch's membrane	+	+	+	+	+	?
Descemet's membrane	+	+	+	+	+	?
Cochlea	+	+	+	+	+	?
Skin	+	+	-	-	+	+
Esophagus	+	+	-	-	+	+
Brain	+	+	+	+	-	-
Lung	+	+	+	+	+	+
Muscle						
Synaptic fiber	(+)	(+)	+	+	?	?
Extrasynaptic fiber	+	+	-	-	?	?
Distribution in X-linked AS (mutations in the α5 chain gene)						
Kidney						
GBM	+	+	-	-	-	-
Bowman's capsules	+	+	-	-	-	-
Distal tubules	+	+	-	-	-	-
Eye						
Anterior lens capsule	+	+	-	-	-	?
Skin	+	+	-	-	-	-
Distribution in autosomal recessive AS (mutations in the α3 and α4 chain genes)						
Kidney						
GBM	+	+	-	-	-	-
Bowman's capsules	+	+	-	-	+	?
Distal tubules	+	+	-	-	+	?
Skin	+	+	-	-	+	?

References: Cheong *et al*. 1994, Gubler *et al*. 1995, Hino *et al*. 1996, Kashtan *et al*. 1996, Kashtan & Michael 1996, Naito *et al*. 1996, Nakanishi *et al*. 1994, Ninomiya *et al*. 1995, Peissel *et al*. 1995, Zhou & Reeders 1996

2.5.5.1 Pathogenesis of Alport syndrome

The fairly late onset of ESRD in Alport syndrome can be explained by a developmental switch between the type IV collagen α1:α2 and α3:α4:α5 networks observed in the GBM. Kleppel and Michael (1990) first provided preliminary evidence for such an event in their studies of fetal human kidneys. Later, Miner and Sanes (1994) described this switch in rodents. Immunohistochemical studies of rat kidneys showed that in the earliest stages of glomerular development, α1(IV) and α2(IV) are present. At the capillary loop stage, α3(IV)-α5(IV) chains are coordinated and colocalized with α1(IV) and α2(IV) chains. When the glomeruli are mature, α1(IV) and α2(IV) chains become concentrated in the mesangium, while α3(IV)-α5(IV) chains continue to accumulate in the GBM. A similar switch can also be seen between some laminin chains during the development of a nephron. The β1 and γ1 chains are present in all basement membranes, but at the capillary loop stage, the accumulation of the β2 chain begins in the developing GBM, and as it matures, the expression of the β1 chain gradually diminishes (Miner 1998).

As discussed earlier, several investigators have reported, that the GBM of Alport patients does not contain type IV collagen α3, α4 and α5 chains. Leinonen *et al*. (1994) first speculated that the lack of the cysteine rich α4 chains may lead to a weakened structure of the GBM. Miner and Sanes (1996) showed that molecular and functional defects in kidneys of mice lacking the α3(IV) chain were very similar to defects in humans with Alport syndrome. They speculated how a mutation in the gene for α3(IV) chain leads to renal disease. The proposal was that the absence of full-length α3(IV) in the GBM prevents the assembly of α4(IV) and α5(IV) chains into a collagen network and results in the degradation of these chains. Again, the network of α1, α2 appearing during the glomerular development remains in the GBM, thus compensating for the lack of α3, α4, and α5 chains. Similarly, Thorner *et al*. (1996) studied the influence of a COL4A5 mutation on the expression of other α(IV) chains, both at the mRNA and protein level, using a naturally occurring canine model for X-linked Alport syndrome (Jansen *et al*. 1986, Zheng *et al*. 1994). They observed that mRNA levels for the α3, α4 and α5 chains were significantly reduced in affected dog kidneys, suggesting coordinated expression of these chains. An immunohistochemical study of kidneys also showed a comparable situation as with humans, a lack of α3, α4 and α5 chains in the GBM. Speculations were addressed, whether the mechanisms of coordinate function include factors acting at the protein assembly and/or at the transcriptional/translational level. Thus, formation of the triple helix or supramolecular assembly of the collagen network might be impaired. Alternatively, there might be some activating or inhibiting elements at the gene or mRNA levels whose functions are hindered because of a mutation in the α5(IV) chain gene. The possible existence of α3, α4 and α5 chains in the same triple helical type IV collagen molecule, or the assembly of these three chains into a supramolecular network, still remaines an open question.

The developmental switch from fetal α1, α2 chains to α3, α4, α5 chains, especially in the kidney, has been studied and discussed by Kalluri *et al*. (1997). They analyzed kidneys from X-linked Alport patients as well as embryonic kidney tissues. It was hypothesized that the developmental switch must be fundamentally important to the long-term stability of the GBM. As a molecular sieve, the GBM is vulnerable to proteolytic degradation, and

the cysteine-rich α3, α4 and α5 chains may have evolved to enhance its resistance to such degradation. Renal basement membranes of X-linked Alport kidneys were shown to be degraded by secreted glomerular cell proteases (collagenase, elastase and cathepsins) more readily than BMs from normal kidneys.

In 1998, Gunwar and co-workers identified a novel disulfide-cross-linked network of α3(IV), α4(IV) and α5(IV) chains. This finding explains that the developmental switch of α1 and α2 chains to α3, α4 and α5 chains happens at the supramolecular level, in which the fetal α1:α2 network is replaced by the adult α3:α4:α5 network. Again, this switch is somehow arrested in Alport syndrome patients, as demonstrated by Harvey *et al*. (1998). Their studies of glomerular development in the X-linked canine model indicated that the α1:α2 network is essential for normal development, whereas the α3:α4:α5 network is essential for long-term stability of the GBM and maintenance of glomerular function. Thus, progression to ESRD, at least in X-linked AS, results from a congenital malformation of the GBM, which is not alone sufficient to cause disease. Instead, the GBM composed of the ”fetal” collagen network is more prone to postnatal, deteriorating processes, which in long term lead to kidney dysfunction.

Studies with the AS animal models, both with the naturally occurring ones (Hood *et al*. 1995, Lees *et al*. 1998), and especially with the X-linked canine model (Harvey *et al*. 1998), as well as with the generated COL4A3 knockout mice (Miner & Sanes 1996, Cosgrove *et al*. 1996, 1998) will help us to better understand the specific mechanisms and regulation of pathological events in Alport syndrome. Additionally, the X-linked canine model provides the opportunity to test, in practice (Harvey *et al*. 1998), possibilities to use gene therapy (Heikkilä *et al*. 1996, Tryggvason *et al*. 1997) to treat Alport syndrome.

2.5.6 Mutations in Alport syndrome

Based on molecular genetic findings, several forms of Alport syndrome are now recognized. An X-linked dominant form is caused by defects in the COL4A5 gene (Barker *et al*. 1990, Zhou *et al*. 1991), while mutations in the COL4A3 and COL4A4 genes cause autosomal recessive or dominant forms of the disease (Mochizuki *et al*. 1994). Furthermore, deletions in the 5' end of the COL4A6 gene extending into the adjacent COL4A5 gene have been recognized to cause Alport syndrome and diffuse leiomyomatosis (DL-AS) (Antignac *et al*. 1992). Even larger deletions, extending far beyond the COL4A5 gene, are involved in a novel AMME syndrome (Piccini *et al*. 1998) which is characterized by Alport syndrome (A), mental retardation (M), midface hypoplasia (M) and elliptocytosis (E). Most patients with Alport syndrome have the X-linked dominant form and almost 300 mutations in the COL4A5 gene have been reported. Autosomal recessive inheritance has been estimated to account for up to 15% of the families, but only a few mutations have been detected so far (Lemmink *et al*. 1997, Boye *et al*. 1998). Large deletions causing DL-AS have been described in about 4% of the AS cases (Lemmink *et al*. 1997, Plant *et al*. 1999), and AMME syndrome has been recognized only in one family (Jonsson *et al*. 1998).

2.5.6.1 Glycine substitutions in collagens

It is generally assumed for all collagens that glycine substitutions affect the stability of a triple helical molecule. This is because glycine is the only amino acid small enough to fit into the center of the triple helix and an uninterrupted Gly-X-Y repeat sequence is essential for the maintenance of the triple helical conformation. The result of a glycine substitution is destabilization of the helix and formation of kinks in the molecule, which may not be tolerated. Although this has not been demonstrated at the protein level for type IV collagen, a number of similar glycine mutations, either inhibiting helix formation or causing kinks in the molecule, have been described in type I collagen in osteogenesis imperfecta (Kuivaniemi *et al*. 1997). However, the region where glycine is substituted, and to what amino acid, may have some importance, since a rather surprising observation was made by Boye *et al*. (1998). They detected mutations in the COL4A4 gene in patients with autosomal recessive AS and found a glycine (545) to alanine substitution in a patient with two nonsense mutations, as well as in a large number of control individuals, which excludes the possibility of pathogeneity of this mutation. Moreover, this substitution was observed in a homozygous state in one control individual. Alanine is the most similar amino acid to glycine, and its substitution could be suspected to be the least harmful. This could explain why glycine to alanine substitutions have not been observed in severe forms of osteogenesis imperfecta (Byers *et al*. 1991). And in X-linked AS, only 5 % (5 out of 91) of glycine substitutions are glycine to alanine substitutions. Results from two *in vitro* studies, where COL7A1 gene defects were analyzed, are also interesting (Bruckner-Tuderman *et al*. 1999). Three glycine substitutions close to the hinge region of the collagen VII molecule interfered with protein folding and caused intracellular accumulation of the mutant molecules, whereas two glycine substitutions within a long uninterrupted Gly-X-Y repeat, rather close to the NC1 domain, remained biologically and clinically silent.

2.5.6.2 X-linked dominant Alport syndrome

Since the first mutations were described in the type IV collagen α5 chain gene in X-linked Alport syndrome (Barker *et al*. 1990), several mutation reports have been published by many research groups. Five extensive studies have been carried out using the SSCP analysis of PCR products (Kawai *et al*. 1996, Knebelmann *et al*. 1996, Renieri *et al*. 1996, Plant *et al*. 1999, Cheong *et al*. 2000), and one using RT-PCR and direct sequencing, (Inoue *et al*. 1999) to search for mutations in the COL4A5 gene. The mutations include large and small gene rearrangements such as deletions, insertions and duplications as well as single base changes. About 85 % of the mutations, however, are small in size. Approximately 45% of the mutations convert a glycine residue in the Gly-X-Y repeat to another amino acid. Furthermore, complete loss of the gene has been reported in two cases (Netzer *et al*. 1992, Antignac *et al*. 1994, Heidet *et al*. 1995). The mutations identified in COL4A5 are highly dispersed in this huge gene. The same mutation has been found in

only a few cases in two or more unrelated kindreds. The dispersion of mutations resembles the situation in some other collagen genes, such as COL1A1, COL1A2, COL2A1 and COL3A1, causing a wide spectrum of diseases of bone, cartilage and blood vessels, respectively (Kuivaniemi *et al*. 1997).

The mutations in Alport syndrome can in most cases explain the structural and pathophysiological changes in the GBM (Tryggvason 1996). They can disturb many of the steps of type IV collagen synthesis and assembly, such as the synthesis of the primary transcript, splicing, or translation of the mRNA. The assembly and folding of three α chains into a triple helical molecule may be affected. Disturbance in both the stability of the helix structure and the formation of cross-links between individual triple helical molecules may occur. Large gene rearrangements can result in the total loss of expression of the gene and small mutations in regulatory elements may have a similar outcome. Abnormal splicing and skipping of exons can cause two types of changes. Firstly, if the exon skipping is in frame, the result is a shortened polypeptide lacking a segment. Secondly, if the exon skipping occurs out of frame, the result is either a protein ending in a nonsense amino acid sequence, or a truncated protein due to a premature translation stop codon.

Since so many mutations have been identified in the COL4A5 gene, it is of interest, from a clinical point of view, to correlate them with the phenotypes they produce. However, it has seemed to be impossible to predict the phenotype based on the type of mutation until a recent publication by Jais and co-workers (2000). They reported a multicentric study of 195 European Alport syndrome families with genotype-phenotype correlations in males. All patients had hematuria and the rate of progression to ESRD and deafness correlated with the type of mutation. The affected male patients with large deletions, nonsense mutations or small mutations changing the reading frame, had a 90% probability of developing ESRD before 30 years of age. The same risk was 70% in patients with splice-site mutations and 50% with missense mutations. The risk of developing hearing loss before the age of 30 was 60% in male patients with missense mutations, and 90% with the other types of mutations.

2.5.6.3 Autosomal Alport syndrome

A distinct form of autosomal recessive AS was suspected, since involvement of the COL4A5 gene was excluded by polymorphism studies in certain pedigrees (Knebelmann *et al*. 1993). It was logical to search for mutations in the COL4A3 and COL4A4 genes in the autosomal form of the disease, as the α3(IV) and α4(IV) chains are also prominent in the GBM. Such mutations have now been found in both the COL4A3 and COL4A4 genes in different Alport kindreds.

The first mutation in the COL4A3 gene was found in a girl with juvenile Alport syndrome accompanied by sensorineural deafness (Mochizuki *et al*. 1994). To date, five different mutations have been found in the COL4A3 gene (Lemmink *et al*. 1994, Mochizuki *et al*. 1994, Ding *et al*. 1995, Knebelmann *et al*. 1995), two of them in two

unrelated patients. These have been found either as homo- or heterozygous mutations in different patients. The parents have been shown to be unaffected carriers, indicating an autosomal recessive pattern of inheritance. Two of the COL4A3 mutations are nonsense mutations, two are small deletions in exons, and one is a splice-site mutation. All five mutations create premature translation stop codons, which lead to a truncated malfunctional polypeptide.

The first mutations in the COL4A4 gene were reported by Mochizuki and co-workers (1994) in two families with AS. One mutation is a glycine substitution in the collagenous domain with the probable impairment of triple helix formation. The other causes a premature stop codon in the collagenous domain and a truncated protein as a result. Recently, Boye *et al*. (1998) analyzed the COL4A4 gene in 31 unrelated autosomal recessive AS patients and found ten novel mutations among eight of them. Five of the mutations result in premature stop codons or a shift in the reading frame, and are therefore potential null mutations. One mutation causes a deletion of two Gly-X-Y repeats in the collagenous region, thereby shortening the protein. Two mutations affect splicing and two are missense mutations, one a glycine substitution in the collagenous region, and the other a highly conserved proline to leucine substitution in the carboxyterminal NC1 domain.

A much less common autosomal dominant form of the disease had already been described in 1985 (Feingold *et al*.), but recently Jefferson *et al*. (1997) performed genetic linkage analysis of a large family with autosomal dominant Alport syndrome. X-linkage was excluded by negative results with COL4A5 markers. Polymorphic markers across the COL4A3/COL4A4 region showed strong linkage. Furthermore, SSCP analysis of five exons from the 3' end of the COL4A3 gene resulted in a band shift in exon 5 which segregated with one of the maternal haplotypes and not with the disease. Sequencing revealed a missense mutation in exon 5, changing the evolutionarily conserved leucine to proline. Lemmink *et al*. (1994) have previously reported this mutation as non-pathogenic in two families; in this case the mutation is not pathogenic in itself, but it may lead to a more severe phenotype in affected patients who carry this abnormality.

2.5.6.4 Alport syndrome associated diffuse leiomyomatosis (DL-AS)

Diffuse leiomyomatosis (DL) is a rare tumorous process usually involving esophageal smooth muscle cells, but sometimes also cells of other organs, such as those of the genital area and the tracheobronchial tree (Antignac & Heidet 1996, Heidet *et al*. 1997). Both sporadic and familial cases have been reported, but in most inherited cases, DL is transmitted as an X-linked dominant trait and is associated with X-linked AS (Garcia-Torres & Orozco 1993). The first report concerning the molecular abnormalities in three male patients with DL-AS revealed in each case a deletion including the 5' end of the COL4A5 gene and extending beyond it (Antignac *et al*. 1992). Later, identification of the COL4A6 gene enabled further characterization of the deletion showing that it was limited only to the first two exons, or to be exact, to exons 1, 1' (which are alternatively transcribed; Sugimoto *et al*. 1994) and 2 of the COL4A6 gene. In fact, this is true in most

cases, since patients with COL4A5/COL4A6 deletions, extending further into COL4A6, display no tumors (Heidet *et al*. 1995). However, mutations in the COL4A6 alone have not been reported to cause only DL or AS (Heiskari *et al*. 1996, Tryggvason 1996).

Mechanisms leading to deletions in DL-AS have been recently proposed by some investigators. Ueki *et al*. (1998) identified the consensus sequences of topoisomerase I and II binding sites at the deletion breakpoints in one patient with DL-AS. Topoisomerase I and II sites have been reported to be present at the breakpoints of translocations or deletions in the dystrophin and steroid sulfatase genes. Thus, it was suggested that topoisomerase I and II activity may also be involved in the mechanisms of DNA breakage and reunion in DL-AS. Additionally, they discovered that the breakpoint on the COL4A5 site was located within the L1 repetitive element. Several deletions at the L1 elements have been reported, including that in the β-globin gene. Segal *et al*. (1999) also noticed the involvement of the L1 elements in two patients with DL-AS. In one patient, L1 element in intron 1 of COL4A5 was fused to intron 2 of COL4A6 by a nonhomologous recombination, resulting in a 13,4 kb deletion. In the other patient, there was an unequal homologous recombination between the same L1 and a colinear L1 element in intron 2 of COL4A6, resulting in a >40 kb deletion.

The exact molecular pathogenesis of DL-AS is still unclear, even if several explanations have been demonstrated. One is that DL could be due to loss of the shared COL4A5/COL4A6 promoter region leading to loss of both α5(IV) and α6(IV) chains, which are normally present in the esophagus. However, this hypothesis was abrogated by Zheng *et al*. (1999) with their study using the X-linked canine model for AS. They showed that due to the mutation in the COL4A5 gene, there is no change at the mRNA level of the α6 chain, but this chain is totally absent at the protein level in smooth muscle. Thus, lack of expression of both α5 and α6 chains would not alone cause DL. And furthermore, the hypothesis of the existence of an abnormal truncated α6 chain, which was favored by the observation that the α5 and α6 proteins were absent (Heidet *et al*. 1997), but α6 mRNA transcript present in esophageal tumors (Heidet *et al*. 1995, 1997), might not be relevant either. Still another hypothesis is that there might be a third, yet unidentified gene and/or its regulatory element(s) involved in the large second intron of COL4A6 (Ueki *et al*. 1998).

It has also been postulated that the loss of the integrin α5 subunit (the expression of which was abnormal in BMs of smooth muscle in a DL patient) and the loss of integrin α5β1 might be peculiar to smooth muscle tumors arising in DL (Thorner *et al*. 1998). Integrin α5β1 binds to fibronectin in the extracellular matrix, and to talin and α-actin within the cell cytoplasm. Disruption of integrin binding results in dissociation of actin and associated proteins from focal adhesion plaques. The α5β1 integrin is involved in the control of cell proliferation, and reduced levels are shown to be associated in tumor cell proliferation and tumor progression. Thus, the COL4A5/COL4A6 mutation may initiate a series of abnormalities leading to cytoskeletal changes and proliferation of smooth muscle via faulty cell signaling.

2.5.6.5 AMME contiguous gene deletion syndrome

An Italian group (Jonsson *et al*. 1998, Piccini *et al*. 1998, Vitelli *et al*. 1999) recently described a novel contiguous gene deletion syndrome including Alport syndrome (A), mental retardation (M), midface hypoplasia (M), and elliptocytosis (E). They identified a large deletion in the Xq22.3-q23 region, comprising the first two exons of COL4A6, the entire COL4A5 gene and extending about 1-2 Mb towards the telomere. This telomeric region is relatively gene-free, but two genes involved in AMME have now been identified in this region. One gene, FACL4 (Piccini *et al*. 1998), encodes a new long chain acyl-CoA synthetase and the other, named AMMECR1 (Vitelli *et al*. 1999), encodes a relatively neutral protein which might be a regulatory factor involved in the development of AMME syndrome. This protein was shown to be very conserved throughout the course of evolution from *C. elegans* and yeast to various microorganisms.

2.6 Role of type IV collagen in other diseases

Expanding knowledge of molecular structure and function of the basement membranes has increased understanding of pathogenetic mechanisms in many diseases. The genes and gene defects underlying several inherited diseases have been recognized. Such laminin associated diseases are junctional epidermolysis bullosa (JEB), in which mutations in the genes encoding the subunits (γ2 and α3 chain) of laminin-5 have been identified (Pulkkinen *et al*. 1994a,b), and congenital muscular dystrophy (CMD), in which mutations in the LAMA2 gene, coding for the α2 chain (present in laminin-2 and laminin-4), are causative for the disease (Helbling-Leclerc *et al*. 1995, Nissinen *et al*. 1996). Since type IV collagen is the major structural component of the basement membranes, many disorders have been linked to it. In the following sections, the role of type IV collagen in certain inherited and acquired diseases is reviewed.

2.6.1 Benign familial hematuria

Benign familial hematuria (BFH), also called thin glomerular basement membrane nephropathy, is inherited as an autosomal dominant trait. These names very clearly reflect the outcome of this disorder, since it is characterized by chronic, usually mild, microscopic hematuria and abnormally thin GBM. However, differentiation between early stages of Alport syndrome and BFH may be difficult. The only microscopic abnormality in young hematuric AS patients may be the thinning of the glomerular basement membrane (Gubler *et al*. 1976, Rumpelt *et al*. 1974), while irregular thickening and multilamination of the GBM develops later in life. Thus, in addition to renal biopsy, a study of family pedigrees should be performed in BFH patients. Firstly, this is to clarify the trait of inheritance,

which is X-linked dominant in AS, and secondly to verify the symptoms of each affected individual, since BFH patients do not develop either sensorineural hearing loss or eye abnormalities indicative for AS. This is also necessary for prognostic purposes and genetic counseling. Renal prognosis in BFH is excellent, about half of the patients develop late-onset hypertension and only some patients late-onset renal insufficiency during the 5th or 6th decade (van Breda Vriesman 1998).

Several groups proposed linkage of BFH to type IV collagen. A Japanese group failed to show linkage to the COL4A3/COL4A4 gene pair, when they used markers from the tip of the chromosome 2 (Yamazaki *et al*. 1995). Furthermore, α1(IV) and α2(IV) chain genes were excluded by Savige (1991). However, Lemmink *et al*. (1996) demonstrated a linkage of BFH with the COL4A3 and COL4A4 genes following the subsequent identification of a mutation in the COL4A4 gene. A glycine was substituted to glutamic acid in the collagenous region, and this mutation was found in all affected family members, but not in either of the nonaffected individuals. Recently, Boye *et al*. (1998) reported a family with a deletion in the COL4A4 gene. The mutation segregated with the presence of microscopic hematuria and thin basement membranes. These data suggested that the patients with benign familial hematuria could be carriers of autosomal recessive Alport syndrome.

2.6.2 Anti-GBM nephritis

Kidney transplantation is generally a good treatment for AS patients who have developed ESRD. However, in some transplanted patients, anti-GBM antibodies are deposited on the GBM of the grafted kidney, and in very rare cases (about 3-4% of the patients), these antibodies destroy the transplant by producing crescentic glomerulonephritis (Gregory *et al*. 1996). This happens usually within a year after transplantation. AS patients who develop anti-GBM nephritis are usually males, mostly deaf, and likely to have reached ESRD before the age of 30. This outcome is indeed true for the majority of AS patients. In contrast, patients with normal hearing or late progression to ESRD, as well as female patients with X-linked AS, are very unlikely to develop anti-GBM nephritis (Kashtan 1998). Patients who suffer from allograft dysfunction should undergo evaluation of anti-GBM nephritis, because it may respond to an appropriate therapy, and in all probability, subsequent allografts are also hit by circulating antibodies. In most retransplanted patients, anti-GBM nephritis has recurred despite prolonged intervals between transplants and the absence of detectable circulating anti-GBM antibodies before retransplantation. Additionally, anti-GBM nephritis may occur in several family members (Kashtan 1998, Kashtan *et al*. 1996).

In 1976, McCoy and coworkers showed by immunohistochemical staining with autoantibodies from Goodpasture syndrome patients, that some Alport patients lacked the Goodpasture antigen. This finding was confirmed later in several studies (Olson *et al*. 1980, Savage *et al*. 1986, Gubler *et al*. 1988). The Goodpasture antigen has been shown to be located in the NC1 domain of the α3(IV) chain (Butkowski *et al*. 1987). This NC1

domain, which can be purified from the GBM after digestion with bacterial collagenase, has a molecular weight of 28 kDa. This led researchers to believe that the α3(IV) chain gene was defective in Alport syndrome. However, as shown later and described above, this could not be the case in the X-linked form of the disease, since the α3(IV) chain gene is located on chromosome 2. Indications for the existence of another as then unidentified component of type IV collagen, which might be involved in Alport syndrome and in anti-GBM nephritis, came from studies by McCoy *et al*. (1982). Later, several other investigators showed that some Alport patients developed antibodies against renal allografts. Some of these antibodies have been shown actually to react with the 28-kDa NC1 domain of the α3(IV) chain (Saus *et al*. 1988), but some identify an NC1 domain-like protein of 26 kDa (Kashtan *et al*. 1990). This 26-kDa component is now known to represent the NC1 domain of the α5(IV) chain (Ding *et al*. 1994a, Kleppel *et al*. 1992).

Recently, Brainwood *et al*. (1998) studied the targets of the alloantibodies in 12 Alport patients who had developed post-transplant anti-GBM disease. Western blot analysis of recombinant NC1 domains showed selective binding to α5(IV)NC1 in nine cases. In one case, the antibody showed binding to both α5(IV)NC1 and α3(IV)NC1, while two cases showed predominant binding to α3(IV)NC1. Seven of the patients who had X-linked inheritance all showed anti–α5 reactivity, and one with α3-reactivity had a COL4A3 mutation. In spontaneous anti-GBM diseases (anti-GBM or Goodpasture syndrome), the major target of autoantibodies is the α3(IV)NC1 domain (Saus *et al*. 1988, Kalluri *et al*. 1995, Dehan *et al*. 1996), but the primary target in most Alport anti-GBM patients is the NC1 domain of the α5 chain of type IV collagen (Brainwood *et al*. 1998).

It has been suggested that transplanted AS patients with large deletions in the COL4A5 gene have higher risk in developing anti-GBM nephritis, because of introduction of an immunogenetically new component (Ding *et al*. 1994b, Lemmink *et al*. 1997). In fact, in a study by Jais *et al*. (2000), three of the 118 transplanted male patients developed anti-GBM nephritis, and all three had a large COL4A5 deletion. Even if some types of mutations may contain higher risk, it is still unclear which factors influence the initiation and elaboration of the immune response to the allograft (Kashtan 1998).

2.6.3 Goodpasture syndrome

Another disease, where circulating antibodies against type IV collagen components are pathogenic, is Goodpasture syndrome. In 1919, an unusual case was described by Ernest Goodpasture. The patient was a young man who had died of an influenzal illness characterized by hemoptysis, alveolar hemorrhage and necrosis, as well as proliferative glomerulonephritis. Since then, hundreds of similar cases have been described, and even if Goodpasture syndrome is generally a very rare disorder, it is the most common manifestation of human anti-basement membrane diseases (Netzer *et al*. 1998). Goodpasture syndrome is characterized by circulating IgG autoantibodies against type IV collagen in the glomerular or alveolar basement membrane (Hudson *et al*. 1989). The target of these antibodies has been identified as the NC1 domain of the α3(IV) chain

(Butkowski *et al*. 1987, Saus *et al*. 1988). Several studies have been carried out to investigate the specific epitope for these autoantibodies (Kalluri *et al*. 1991, Levy *et al*. 1997, Penades *et al*. 1995, Ryan *et al*. 1998). Two major conformational epitope regions were identified by Netzer *et al*. (1999), and recently, Borza *et al*. (2000) showed that these regions contain multiple cryptic epitopes recognized by heterogeneous population of Goodpasture antibodies.

The cause of Goodpasture syndrome is unknown. Different initiating events, such as infection, inhalation of gasoline or other hydrocarbon fumes, smoking, renal injury or ischemia may promote the disease by damaging the basement membrane and exposing the cryptic epitope (Bolton 1996, Netzer *et al*. 1998). However, T-cells most probably have a role in the autoimmune response, because NC1-specific T-cells have been isolated from Goodpasture patients (Derry *et al*. 1995, Merkel *et al*. 1996). Association of certain HLA antigens with the disease has also been noted, thus implying a genetic susceptibility for the disease (Fisher *et al*. 1997).

2.6.4 Diabetic nephropathy

Diabetic nephropathy is a devastating secondary manifestation of diabetes. About 30-40% of insulin-dependent and 10% of non-insulin-dependent diabetics will develop renal insufficiency and renal failure. In the USA, diabetic nephropathy accounts for about 25% of all new ESRD cases (Rohrbach & Murrah 1993). Diabetic nephropathy is characterized by expansion of the mesangial matrix and thickening of the glomerular and tubular basement membranes, abnormalities leading to increased proteinuria and loss of the selective filtration capability of the GBM (Kanwar 1984). These morphological changes and functional deficiency of the GBM are mainly thought to result in nonenzymatic glycosylation of type IV collagen. Nonenzymatic modifications of the 7S domain and the NC1 domain of type IV collagen have been demonstrated, including both *in vitro* (Tsilibary *et al*. 1988, Raabe *et al*. 1996) and *in vivo* (Raabe *et al*. 1998) studies.

Nonenzymatic glycation involves the reaction of reducing sugars with free amino groups of proteins following the formation of Amadori intermediates, of which some are further modified to advanced glycation end products (AGEs) (Brownlee *et al*. 1984). The accumulation of AGEs in turn clearly correlates with the development of long-term complications in diabetes (Monnier *et al*. 1986). These may be due to interference in normal cell-matrix interactions as well as in cell signaling (Krishnamurti *et al*. 1997). Again, regulation of expression of certain genes may be affected (Cohen *et al*. 1997). However, the detailed mechanisms by which nonenzymatic glycation finally leads to diabetic nephropathy are still unclear.

3 Aims of the present study

The basement membrane specific type IV collagen is involved in certain hereditary and acquired diseases. Three chains, α3(IV), α4(IV) and α5(IV) chains, which have restricted tissue distribution, have been shown to be mutated in Alport syndrome. This is a heterogeneous kidney disorder that progresses to end-stage renal failure. About 85% of the patients have the X chromosome-linked dominant form of this disease caused by mutations in the α5(IV) chain gene. In contrast, the α3(IV) and α4(IV) chain genes are mutated in autosomal AS. Exact diagnosis of Alport syndrome has been a subject of controversy among physicians, and it is clinically important to identify the mutations, especially in the COL4A5 collagen gene.

A minority of Alport patients develops antibodies against the transplanted allograft, and the primary targets of these antibodies have been shown to be the noncollagenous domains of the α3 and α5 chains of type IV collagen. Goodpasture syndrome, a rare autoimmune disease characterized with progressive glomerulonephritis and pulmonary hemorrhage, is caused by circulating α3(IV) antibodies. To better understand molecular pathology of Alport syndrome, the following specific aims were set for this study:

1. To further characterize the COL4A5 gene and establish conditions for PCR amplification as well as direct sequencing of all exon regions and the promoter region of this gene.

2. To facilitate exhaustive analysis of mutations in the COL4A5 gene and provide the basis for an automated detection system.

3. To produce recombinant type IV collagen for autoantibody detection in anti-GBM nephritis.

4 Materials and methods

4.1 Patient samples (I, II, III, IV)

In the mutation analyses (I, II, III) the diagnostic criteria for suspicion of Alport syndrome varied from one nephrologist to another, including glomerular renal disease in combination with one or two of the following findings: positive family history, hearing loss, opthalmological changes of AS, or typical splitting of the glomerular basement membrane in electron microscopic examination. Patient DNA was obtained, or it was isolated from whole blood in an ABI 340A DNA extractor or by standard manual methods. The samples (from 107 unrelated patients) were from Denmark, Finland, Germany, Iceland, Sweden and the USA. Serum samples for antibody detection (IV) were obtained from Denmark and Finland.

4.2 Characterization of the COL4A5 gene (I, III)

To determine previously unknown intron sequences flanking exons 2 and 37, two genomic clones were characterized. Screening of the human chromosome X specific cosmid library LL0XNC01 with exon 2 specific oligonucleotides resulted in isolation of clone U238C7. The 40 kb clone was isolated with the standard alkaline lysis method (Sambrook *et al*. 1989) and directly sequenced using the T7 DNA Sequencing Kit (Pharmacia, Uppsala) (I).

The Genome Systems, Inc. (St Louis, MO) screening service was utilized and exon 37 specific oligonucleotides were supplied to screen the human P1 library. The delivered clone DMPC-HFF#1-1049-5A was isolated with standard CsCl-method (Sambrook *et al*. 1989). The cosmid clone was sequenced directly from DNA using ABI Prism Dye Terminator Labeling kit (Perkin-Elmer) (I).

Two previously isolated genomic clones containing the entire intron 41, cosmid clone DMPC-HFF#1-1049-5A (above) and lambda clone F7 (Zhou *et al*. 1994b) were directly sequenced with the Dye termination cycle sequencing kit (Perkin-Elmer) using an Abi Prism 310 DNA sequencer (Perkin-Elmer) (III).

4.3 PCR and sequencing (I, II, III)

4.3.1 Oligonucleotides

Oligonucleotides for PCR amplification and sequencing were synthesized with an ABI 381A DNA synthesizer. One of the PCR primers was labeled with biotin during the oligonucleotide synthesis (biotin amidite Biodite, Pharmacia) to enable the purification of the PCR products. The primers for nested sequencing were end-labeled with fluoresceine amidite FluorePrime (Pharmacia) during the synthesis (I, II).

4.3.2 PCR amplification

In mutation analyses, PCR amplification reactions were carried out using the GeneAmp 9600 thermocycler (Perkin-Elmer) in a reaction volume of 50 µl with 100 ng of genomic template DNA, 5 pmol of primers, 0.2 mM of dNTP, 1.5 mM $MgCl_2$ and 1 U of AmpliTaq or AmpliTaq Gold DNA polymerase (Perkin-Elmer). Annealing temperatures varied depending on each exon (I, II).

For RNA preparation, lymphocytes from two patients were transformed with Epstein-Barr virus. Total RNA was extracted from cultured cells using the acid guanidinium thiocyanate-phenol method (Chomszynski & Sacchi 1987). The first strand cDNA was synthesized using 20 U MuMLV reverse transcriptase and 5 pmol of COL4A5 cDNA specific primer (II).

PCR amplification of one microliter of first strand cDNA synthesis mix (II), or two microliters of 1:10 dilution of each cDNA library (III) consisted of a 12 minute denaturation at 95°C, and 35 cycles including 45 seconds at 95°C, 1 minute at 55°C/58°C, and 1 minute at 72°C followed by a 10 minute extension at 72°C.

4.3.3 Purification of the PCR products

The PCR amplified genomic DNA was purified with the help of streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin Dynal, Norway). The biotin-labeled PCR-amplified double-stranded DNA fragments bind to this coated solid phase (Hultman *et al*. 1991) and a neodymium-iron-boron permanent magnet (Dynal MPC-96, Dynal, Norway) was used for bead sedimentation. During the purification procedure, magnetic beads (200 µg) were mixed with the PCR amplification products and incubated for at least 15 minutes at room temperature. The beads were washed with WB-buffer (washing and binding buffer: 0.1 M TrisHCl pH 7.5, 1 mM EDTA, 1 M NaCl). The non-biotinylated strands were melted off with 0.1 M NaOH at room temperature for 15 minutes, after which they were removed by washing first with 0.15 M NaOH and then with WB-buffer. The beads containing the single-stranded DNA were suspended in water (I, II). The samples to be sequenced with the Abi Prism 310 DNA sequencer or Abi Prism 377 DNA sequencer (Perkin-Elmer) were purified with the Wizard PCR purification kit (Promega) (II, III).

4.3.4 Direct sequencing of the PCR products

The specific fluorescein-labeled nested sequencing primers (1.0 - 15.0 pmol) were annealed to the immobilized single-stranded template DNA in 1 M TrisHCl pH 7.6, 100 mM $MgCl_2$, 160 mM DTT. Sequencing was subsequently carried out using the AutoRead Sequencing Kit (Pharmacia) with the automated Laser Fluorescence DNA sequencer A.L.F. (Pharmacia) (I, II). The PCR-amplified cDNA synthesis mix (II) was sequenced with nested COL4A5 specific primers, and the cDNA libraries (III) with the PCR primers using Abi Prism 310 DNA sequencer or Abi Prism 377 DNA sequencer (Perkin-Elmer) and the Dye termination cycle sequencing kit (Perkin-Elmer) (III).

4.4 Construction, expression and purification of the recombinant proteins (IV)

Constructs for recombinant protein production were prepared by PCR. For α1(IV), α2(IV), α5(IV) and α6(IV), cDNA clones were used as templates (Soininen *et al*. 1987, Hostikka & Tryggvason 1988, Zhou *et al*. 1992a, Zhang *et al*. 1996), and for α3(IV) and α4(IV) kidney mRNA was first reverse transcribed and then PCR amplified (Mariyama *et al*. 1994, Leinonen *et al*. 1994). The PCR oligonucleotides introduced *Nhe*I and *Bam*HI sites to the 5' and 3' ends of the each amplified NC1(IV) domain. For the *Vaccinia* system, the α5(IV) signal peptide and consecutive 23 Gly-X-Y repeats were amplified to insert *Sal*I and *Nhe*I sites to the 5' and 3' ends of the fragment. The sequence coding for 6 histidines was inserted and these fragments were then ligated to the pBluescript II SK+

vector, and again to the *Vaccinia* pSC65 transfer vector. Constructs for bacterial expression were prepared by ligating the NC1(IV) domains to the QIAexpress pQE-31 vector (Qiagen).

Bacterial proteins were expressed using the QIAexpressionist high level expression and protein purification system (Qiagen) and cultures were grown at the conditions suggested by manufacturers for toxic genes and proteins. In the protein purification, the 6Xhis-tag affinity to Ni-NTA (nickel-nitrilo-tri-acetic acid) resin was utilized and all purification steps were done according to the manual for the denaturing-purification of insoluble proteins. Mammalian protein production was performed in HeLa cells according to Ausubel *et al*. (1989). The Ni-NTA chromatography was used for purification of the proteins.

4.5 SDS PAGE and Western blotting (IV)

Bacterial cell extracts were electrophoresed on 12 % SDS gel under reducing conditions and stained with CBB (Coomassie Brilliant Blue). Mammalian proteins were metabolically labeled with ^{35}[S]-Methionine and the culture media was run on SDS gel. Visualization was performed by En^{3}hance* (Du Pont) and autoradiography.

For the Western blotting procedure, about 5 μg of purified mini-α3(IV) and mini-α5(IV) proteins were separated by 12% SDS-gel electrophoresis under reducing conditions (10 mM ditiothreitol), transferred onto a PVDF (polyvinylene difluoride) membrane (Hybond-P, Amersham) and incubated with a 1:100 dilution of patient sera. Peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) was used as the secondary antibody. The serum sample of Goodpasture patient PL1000 was used as the positive control in α3(IV) antibody detection. The protein A-purified serum sample of anti-GBM disease patient (patient c), kindly provided by Dr. A.N. Turner, was used as the positive control in both α3(IV) and α5(IV) antibody detection.

4.6 ELISA detection (IV)

The amount of 100 ng of purified proteins was coated on Nunc-Immuno Maxisorp plates. Bacterial proteins were coated in 6 M urea and mammalian proteins in PBS. Patient sera (1:100 dilutions) were added and peroxidase-conjugated anti-human horseradish IgG (Chemicon) was used as the secondary antibody. In the detection of COL4A3 antibodies in Goodpasture sera, measurements were performed against standards with IgG levels of 1, 3, 9, 27, 81 and 243 mg/ml. The serum sample of Goodpasture syndrome patient (PL1000) was used as the positive control.

5 Results

5.1 Characterization of the COL4A5 gene (I, III)

The complete exon size and distribution pattern of the human type IV collagen α5 chain gene (COL4A5) was determined in 1994 by Zhou *et al*. (1994b), with the exception of exons 2 and 37 and their flanking regions. Two genomic clones containing these regions were isolated in this study and the previously unknown intron flanking sequences were determined by direct sequencing. In addition, previously known 20 bp sequences flanking exons 1, 3 through 36, and 38 through 51 were expanded to 35 to 190 bp (I) by sequencing several previously isolated genomic clones (Zhou *et al*. 1994b).

The complete intron 41 was directly sequenced from two genomic clones to identify possible exon(s) coding for a previously unknown 18 bp sequence, reported to exist in kidney mRNA (Guo *et al*. 1993). The size of this intron was shown to be 9260 bp and to contain two separate 9 bp exons coding for two Gly-X-Y repeats. The intron boundaries of both exons have consensus GT and AG nucleotides at the donor and acceptor sites, respectively. The segment between the two exons is 4.5 kb, exon 41A being about 1.5 kb downstream from exon 41. The intron between exons 41B and 42 was shown to be about 3 kb (III).

5.2 COL4A5 mutation analyses (I, II, III)

All previously known 51 exons, together with 820 bp of the core promoter region upstream of the COL4A5 gene (I), as well as the novel exons 41A and 41B (II, III), were PCR amplified from the DNA of patients with suspected Alport syndrome and sequenced. This resulted in the identification of 67 different mutations of which seven were found in several unrelated patients, the total number of mutations being 76 (Table 4**)**. None of the

mutations involved the promoter region or the novel exons 41A and 41B. After identification of a mutation, the segregation was studied within in a family if samples were available. We found 25 missense mutations of which 24 were glycine substitutions. Additionally, ten nonsense mutations, fourteen splice-site mutations and twelve deletions were identified. One of the deletions was very large and seven of them only one base pair deletions. Three of the deletions were in-frame deletions. Four one base pair insertions were found, and two mutations involved both deletion and insertion. In two patients, a potential splicing mutation was characterized in detail from mRNA (II).

About one-third of the mutations were missense mutations (24 out of 67) converting a glycine to another amino acid. Substitution of glycine-624 to aspartic acid was detected in five unrelated patients, and glycine-869 to arginine in two unrelated patients. One missense mutation was found in the non-collagenous domain (NC1 domain) converting conserved leucine to arginine (L1649R) in two unrelated patients. Additionally, one sequence variation was found in the collagenous domain converting unconserved lysine to asparagine (K664N) in three unrelated patients.

Ten nonsense mutations were found. All of them caused a premature stop codon, and two of these mutations (R373X and R1677X) were found in two unrelated patients.

We could detect fourteen different mutations concerning splice-sites. One included deletion of 32 base pairs and duplication of six base pairs, thus deleting the splice signal. Other mutations were single base substitutions. Four of the mutations were found at the 5' donor splice-site within the gt-consensus sequence changing g to another nucleotide. Two splice-site mutations concerning the 3' acceptor site changed the ag-consensus sequence, and in six cases, alternative splice-sites were formed upstream because of nucleotide changes. In one case, the 3576-11C was changed to A, leading to an at-sequence at the 3' acceptor site. Two splicing mutations were characterized at the mRNA level. Mutation 3756-3C->G caused skipping of an exon and mutation 3657-9A->G led to the insertion of an eight-base-pair intronic sequence (II).

Twelve deletions were detected. Seven of them were single base deletions and one four-base-pair deletion. Three deletions were in frame mutations. One patient had a very large deletion including exons 2 to 8. This was demonstrated by the fact that these exons could not be PCR amplified.

Insertions were found in six patients, four of them being single base insertions. In two patients we found a mutation involving both deletion and insertion. In one case, eight bases between codons Pro812 and Gly824 were deleted in exon 30, and this deletion was substituted by a 30-base-insertion. In another case, ten base pairs were deleted between Ala1642 and Tyr1645 in exon 50, and fourteen bases were inserted.

The mutations in table 4 have been designated according to Beaudet and Tsui (1993) and are explained with the examples as follows: missense and nonsense mutations; G96R – the amino acid glycine number 96 is converted to arginine, X indicates a premature stop codon. Frameshift mutations; 648delC – the nucleotide C number 648 is deleted, 4935del4 – four nucleotides after nucleotide number 4935 are deleted. Splicing mutations; 641-4A->G – the noncoding nucleotide A (*i.*e. in intron) is converted to G four nucleotides before the coding nucleotide number 641.

Table 4. Mutations in the COL4A5 gene, phenotypes and major clinical features of the patients in this study.

Mutation	Effect on coding sequence	Predicted effect on protein	Phenotype				Ref.
			age of ESRD	hearing loss	eye lesions	GBM splitting	
Missense							
G96R[1]	Gly->Arg at 96	G-X-Y int[2]	n.a.[3]	n.a.	n.a.	n.a.	UD[4]
G216R	Gly->Arg at 216	G-X-Y int	23	+	-	+	II
G409S[1]	Gly->Ser at 409	G-X-Y int	n.a.	-	n.a.	n.a.	UD
G415R[1]	Gly->Arg at 415	G-X-Y int	-	-	-	+	II
G420V	Gly->Val at 420	G-X-Y int	n.a.	-	-	n.a.	I
G573D	Gly->Asp at 573	G-X-Y int	20	+	n.a.	n.a.	I
G624D[1]	Gly->Asp at 624	Unknown[5]	-	-	-	n.a.	UD
G624D[1]	Gly->Asp at 624	Unknown	-	-	-	+	UD
G624D[1]	Gly->Asp at 624	Unknown	-	-	-	-	UD
G624D	Gly->Asp at 624	Unknown	-	+	n.a.	n.a.	UD
G624D	Gly->Asp at 624	Unknown	-	-	-	n.a.	I
G635D	Gly->Asp at 635	G-X-Y int	18	+	+	+	I
G766D	Gly->Asp at 766	G-X-Y int	n.a.	n.a.	n.a.	n.a.	UD
G834R	Gly->Arg at 834	G-X-Y int	n.a.	n.a.	n.a.	n.a.	UD
G860D	Gly->Asp at 860	G-X-Y int	n.a.	+	n.a.	n.a.	UD
G869R	Gly->Arg at 869	G-X-Y int	>17[6]	-	-	+	I
G869R[7]	Gly->Arg at 869	G-X-Y int	28	+	-	n.a.	I
G941C	Gly->Cys at 941	G-X-Y int	40	+	n.a.	n.a.	I
G1030S	Gly->Ser at 1030	G-X-Y int	37[6]	+	n.a.	n.a.	I
G1045E	Gly->Glu at 1045	G-X-Y int	17	+	+	n.a.	II
G1066S[7]	Gly->Ser at 1066	G-X-Y int	17	+	-	n.a.	I
G1086D[1]	Gly->Asp at 1086	G-X-Y int	-	-	-	n.a.	II
G1107R	Gly->Arg at 1107	G-X-Y int	-	+	+	n.a.	UD
G1143D[7]	Gly->Asp at 1143	G-X-Y int	25	-	-	+	I
G1167S	Gly->Ser at 1167	G-X-Y int	33	-	-	+	II
G1196R	Gly->Arg at 1196	G-X-Y int	18	+	-	+	I
G1205D	Gly->Asp at 1205	G-X-Y int	-	n.a.	n.a.	n.a.	UD
G1261E[7]	Gly->Glu at 1261	G-X-Y int	23	+	-	n.a.	I
G1357S	Gly->Ser at 1357	G-X-Y int	<30	+	-	n.a.	I
L1649R[1]	Leu->Arg at 1649	Unknown	-	n.a.	n.a.	n.a.	UD
L1649R	Leu->Arg at 1649	Unknown	-	-	-	+	I

Table 4. continued

Mutation	Effect on coding sequence	Predicted effect on protein	Phenotype				Ref.
			age of ESRD	hearing loss	eye lesions	GBM splitting	
Nonsense							
Y30X	Tyr->stop at 30	Shorter pr[8]	20	+	+	n.a.	II
R266X	Arg->stop at 266	Shorter pr	-	n.a.	n.a.	n.a.	UD
R373X[7]	Arg->stop at 373	Shorter pr	-	-	-	n.a.	UD
R373X	Arg->stop at 373	Shorter pr	20	+	+	+	II
Q407X	Gln->stop at 407	Shorter pr	18	+	n.a.	n.a.	I
G1060X	Gly->stop at 1060	Shorter pr	21	+	+	n.a.	I
S1071X	Ser->stop at 1071	Shorter pr	18	+	-	n.a.	I
K1097X	Lys->stop at 1097	Shorter pr	22	+	+	+	I
Q1383X	Glu->stop at 1383	Shorter pr	n.a.	n.a.	n.a.	n.a.	UD
Y1597X	Tyr->stop at 1597	Shorter pr	>17[6]	+	-	+	I
R1677X	Arg->stop at 1677	Shorter pr	26	+	+	n.a.	I
R1677X	Arg->stop at 1677	Shorter pr	>11[6]	+	-	+	I
Frameshift							
648delC	Frameshift at Pro149	Shorter pr	21	+	+	n.a.	I
752insC	Frameshift at Leu184	Shorter pr	22	+	-	n.a.	I
1147insT[7]	Frameshift at Pro315	Shorter pr	27	+	-	n.a.	I
1684delT[1]	Frameshift at Gln495	Shorter pr	22	+	-	+	II
2349delG[7]	Frameshift at Gly716	Shorter pr	22	+	+	+	I
2495/2496delC	Frameshift at Pro765	Shorter pr	n.a.	n.a.	n.a.	n.a.	UD
2549delC	Frameshift at Pro783	Shorter pr	n.a.	n.a.	n.a.	n.a.	I
2636del18/ ins30	Frameshift at Pro812	Shorter pr	18	+	+	n.a.	I
3004insT	Frameshift at Gly935	Shorter pr	26	+	n.a.	n.a.	I
3395/3397delA	Frameshift at Gly1066	Shorter pr	-	n.a.	n.a.	n.a.	UD
3528insT	Frameshift at Pro1109	Shorter pr	n.a.	n.a.	n.a.	n.a.	UD
3728/3729delG	Frameshift at Gly1176	Shorter pr	22	+	+	n.a.	I
4935del4[1]	Frameshift at Glu1579	Shorter pr	-	n.a.	n.a.	n.a.	UD
5126del10/ ins14	Frameshift at Ala1642	Shorter pr	-	+	-	n.a.	UD
del ex 2 to 8	Unknown	Shorter pr	15	-	-	n.a.	I
In-frame deletion							
1567del9[7]	del Pro456-Pro458	Shorter pr	38	+	+	n.a.	I
2605del18	del Gly802-Pro807	Shorter pr	27	+	-	+	I
2791del36	del Ser864-Gly875	Shorter pr	-	+	-	n.a.	II

Table 4. continued

Mutation	Effect on coding sequence	Predicted effect on protein	Phenotype				Ref.
			age of ESRD	hearing loss	eye lesions	GBM splitting	
Splicing							
641-4A->G	3' splice signal	Incorr sp[9]	n.a.	n.a.	n.a.	n.a.	UD
749-2A->G	3' splice signal	Incorr sp	n.a.	n.a.	n.a.	n.a.	UD
749-11del32/ dup6	3' splice signal	Incorr sp	-	-	-	n.a.	UD
1625+1G->A	5' splice signal	Incorr sp	>35[6]	+	+	+	I
1789+1G->A	5' splice signal	Incorr sp	28	+	+	n.a.	I
2243+1G->T	5' splice signal	Incorr sp	n.a.	+	-	+	I
2349-3C->G[1]	3' splice signal	Incorr sp	-	-	-	+	I
2447-1G->A	3' splice signal	Incorr sp	22	+	+	n.a.	I
3309-2A->G	3' splice signal	Incorr sp	10	+	n.a.	n.a.	I
3576-11C->A	3' splice signal	Incorr sp	-	+	-	n.a.	II
3656+1G->T[1]	5' splice signal	Incorr sp	26[10]	+	-	n.a.	I
3657-9A->G	3' splice signal	Incorr sp[11]	37	+	+	n.a.	II
3756-9C->G	3' splice signal	Incorr sp	19	+	+	n.a.	I
3756-3C->G	3' splice signal	Incorr sp	33	+	+	+	I
3756-3C->G	3' splice signal	Incorr sp[12]	28	+	+	+	II

1) Female patient
2) Gly-X-Y interruption
3) "n.a." indicates information not available
4) "UD" means unpublished data
5) Substitution in Gly-X-Y interruption
6) The mean age of ESRD in family men, patient younger
7) No family history of hematuria
8) Shorter protein
9) Incorrect splicing
10) ESRD age of brother
11) Insertion of 8 bp before exon 39
12) Exon 40 spliced out

5.3 Sequencing of the PCR amplified fragments from different cDNA libraries (III)

In order to investigate if the 18 bp segment of COL4A5 reported in kidney mRNA (Guo *et al*. 1993) is also present in some extrarenal tissues, cDNA libraries from several tissues such as kidney, liver, placenta, spleen and epithelia (of a thymus) were PCR amplified. The presence of two mRNA transcripts with and without the 18 bp sequence was demonstrated in kidney, liver, placenta and spleen. However, in epithelial cDNA, only the sequence of exon 41B was present.

PCR from a cDNA library most probably results in amplification of transcripts in the right proportions. In the case of the kidney, the dominant transcript contains the 18 bp sequence. This codes for the amino acids Gly-Pro-Thr-Gly-Phe-Gln. In contrast, the sequence data obtained from either one (Gly-Phe-Gln) or two extra Gly-X-Y repeats in the epithelial and spleen cDNAs is very weak, but can still be followed. In the liver and placenta, both transcripts seem to be present, since equally high peaks can be seen in the sequence data.

5.4 Production and purification of the recombinant mini-collagens and NC1 domains (IV)

All constructs for recombinant protein production were sequenced without finding any mistakes, and proper ligation to expression vectors was also confirmed. All mini-collagens were secreted into the media, but the α4(IV) very poorly. The sizes of the proteins corresponded to calculated sizes of the mini-collagens, *i.e*. 36 kDa for the α3(IV) and α4(IV), and 35 kDa for the others. One culture was infected with the wild type virus, and comparison of the sizes excluded the possibility of confusion with wild type thymidine kinase protein. In bacterial protein production, SDS gel electrophoresis revealed overproduction of the proteins with the sizes corresponding to those of NC1(IV) domain monomers (26-28 kDa).

Both mini-collagens and NC1 domains were purified with an Ni-NTA column. Yields of about 200μg/liter pure protein were obtained for mini-collagens. In contrast, yields of bacterial NC1 domains were about 10-50 mg/liter.

5.5 ELISA method for detection of antibodies against type IV collagen in patient sera (IV)

To detect antibodies in sera from Goodpasture patients, recombinant α3(IV) mini-collagens were coated on microtiter plates. In three samples, relatively high levels of antibodies, ranging from about 260-280 mg/l, could be seen. This was also the case in two

separate samples which were taken from the same patient within a three-week-period. Again, one sample included as much as about 750 mg/l IgG.

Because of poor yields in the mammalian expression system, all six recombinant NC1(IV) domains made in *E.coli* were used in ELISA to analyze serum samples of 30 Alport syndrome patients, as well as 39 normal serum controls. This was done to see if Alport patients (without having developed an anti-GBM nephritis) have some circulating antibodies against type IV collagen. Surprisingly, one patient showed very strong reactivity against the α3(IV) NC1 domain and two other patients seemed to have some antibodies against α5(IV). Additionally, one normal control also reacted relatively strongly with the α3(IV) NC1.

5.6 Western blot analysis (IV)

The sera from the three patients having α3(IV) and α5(IV) antibodies were crosschecked with Western blotting. Serum samples from one Goodpasture patient, one patient with anti-GBM nephritis, known to have some α3(IV) and mainly α5(IV) antibodies, as well as one normal serum sample showing reactivity against α3(IV), were included as controls. Western blotting was performed with purified *Vaccinia* mini-collagens. Positive results in ELISA were confirmed in the case of a patient with anti-α3 antibodies and in another patient with anti-α5 antibodies. Possibly the amount of antibodies of the patient with positive reaction of anti-α5, as well as in the control serum, were so low that reaction in Western analysis was negative. The Goodpasture patient (control) was positive for α3, but negative for α5, as expected. Again, the patient with anti-GBM disease was negative for α3, but positive for α5.

6 Discussion

6.1 Exon-intron structure of COL4A5

The results of the present work (I, III) provide the complete exon/intron structure of the human COL4A5 gene. The intron sequences flanking exons 2 and 37 were determined for the first time. Two novel exons 41A and 41B, encoding the 18 bp sequence described in kidney mRNA (Guo *et al*. 1993), were identified and their flanking intron sequences were determined. Additionally, intron sequences surrounding exon 1, exons 3 through 36, and exons 38 through 51 were extended. These results are important as they allow, for the first time, a complete mutation analysis of all exon regions of the COL4A5 gene in Alport syndrome.

6.2 Mutation analyses in X-linked Alport syndrome

The present work provides information that enables detection of over 80% of mutations in X-linked AS using PCR of exons and the promoter region and direct sequencing. Several different methods have been used to detect mutations in the COL4A5 gene in X-linked AS patients. Southern blot analyses are used for the detection of major gene rearrangements (Barker *et al*. 1990, Antignac *et al*. 1994), but these account for only about 5 to 15% of the mutations in X-linked Alport syndrome, depending on the population (Tryggvason 1996). Therefore, the vast majority of disease-causing mutations is small in size. A search for small mutations has mainly been carried out by the PCR-amplification of exons, identification of sequence variants by chromatographic methods such as DGGE (Zhou *et al*. 1992b, Netzer *et al*. 1996) or SSCP (Kawai *et al*. 1996, Knebelmann *et al*. 1996, Renieri *et al*. 1996, Plant *et al*. 1999, Cheong *et al*. 2000), followed by the nucleotide

sequencing of abnormally migrating PCR products. This approach has been partially hampered by the fact that sequences flanking some of the exons have not been available and, therefore, some exon regions have been impossible to analyze. In this study intron sequences flanking all 53 exons of the human COL4A5 gene were significantly extended. This enables PCR amplification of all exons in such a way that it is possible to derive exon flanking sequences long enough to probably contain most of the sequences needed for mRNA splicing. The availability of these sequences will significantly facilitate screening for mutations of this gene in Alport syndrome. Previously, only 20 base pairs of intron sequences flanking exons 1, 3-36 and 38-51 had been available (Zhou *et al*. 1994b). The sequences of introns flanking exons 2 and 37 were not available at all, and exons 41A and 41B had not been identified.

The mutation detection rate observed with the method we have used is considerably higher than with the chromatographic methods reported. In fact, previous studies have implied several difficulties in identifying mutations in the COL4A5 gene in X-linked Alport syndrome (Table 5). In a study by Knebelmann *et al*. (1996), SSCP analysis of 48 of the 51 exons in 131 unrelated Alport syndrome patients resulted in the identification of 63 different mutations. This represents a detection rate of 50%. Kawai *et al*. (1996) analyzed all 51 exons by PCR-SSCP analysis, detecting mobility shifts in the PCR products from 22 out of 60 Japanese Alport patients, a detection rate of only 37%. Renieri *et al*. (1996) analyzed all 51 exons (excluding intron regions 2 and 37) by PCR-SSCP in 201 Italian Alport syndrome patients finding mutations in only 45% of certain X-linked cases. Plant *et al*. (1999) performed SSCP analysis for 153 British families, which resulted in the finding of a mutation in 77 families, with a detection rate of 50%. Furthermore, Cheong *et al*. (2000) identified 10 mutations in 25 unrelated Korean patients (detection rate 40%). The reasons for the low detection rates in the above-mentioned studies may be mutation locations outside the immediate exon region, existence of other X-chromosomal gene(s), or lack of sensitivity of the SSCP method. Additionally, definitions of X-linked AS varied from certain to unlikely, or even certain autosomal inheritance. Recently, a Japanese group (Inoue *et al*. 1999) reported a mutation analysis of the COL4A5 gene in 22 AS patients by performing reverse transcription polymerase chain reaction (RT-PCR) and direct sequencing using leukocytes. The mutation detection rate was 92% in males and 56% in females (overall detection rate 77%). Stringent criteria for X-linked AS may partly relate to the high detection rate in that study. The present study describes the identification of a mutation in 76 unrelated patients out of 107 studied, providing a detection rate of 71% by sequencing analysis of all exons. This implies a good potential for being able to find a mutation in most AS patients because, if unlikely X-linked cases are not included, the ”corrected” detection rate is 83% (Table 5). As mentioned earlier, we could analyze longer exon-flanking sequences than in the other studies. About 20% of the found mutations involved splicing sequences. In studies with the SSCP method, percentages of splicing mutations have varied from 10 (Renieri *et al*. 1996, Plant *et al*. 1999) to 25% (Knebelmann *et al*. 1996). Even if only one-fifth of the found mutations in this study were splice-site mutations, four of them, however, concerned positions at more distant locations than reported before in AS. Furthermore, the effect of a mutation at location -9 was identified at the mRNA level.

Table 5. Mutation detection rates in extensive analyses of the COL4A5 gene in Alport syndrome.

Reference	Patients[1]	Mutation found[1]	Detection rate	Mutation not found in patients with diagnosis of X-linked AS[1]			"Correct" detection rate[2]
				certain	likely	unlikely	
Kawai *et al*.	60	22	37%	?	?	?	?
Knebelmann *et al*.	131	63	50%[3]	22	29	17	58%
Renieri *et al*.	201	60	30%[4]	53	25	63	43%
Plant *et al*.	153	77	50%	17	20	39	68%
Inoue *et al*.	22	17	77%	5	-	-	77%
Cheong *et al*.	25	10	40%	12	3	-	40%
This study	107	76	71%	-	13	15	83%

[1]Number of patients
[2]Patients with unlikely X-linked AS have been left out from the total number of patients in the study
[3]Noted that 48 out of 51 exons studied
[4]Detection rate of 45% in article was calculated from certain X-linked cases

As mentioned above, it has been speculated that mutations might involve locations outside the immediate exon regions. We extended our study to concern two previously unstudied regions. Conditions for PCR and direct sequencing of the 820 bp promoter region (Sugimoto *et al*. 1994) were optimized, and this promoter was sequenced. Additionally, two novel 9 bp exons were identified in intron 41, and also these exons and exon flanking introns were sequenced. However, patients, in whom no mutations were found in any of the 51 exons, had no sequence variations in these regions either.

6.3 Mutations in the COL4A5 gene and genotype-phenotype correlations

Hundreds of DNA samples from patients and family members suspected of having Alport syndrome have been collected during the past decade. In addition to studies I and II, mutation detection was performed in samples which have not been studied earlier (Heiskari *et al*. 1996), or of which enough DNA was available. A total of 107 such samples were studied, including the 67 from studies I and II. Unfortunately, despite several requests, clinical data of some patients could not be obtained. Thus, phenotypes and clinical data are missing for a number of patients in table 4 (section 5.2.), where results have not been published (UD).

We could find 76 mutations of which six were found in two or more unrelated patients. We never found more than a single mutation in the same patient. As expected, mutations were dispersed all over the gene. However, the majority of the mutations was found in the central part of the gene, in exons 20 to 40 (Fig 4). Additionally, the 820 bp promoter region, as well as two novel 9 bp exons, exons 41A and 41B, were sequenced from patients without mutations in any of the 51 exons, but no mutations were found in these regions.

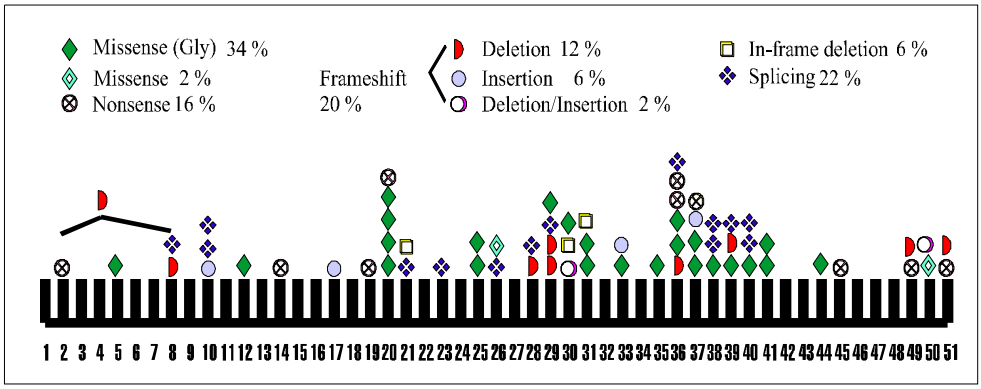

Fig 4. Schematic illustration of the distribution of different mutations found in the COL4A5 gene in this study. Numbered black bars describe 51 exons.

A missense mutation was identified in 31 patients. In all but 2, this affected the codon for glycine. One substitution other than glycine was a conserved leucine changing to arginine (L1649R), found in two unrelated patients. The L1649R substitution is common in the Western United States, causing adult type AS with tardive hearing loss (Barker *et al*. 1996). Both of the patients in our study were from the USA, but from different states. However, they were most probably descendants of a common ancestor. We also found a sequence variation, an unconserved lysine substituted by asparagine (K664N), in three unrelated patients. No other sequence variants were found in these patients, and segregation was seen in one family. The K664N has been previously described by Knebelmann *et al*. (1996) in two families. It did not segregate with the disease and it was found from a healthy individual as well. Additionally, it was associated with a causative mutation in one case. Thus, the K664N alone cannot be considered causative for AS.

Glycine substitutions were found in over one-third of the patients. As discussed earlier, they are thought to cause kinks in the collagen molecule, and these in turn interfere with the normal assembly of the molecules into the network. However, one of the glycine substitutions in this study was found in the short 12th noncollagenous conserved interruption in exon 25. These interruptions, again, are thought to give natural flexibility to the collagenous structures. Maybe this is why the conversion of glycine624 to aspartic acid only causes a fairly mild phenotype, because none of the patients had reached ESRD.

However, three of them were females, and the age of two other male patients was not known. The G869R mutation was found in two unrelated patients. It could be stated that this mutation is the most common in AS, because it has now been found in ten unrelated European patients (Boye *et al*. 1995, Knebelmann *et al*. 1996, Lemmink *et al*. 1997, Plant *et al*. 1999). Others have also identified many of the glycine substitutions found in our study. These are G635D and G1357S found in British patients (Plant *et al*. 1999,) and G1107R found in British and Japanese patients (Plant *et al*. 1999, Inoue *et al*. 1999). Instead, some glycines have been found to be substituted by variable amino acids. Such substitutions are G409D (Renieri *et al*. 1996), G420Q, G1066R (Knebelmann *et al*. 1996), G1143S (Renieri *et al*. 1994) and G1205C (Knebelmann *et al*. 1996). Major differences in phenotypes can be seen in the case of glycine1143, since substitution to asparagine causes juvenile type AS without hearing loss or eye signs, and substitution to serine causes adult type AS with both hearing loss and eye abnormalities.

We could detect 12 nonsense mutations. Two of these were found in two unrelated patients. Both patients with the R373X mutation were from Finland, and might be distantly related. However, in another case, there was no family history of hematuria. The mutation has also been found in French (Knebelmann *et al*. 1996) and Italian (Renieri *et al*. 1996) patients, and in all cases it caused juvenile type AS, which is most probable also in the case of the Finnish patient without ESRD, because the patient is very young. Hearing loss and eye abnormalities are associated in two cases, in which the patients are older. The R1677X was also found in two unrelated patients. This mutation was first described by Barker *et al*. (1997) in three Ashkenazi-American families with a common ancestor. In our study, one patient was an American and might be a distant relative of the above-mentioned families, but another was from Denmark. In Ashkenazim, this mutation causes a fairly mild adult type AS with hearing loss of variable severity and without eye abnormalities, and may not significantly decrease the reproductive fitness. However, in the Danish patient, the phenotype is juvenile AS with hearing loss and eye signs, indicating a possible second mutation in an undetectable area of the gene.

About one-fourth of the mutations were in-frame deletions and frameshift mutations including deletions, insertions and two mutations with both deletion and insertion. One deletion was large, involving exons 2 to 8, accompanied by a juvenile form of AS. It could not be determined whether this deletion happens in frame or not (exons could not be PCR amplified). The deletion breakpoints, however, most probably lie in the introns, thus not causing a shift in reading frame. Juvenile form of AS is in most cases associated with large gene rearrangements or frameshift mutations (Jais *et al*. 2000). In this study, eleven of the frameshift mutations were only one base pair alterations, always causing a juvenile type AS. This was also the case in all other frameshift mutations. A nine base pair deletion in the collagenous sequence in exon 21, instead, caused an adult onset ESRD in one patient. This same mutation has been found in an Italian female patient without onset of ESRD (Renieri *et al*. 1996). There is also another report of an in-frame deletion of 27 bp of the collagenous sequence in exon 34, which causes adult type AS (Plant *et al*. 1999). It has been shown that small in-frame mutations usually cause the adult type of AS (Jais *et al*. 2000).

Fifteen of the mutations involved splice-sites. In one case, 32 base pairs were deleted from the intron-exon junction and 6 adjacent base pairs were then duplicated. This patient was a young male, and his mother was a carrier for this mutation. In at least three cases

where the age of onset of ESRD was known, incorrect splicing caused adult type AS with hearing loss and eye abnormalities, thus showing conformity with the findings by Jais *et al*. (2000). However, the mutation 1789+1G->A has also been found in a British patient (Plant *et al*. 1999) and the 3656+1G->T in a German patient (Netzer *et al*. 1993). The phenotype is juvenile AS with hearing loss and eye abnormalities in the former cases, and without eye abnormalities in the latter cases. The possible reason for juvenile onset of AS in these two cases could be explained by the different consequence of a splice-site mutation as discussed in the next paragraph.

It is often difficult to predict the consequence of a splicing mutation, as demonstrated in two cases of this study. In one case, an exon was skipped due to a point mutation at the -3 position at an acceptor splice-site. This is not a surprise, but one could also have expected the insertion of a two base-pair intronic sequence as a result of a new ag splice-site, as shown in another case described below. A closer look at the intron sequences around all 53 exons of the COL4A5 gene revealed that nucleotide g at position -3 cannot be found in any introns. In fact, nucleotide c is present at the -3 position in 74% of human genes (Krawczak *et al*. 1992). Thus, the consensus sequence is possibly not recognized and causes skipping of the exon. In this case, the reading frame was retained, resulting most probably in a shortened collagen molecule. In contrast, the mutation at position -9 in intron 38, generating a new ag-consensus splice-site, was shown to cause the insertion of an intronic sequence between two exons in mRNA. This is the first time reported in AS that a point mutation at this distant location causes the alternative splice-site to be used. The following insertion of eight base pairs causes a shift in reading frame, and furthermore a premature stop codon.

6.4 Splicing variants of one or two Gly-X-Y repeats

Sequencing of the large intron 41 of the COL4A5 gene resulted in identification of two novel 9 bp exons about 4.5 kb from each other. The amino acid sequence coded by these exons contains two Gly-X-Y repeats, *i.e*., Gly-Pro-Thr-Gly-Phe-Gln. PCR-amplification of cDNA libraries revealed that either one or two of these exons are alternatively spliced depending on the tissue. Also, the amount of different splice products was shown to be variabe when PCR products were sequenced.

The extra 18 bp sequence has been reported earlier to be present only in kidney mRNA. However, in this study, the 18 bp sequence was shown to be present in all the tissues examined, except in epithelia, where only exon 41A seemed to be expressed.

6.5 Autoantibodies in Alport and Goodpasture syndromes

Recombinant NC1 domains of all six different α chains (α1-α6) of type IV collagen were produced with two expression systems. In the mammalian system, secretion of the proteins was fairly poor and, in fact, in the case of α4(IV) mini-collagen, the product almost totally remained in the cells. Possibly, this is because α4(IV) is rich in cysteines, which make it very insoluble. Due to low yields with the mammalian system, the six NC1 domains were also produced in *E. coli*, with much better yield.

Synthetic peptides have been used in studies to identify the Goodpasture epitope, suggesting that the epitope is linear (Kefalides *et al.* 1993, Hellmark *et al.* 1996). However, some studies have demonstrated that the Goodpasture antigen might be sensitive for conformation in binding antibodies, because reduction of the disulfide bonds results in loss of reactivity (Wieslander *et al*. 1985, Kalluri *et al*. 1991). Furthermore, it is suspected that the specific epitope for antibodies is not the same in transplanted Alport patients who have developed anti-GBM disease (Turner & Rees 1996). And, in addition to antibodies against α3(IV) NC1, those against α5(IV) NC1 has also been found in anti-GBM disease patients (Kalluri *et al*. 1991, Dehan *et al*. 1996, Brainwood *et al*. 1998). Our results showed that binding of Goodpasture antibodies to the recombinant α3(IV) mini-collagen was not reduced by denaturing agent.

Bacterial NC1 domains were used in ELISA to detect antibodies against all six chains in Alport syndrome patients, who have not developed anti-GBM disease. Such patients have not been previously studied. In one transplanted female patient, surprisingly strong reaction against α3(IV) chain was observed and two of the transplanted male patients seemed to have, to some extent, antibodies against α5(IV). These results indicate that some Alport patients tend to have small amounts of circulating autoantibodies against type IV collagen without developing an anti-glomerular basement membrane disease after transplantation. It can be stated that the use of ELISA in the follow-up of Goodpasture and other, e.g. kidney transplantation patients, is strongly encouraged, and is supported by these results.

6.6 Conclusions

Genetic and immunologic abnormalities of type IV collagen were studied in this thesis. The COL4A5 gene was further characterized and the basis for an automated sequencing method for detection of mutations in X-linked Alport syndrome was developed. Additionally, two novel small exons were identified and their presence in different tissues was studied. Furthermore, all six recombinant type IV collagen NC1 domains were produced in two different systems and an ELISA method was developed to detect autoantibodies in Alport and Goodpasture syndromes.

Mutation analysis of the COL4A5 gene with the automated sequencing method resulted in an overall high detection rate of over 70%, when compared to 50% at its best in

other studies using the SSCP method. This is encouraging when commercial applicability of this method is considered. There is no service available at the moment for the screening of mutations in all exons, because it is time consuming and expensive. However, several questions have been addressed in this matter by Alport syndrome patients and their families on Alport syndrome home pages (http://www.cc.utah.edu/~cla6202/ASHP.htm). Thus, there seems to be a real need for such a service. It can be stated that if this method is switched over to a Perkin-Elmer sequencer, in which 72 samples can be processed simultaneously, a rough estimation for the optimum time of performing one analysis (*i.e.* one patient, all exons) will be three days, when DNA is the starting material.

Several studies have proposed conformation specificity for the Goodpasture antigen in the binding of autoantibodies of Goodpasture patients. However, the specificity of the target of autoantibodies in Alport patients after transplantation seems to differ. Goodpasture antibodies were detected with mammalian α3(IV) NC1 domain under nondenaturing, but not under denaturing conditions. However, bacterial recombinant NC1 domains detected antibodies in AS patients (who have not developed an anti-GBM disease) under reducing conditions. Results were furthermore confirmed with Western analysis using mammalian recombinant proteins. Thus, it can be concluded that bacterial recombinant proteins are appropriate for detection of autoantibodies in Alport syndrome patients. The overall results have direct clinical benefit, as they facilitate DNA-diagnosis of Alport syndrome.

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