Regulation of the key molecules of glomerular ultrafiltration in proteinuric models

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Academic Dissertation

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Yliopistopaino
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If language were liquid
It would be rushing in
Instead here we are
In a silence more eloquent
Than any word could ever be

These words are too solid
They don't move fast enough
To catch the blur in the brain
That flies by and is gone
Gone
Gone
Gone

By Suzanne Vega, 1985

CONTENTS

ABBREVIATIONS

A	adenine
aa	amino acid
ACTN4	gene encoding alpha-actinin-4
ATP	adenosine triphosphate
bp	basepair(s)
BB	brush border
CD2AP	CD2-associated protein
cDNA	complementary DNA
CNF	congenital nephrotic syndrome of the Finnish type
CNS	congenital nephrotic syndrome
C-terminus	carboxyterminus
DG	dystroglycans
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EM	electron microscopy
FCS	fetal calf serum
FITC	fluoresceinisothiocyanate
GBM	glomerular basement membrane
GAPDH	gene encoding glyseraldehyde-3-phosphate dehydrogenase
gp	glycoprotein
$HgCl_2$	mercuric chloride
HSPG	heparan sulphate proteoglycan
HN	Heymann nephritis
HRP	horse radish peroxidase
IF	immunofluorescence
IGF	insulin-like growth factor
kDa	kilodalton
KTS	amino acids lysine (K), threonine (T), serine (S)
L-NAME	L-N^G-nitroarginine-methylester
mAb	monoclonal antibody
MAC	membrane attack complex
Mab 5-1-6	monoclonal antibody 5-1-6
MCN	minimal change nephropathy
MN	membranous nephropathy
mRNA	messenger RNA
NPHS1	gene encoding nephrin
N-terminus	aminoterminus
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PA	puromycin aminonucleoside
PAX	paired pox
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
SA	sialic acids
SDS	sodium dodecyl sulphate
SD	slit diaphragm
TGFβ	transforming growth factor β
TJ	tight junction
ZO-1	zonula occludens-1
WT1	Wilms´ tumor 1
Å	ångström, 1 Å = 0,1 nm

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals:

I Ahola H, Wang S-X, **Luimula P**, Solin M-L, Holzman LB, and Holthöfer H: Cloning and expression of the rat nephrin homolog. Am J Pathol 155: 907-913, 1999

II **Luimula P**, Ahola H, Wang S-X, Solin M-L, Aaltonen P, Tikkanen I, Kerjaschki D, and Holthöfer H: Nephrin in experimental glomerular disease. Kidney Int 58: 1461-1468, 2000

III **Luimula P**, Aaltonen P, Ahola H, Palmén T, and Holthöfer H: Alternatively spliced nephrin in experimental glomerular disease of the rat. Pediatric Research 48:759-762, 2000

IV **Luimula P***, Sandström N*, Novikov D, and Holthöfer H. Podocyte-associated molecules in puromycin aminonucleoside nephrosis of the rat. Lab Invest 82: 713-718, 2002

V Lehtonen S*, **Luimula P***, Palmén T, McQuistan T, Holthöfer H, and Lehtonen E: Development of experimental acute nephrosis is associated with dysregulation of CD2-associated protein, submitted

* These two authors have contributed equally to the study

In addition to these, some unpublished results are included.

ABSTRACT

The abnormal permselectivity within the glomerulus manifests itself as proteinuria. The molecular mechanisms of proteinuria have remained obscure while knowledge of the structure and function of the glomerulus has steadily grown in the last forty years. These data have been largely obtained utilizing experimental animal models. After discovery of the NPHS1 gene, and the mutations within this gene causing congenital nephrotic syndrome of the Finnish type (CNF), the study of the molecular mechanisms of proteinuria has reached a new impetus.

Puromycin aminonucleoside (PA) nephrosis of the rat mimics human minimal change nephropathy (MCN) and is widely used as an animal model of proteinuria. The effacement of podocyte foot processes is characteristic of these two conditions. Recent studies have suggested that the slit diaphragm, spanning between the epithelial cell foot processes, is an essential constituent of the permeability barrier. In this thesis the rat homologue of the human nephrin complementary DNA (cDNA) was cloned to obtain tools for the molecular analysis of the experimental diseases. Additionally, the correlation between nephrin messenger RNA (mRNA) and protein as well as the regulation of an alternatively spliced nephrin mRNA variant in the experimental models were studied. Finally, the distinct alterations of other podocyte-associated molecules in the PA nephrosis model were determined.

Cloning of the cDNA for the nephrin rat homologue revealed, in addition to the full-length nephrin cDNA, the spliced forms, termed α and β. Full-length nephrin and its α splicing variant mRNAs were shown to be down-regulated in PA nephrosis. The study of the rat nephrin protein also revealed reduction after puromycin treatment. Nephrin appeared in the urine 6 and 9 days after induction of PA nephrosis. Study of other podocyte-associated molecules revealed a set of distinctly regulated molecules. Expression of podocin and CD2-associated protein (CD2AP) closely resembled to that of nephrin, whereas β1 integrin was up-

regulated after administration of PA. In the chronic Heymann nephritis model the expression levels of the studied molecules, nephrin and CD2AP, were not significantly altered during the course of the disease.

INTRODUCTION

Proteinuria is a central clinical feature of renal diseases. In most cases the cause of proteinuria is a defect in glomerular permeability. The pathogenesis of proteinuria has been studied intensively for more than a hundred years; however, it still remains unknown. In the late 1950s and the beginning of the 1960s, experimental animal models of glomerular damage were introduced and became part of routine research. Ever since they have been used to explore the mechanisms of kidney damage. The PA model of the rat is a well-defined and extensively studied model of acute proteinuric disease (1) and it also mimics human minimal change nephropathy (MCN). Another valuable kidney disease model is Heymann nephritis (HN), a model of human membranous nephropathy (MN) (2). In this immunocomplex glomerular disease, antibodies against podocyte antigens result in a gradually progressing disease. In 1998 a protein molecule called nephrin was identified. In a genetic disease, congenital nephrotic syndrome of the Finnish type (CNF, NPHS1), the gene coding for nephrin is mutated (3). Mutation in *NPHS1* leads either to the lack of the whole protein product or the expression of truncated forms impairing the specific structure of the foot processes of glomerular podocytes, and results in proteinuria. In addition to nephrin, other important podocyte-associated molecules, including CD2-associated protein (CD2AP) (4) and podocin (5), have since been identified. These discoveries have allowed a new approach to investigate molecules involved in the pathogenesis of proteinuria. In this study two experimental models of proteinuria, PA nephrosis and HN, were employed to study the functional genetics of nephrin and other podocyte-associated molecules.

REVIEW OF THE LITERATURE

1. Glomerulus as a unique structure for filtration of blood

The functional unit of the kidney is a nephron, of which there are approximately one million in a human kidney. The main domains of the nephron are the glomerulus, the proximal tubulus, the loop of Henle, the distal tubulus and the collecting duct. All these segments can further be subdivided into smaller functional units by morphology. The glomerulus, also termed the renal corpuscule, is responsible for ultrafiltration of blood. Glomerulus consists of a branching capillary tuft that is surrounded by a parietal epithelium known as Bowmann´s capsule. The filtration barrier of glomerulus, through which the ultrafiltration of blood takes place, is composed of three components: the endothelium, the basement membrane, and the epithelium (6).

1.1 Development of the glomerular structures

To understand the normal and diseased function of the glomerulus, I next briefly introduce the development of the kidney. The morphogenesis of the permanent mammalian kidney, the metanephros, is a reciprocal spatial and temporal phenomenon with several interactions between epithelial and mesenchymal cells (7, 8). A variety of regulatory factors participate in the development of the permanent kidney (9). Metanephros developes from two tissue components: the mesenchymal nephric cord (blastema) and the epithelial, originally mesoderm-derived, Wolffian duct. First, metanephrogenic mesenchyme induces the branching of nephric ducts that form the epithelial tubes called ureteric buds. These buds invade into metanephrogenic mesenchyme and in turn induce mesenchyme to differentiate into individual nephrons. Developing mesenchyme further induces ureteric bud to elongate and branch, and it eventually develops into collectiong ducts and the ureter (7, 10).

Nephrogenesis consists of six distinct stages. The first two stages include the first induction and condensation, during which mesenchyme cells adhere and condense around the tip of the ureteric bud. At the third and the fourth stage the condensate mesenchyme gives rise to renal vesicles, which further develop into the comma-shape body. In the fifth and sixth stages the structure known as a S-shaped body forms and the tubular part elongates. The farthest cells from the collecting duct, the future glomerulus, polarize and form lower glomerular cleft from which the visceral epithelial cells (podocytes) originate. Capillaries diffuse this cleft and the opposite end of the tubule elongates to form the proximal and distal tubules and the structures of the loop of Henle (Figure 1) (7).

Differentiation of the metanephros is tightly regulated by a variety of transcription factors, growth factors and extracellular matrix components (11). Examples of major transcription factors involved include the paired box gene 2 (PAX2) (12), homeobox genes (HOX), Wilms´ tumor gene (WT-1) (13), and proto-oncogenes N-myc and c-myc (9). Additionally, the growth factors involved in the regulation of nephron development include e.g. the insulin-like growth factor (IGF) 1 and 2 (14), vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) (15). Other molecules, such as the homologue of Drosophila wingless gene 4 (Wnt4), glial cell line-derived neurotrophic factor (GDNF) and its receptor cRET, as well as some extracellular matrix components have a distinct role in the development of the metanephros (15).

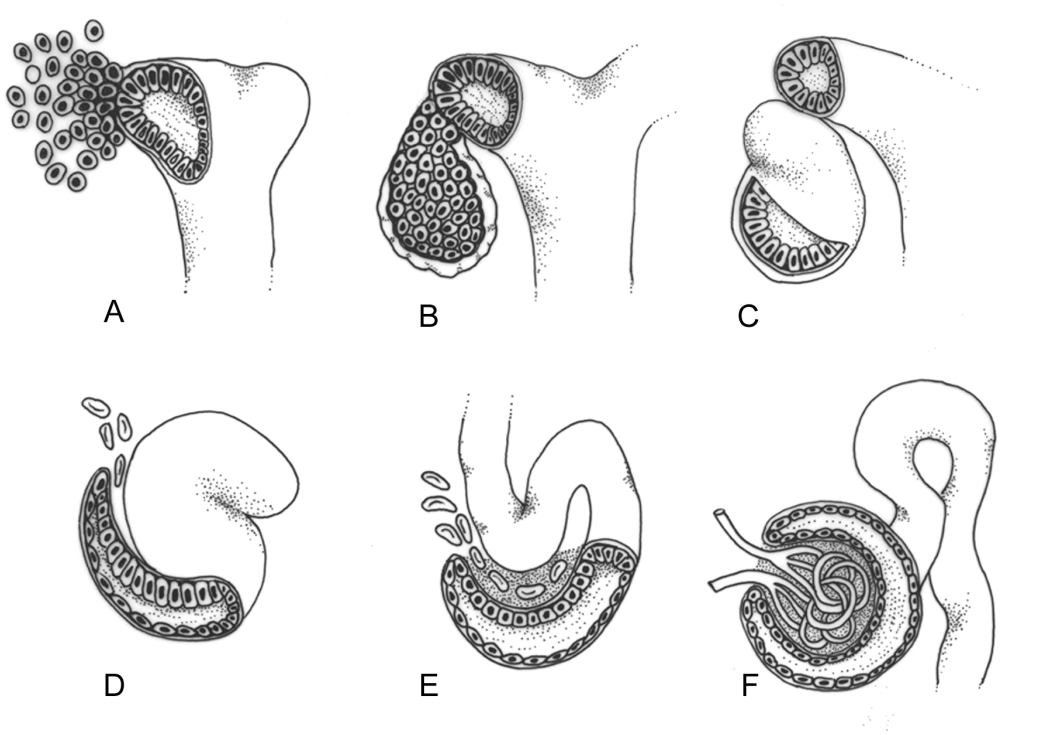

Figure 1. Different stages in nephrogenesis. Mesenchyme is aggregated around the tip of the ureteric bud (A) and further condensates forming a vesicle (B). This vesicle then develops into comma- (C) and S-shaped bodies with upper and lower cleft (D). Capillaries invaginate into the lower cleft and start to form a capillary tuft (E) and further develop into glomerulus (F). (After Saxen, 1987)

1.2 Endothelial cell layer

The blood is brought to the glomerulus through the afferent arteriole that is eventually split up into smaller capillaries. The thin endothelial cell layer lines the inside of the capillary wall and is openly fenestrated (Figure 2). Endothelial cells together with epithelial cells contribute to the formation of the glomerular basement membrane (GBM) (16). In the rat, the fenestrae constitute approximately 54% of the total endothelial surface area (17). This endothelium does not serve as an appreciable barrier to macromolecules; however, it prevents blood cells from escaping the circulation. Endothelial cells are negatively charged due to the abundancy of sialic acid (SA)-rich glycoproteins and -lipids. A glycoproteins termed podocalyxin is one of the known components comprising the polyanionic surface (18).

1.3 Glomerular basement membrane (GBM)

The GBM, the meshwork of extracellular matrix derived from both endothelium and epithelium, has three layers: *lamina rara interna*, *lamina densa and lamina rara externa* (Figure 2). The thick *lamina densa* prevents particles equal to the size of albumin or larger from entering the primary urine, while the lamina rara interna and externa, which contain anionic sites, prevent charged particles from going through the barrier (19). In addition to its main components type IV collagen and laminin, the GBM contains nidogen/entactin, fibronectin, and heparan sulphate (20, 21).

1.4 Epithelial cell layer and slit diaphragm (SD)

The glomerular basement membrane is lined from the urinary space aspect by visceral epithelial cells that are called podocytes (6). The unique structure of podocytes is divided into three parts: nucleus-containing cell body, major (primary) processes and foot (secondary) processes. The latter processes are embracing the capillary loop. The extremely negatively charged cell membrane of the foot processes is polarized and contains two membrane

domains, the basal and apical membrane. Between these two domains locates the slit diaphragm (SD) that connects two foot processes together. The soles of the foot processes are the only part of podocytes that are in direct contact with the GBM (Figure 2). This basal membrane part of podocytes has been shown to have intense endocytotic activity (22). The apical membrane of foot process is covered by glycoproteins and glycolipids that maintain the separation between foot processes and podocytes to parietal epithelial cells. Between these interdigitating foot processes locate the slit diaphragm (SD) that are 25- 40 nm wide and 6 nm thick. SD functions as the final selective apparatus in the filtration mechanism (Figure 2). Rodewald and Karnowsky have suggested, by analysing EM micrographs, that SD is a zipper-like construction (23). The exact molecular composition of slit diaphragm was shrouded in mystery almost twenty years but recently, several studies have revealed new details on structure and function of SD (See 4. Molecular composition of podocytes).

1.5 Mesangial cells

In addition to endothelial cells and epithelial podocytes the glomerulus contains mesangial cells that are situated between capillaries and provide support to the glomerulus. Mesangial cells have also many important functional properties (24) and resemble smooth muscle cells in many aspects (25). They synthesize matrix components, have phagocytic activity and participate in the modulation of glomerular filtration. They respond and synthesize vasoactive peptides, growth hormones, cytokines, bioactive lipids and enzymes (26, 27).

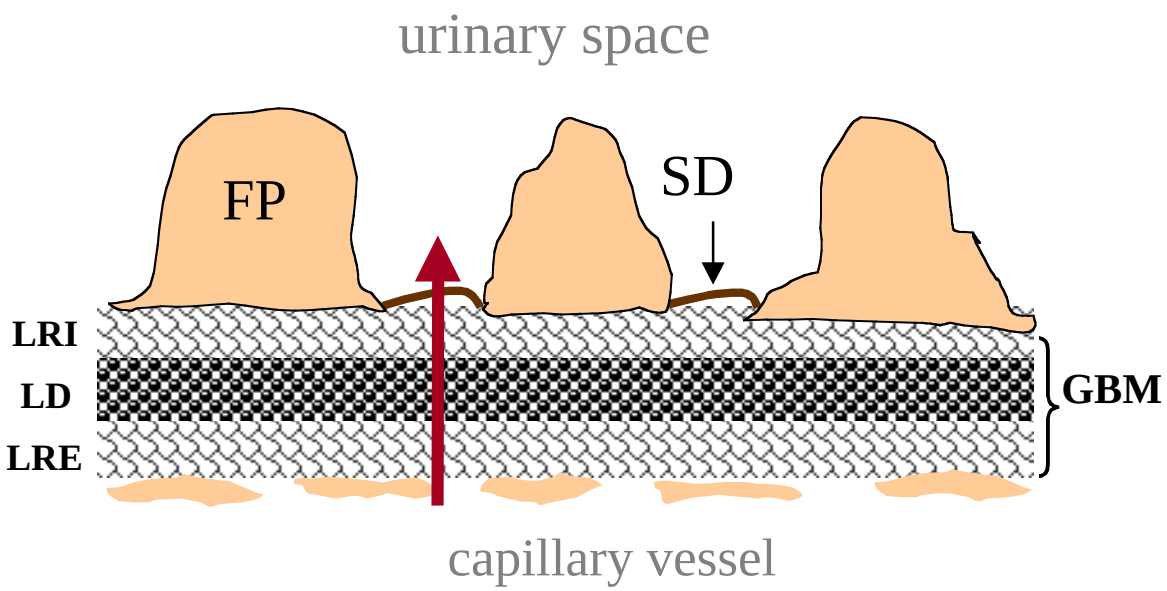

Figure 2. Schematic presentation of the filtration barrier in the glomerulus. FP; foot processes, SD; slit diaphragm, LRI; lamina rara interna, LRE; lamina rara externa, LD; lamina densa; GBM; glomerular basement membrane. The arrow represents the direction of the ultrafiltration of blood.

2. Proteinuria

The glomerular ultrafiltration of blood, allowing plasma solutes and water to enter the urinary space but keeping still cells and proteins in circulation, is the first and most important stage in formation of urine. In human, approximately 150 liters of primary urine is formed daily from plasma through the glomerular filtration barrier. Some proteins (80 mg/24h) of small molecular weight always enter the primary urine according to their size and charge. Studies performed with dextrans and other tracer molecules have shown that small (radius under 20 Å) and neutral molecules, such as inulin, enter freely the urinary space. The glomerular filtration barrier is less permeable to uncharged molecules ranging between radii 20-50 Å (28,

29). Albumin, with its heavy negative charge and a radius of 36 Å, is predominantly retained in circulation but often appears in urine in various disease states (28-31). Proteinuria is defined as a leakage of proteins into the urine. It is measured by the amount and/or content of proteins in urine, and is of either glomerular or tubular origin, glomerular injury being a primary reason for proteinuria. In glomerular injury, the permselectivity of a glomerulus is altered, and the plasma proteins leak to primary urine (32, 33). Alterations in any structural or functional component of the glomerular filtration barrier can lead to proteinuria (31, 34). Normally small amounts of proteins leak into the urinary space, but these proteins are reabsorbed by proximal tubular cells. The reabsortion can fail, for example, due to metallic ions and cause tubular proteinuria (35).

2.1 Causes of proteinuria

Proteinuria, being the one of the hallmarks of nephrotic syndrome, may be caused by various mechanisms including immunological, toxic, metabolic or hereditary (36). In immunological mechanisms, inflammatory tissue damage is mediated e.g. by circulating and infiltrating macrophages, lymphocytes and platelets (34, 37). These circulating cells can also cause the glomerular injury via reactive oxygen species (ROS) and cause glomerular hypercellularity. Non-inflammatory immune damage includes antibodies against GBM and glomerular cell membranes with or without the involvement of the components of complement (34, 37). Immune injury starts with the formation of immune deposits and can lead to the activation of complement that finally end up in the assembly of the membrane attack complex (MAC, C5b-9). MAC promotes the epithelial cell and GBM damage as seen in membranous nephropathy (MN), the human autoimmune disease, and minimal change nephropathy (MCN), common childhood proteinuric disease (34, 37, 38).

Transient or haemodynamic proteinuria can be observed after heavy exercise, in patients with heart failure and with fever condition (35). Massive and persistent proteinuria, the excretion

rate of which exceeds over 3.5 g/24 hours protein in urine, lowers the concentration of plasma proteins, including albumin, which is one of the most important components maintaining the osmotic balance. The results are hypoalbuminemia and reduction of oncotic pressure of plasma, which in turn, leads to edema. Activation of the rennin-angiotensin system, increased aldosterone secretion and cholesterol synthesis, i.e. hyperlipidemia, are consequences. All these together give rise to the nephrotic syndrome (35).

2.2 Congenital nephrotic syndromes

Congenital or infantile nephrotic syndromes are rare renal diseases already manifested during the first years of life. Symptoms, including proteinuria, hypoproteinemia and edema, are signs of congenital nephrotic syndrome (CNS), and become manifest soon after birth and before the age of three months. CNS, a heterogeneous group with a variety of kidney disorders, is classified as primary or secondary, e.g. acquired, inherited, or sporadic, diseases. One example of primary nephrotic syndrome occurring within first 3 months of life is the congenital nephrotic syndrome of the Finnish type (CNF). When compared with other nephrotic syndromes that occur in the later ages, the prognosis of CNS is poor with a need of nephrectomy and renal transplantation (39, 40).

Secondary nephrotic syndrome can be caused by systemic diseases, such as diabetes mellitus and amyloidosis, microbial infections or toxins (36, 41).

2.3 Congenital Nephrotic Syndrome of the Finnish type

In 1956 Hallman et al. described for the first time the congenital nephrotic syndrome of the Finnish type (CNF, NPHS1) that belongs to the Finnish disease heritage (42). Compared with other congenital nephrotic syndromes CNF has not only the common symptoms but also has distinctive features and pathological findings. It is an autosomal recessive disease with an

incidence of 12 per 10000 births in Finland (43). Few sporadic cases have been reported randomly in the world, especially in the United States (44-46).

2.3.1 Clinical aspects

The most important clinical hallmark of CNF patients is massive proteinuria, starting already *in utero*. The affected infants are born prematurely by the 36th gestational week and the placenta is typically enlarged (47). These newborn patients present edema, hypoalbunemia, and hyperlipidemia (43). Additional symptoms include abdominal distension, ascites, umbilical hernias and developmental retardation. The loss of immunoglobulins predisposes to frequent infections (44) and before 1980s the outcome of CNF was invariably fatal. Later, substitute treatment with proteins and nutrients, followed by bilateral nephrectomy and ultimately renal transplantation has prolonged the life expectancy of CNF patients and dramatically changed the disease outcome: the patients do not appear to develop additional symptoms in other organ systems (48).

2.3.2 Pathology of CNF

The pathology of CNF has been under intensive research for decades but the molecular mechanism of the disease has remained unknown until recently (see Genetics of CNF). In foetal CNF kidneys the organogenesis and nephrogenesis do not seem to differ from normal kidneys (49). However, the representative morphological changes are that CNF neonates have 2-3 -fold larger kidneys than normal, with increased number and relative volume of glomeruli (49).

In several light microscopy studies the apparent morphological changes in CNF are mesangial cell hyperplasia, including accumulation of mesangial matrix, dilatation of proximal tubules and tubular atrophy (40, 49, 50). Out of these, particularly, dilated proximal tubuli are the consequence of protein leakage into urine (42). In electron microscopy studies, flattening of

podocyte foot processes has been constantly observed (51, 52). In additon, an increased number of endocytotic droplets has been observed in endothelial cells and electron lucent vacuoles in mesangial cells (52). Reports on the thickness of GBM in CNF patients are contradictory. While Autio-Harmainen and Rapola reported thinning of GBM (53), Ljungberg et al. could observe light thickening of GBM (54). The constant proteinuria eventually leads to the fibrotic kidneys in the course of nephrosis (55).

2.3.3 Diagnosis and treatment

The prenatal diagnosis was developed to measure the high concentrations of α-fetoprotein in maternal serum or amniotic fluid (56, 57). However, raised α-fetoprotein levels imply not only CNF but also other foetal disorders such as defects in neural tube or abdominal wall. Poor screening methods have given false positives and led to abortions of healthy foetuses. The identification of the *NPHS1* gene and its localization to chromosome 19 has allowed a more accurate diagnosis of the disease that can be performed on the 12^{th} or 13^{th} gestational week by PCR-restriction fragment length polymorphism (PCR-RFLP) and a dual colour oligonucleotide ligation assay (OLA) (58, 59).

The diagnosis of CNF can be set postnatally by large placenta, occurrence of proteinuria, exclusion of other types of CNS, positive family history and normal glomerular filtration rate during the first six months of life (48).

The only life-saving treatment for the CNF patients is early bilateral nephrectomy and subsequent renal transplantation (44, 48). Following nephrectomy the patients must remain on continuous cyclic peritoneal dialysis and nutritional correction until their body weight reaches 7 kg, after which they are suitable for transplantation. The regular transplantation treatment for CNF began in 1985 in Finland (44, 48).

2.3.4 Genetics of CNF

Several basement membrane components (54, 60) as well as embryonic developmental and differentiation factors (61) have been studied as potential gene defects causing CNF. Heparan sulphate proteoglycans (HSPG) partly produce anionic charge of GBM, but a defect in HSPG has been excluded as a potential cause of CNF (54) as well as *PAX2* that is coding for a paired-box transcription factor expressed during the development of the kidney (61-63).

The gene defect of CNF was localized to the long arm of chromosome 19, location at 19q13.1, with disequilibrium analysis (64, 65). In 1998, a 26-kb sequence encoding nephrin (*NHPS1)* was identified by Kestilä *et al.* (3). 94% of CNF cases in Finland are caused by two nephrin mutations: FIN_{major} (nt121delCT) results in an early stop codon at exon 2 causing a frameshift and a failure to express the full-length molecule. FIN_{minor} (R1109X) causes a nonsense mutation in exon 26 encoding the intracellular part of nephrin in residue 1109 (66, 67). In addition to these, roughly 50 different point mutations in the coding region of *NPHS1* have been reported (68, 69).

2.4 Minimal change nephropathy

Minimal change nephrotic syndrome (MCN), previously also called lipoid nephrosis (70), is the most common primary nephrotic syndrome of childhood worldwide. The disease manifests usually in early childhood, from 2 to 6 years of age with a incidence of 1.8-5 cases/1000000/year. For unknown reasons, MCN is more common in Asian and Arab populations (36).

The main clinical finding of MCN is significant proteinuria that exceeds 40 mg/hr/m^2. Microscopic hematuria is found in 1:5 of cases. Raised serun lipid concentrations including cholesterol, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and triglycerides, are usually observed (36). When studied by light microscopy the overall

morphological changes appear negligible. In EM studies a profound change is the effacement of podocytes while GBM is mostly unaltered. Lipid droplets are found in proximal tubules. The risk to develop MCN is higher if the child is atopic and has HLA B12 antigen. Systemic diseases, e.g., syphilis, HIV and solid tumours are known to cause minimal change lesions (36).

Little is known about the pathogenesis of MCN. It is assumed that autoimmune mechanisms cause this kidney disease, since also abnormal function of T-lymphocytes can be the cause of the symptoms (71). In most cases the MCN responds to corticosteroids and therefore minimal change patients are mainly treated with corticosteroids. Sometimes alkylating cytotoxic drugs are required (72).

3. Experimental models of proteinuria

Cell culture approaches have given a great amount of useful information about the function and structure of the kidney but they do not provide information on the complex physiological interactions *in vivo*. Over time, the cultured renal cells lose their cell-specific markers and morphology. Glomerular podocytes are particularly difficult to culture. In addition, the lack of fresh human kidney material, obtained from biopsies or autopsies, has forced researchers to look for other ways of studying kidney diseases. More than forty years ago a number of animal models of human renal diseases was taken into routine use. The reproducibility has made experimental models indispensable in renal disease research. The PA-induced model of acute nephrosis, and brush border (BB) antigen-induced HN, a chronic model of nephritis, are the most used and well-defined.

3.1 Puromycin aminonucleoside nephrosis

In 1959 Vernier et al. reported on changes in kidney glomeruli in the aminonucleoside nephrosis of the rat (73). This classical PA nephrosis model of the rat mimics human MCN by

its histology and ultrastructure. PA nephrosis can be induced with either a single or repeated PA injections that result in bulky proteinuria in rats starting around day 3-4 post-injection; proteinuria peaks around day 10 and resolves by day 28 (74). In addition to proteinuria in PA nephrosis, morphological alterations in podocytes are detected in glomeruli by histochemistry, immunohistochemistry and immunoelectron microscopy.

3.1.1 Morphological changes in PAN

In general the dilatation of the proximal, distal and collecting tubules and, furthermore, thickening of the GBM are detected under light microscopy studies. In a more detailed study, the effect of PA on epithelial visceral cells is drastic, including reduction in the number of foot processes resulting from fusion or retraction of the processes (73, 74). In addition to this early change, SDs are altered, dislocated or lost. At the same time, the appearance of occluding-type junctions is evident. The remaining SDs are located above these junctions (75). Later, during the course of the disease, formation of vacuoles due to increased endocytotic and lysosomic activity is seen. Further on, the number of phagosomes is increased and the epithelium is detached from GBM (75). The growing cytoplasmic balloon-like vacuoles finally rupture allowing GBM a direct contact with the urinary space (76). The difference in the course of PA nephrosis between continuous daily administrations and a single intravenous injections of the drug is seen in the early and late changes of morphology.

3.1.2 Pathogenesis and molecular changes in PAN

The target of puromycin aminonucleoside in the kidney is podocyte-specific. Although several studies have been performed concerning the molecular pathogenesis, the exact molecular mechanism through which PA influences the podocytes is still unknown.

The role of reactive oxygen species (ROS) and lipid peroxidation mediating the glomerular injury in PA nephrosis has been under extensive study. *In vitro* analysis shows generation of

O_2^-, H_2O_2 and OH^- in glomerular epithelial cell culture and rat kidney slices (77, 78). The involvement of the xanthine pathway as a mediator of PA nephrosis has been debated (79). Fawcett et al. and Pedraza-Chaverri and colleagues showed a connection between lipid peroxidation and proteinuria in PAN (80, 81) and it was shown that antioxidants, for example probucol, were shown to significantly reduce renal injury and proteinuria (79, 82-84)

In the course of PAN, rats show overall molecular changes in glomerular podocytes. Earlier studies have concentrated mainly on changes of the molecular architecture in GBM that consists mainly of HSPG. In 1986, Mahan et al. showed reduction in anionic charge sites of HSPG in PAN treated rats (85). In addition, a significant 75% reduction of SA on plasma membrane was detected after induction of PA nephrosis (86). Whiteside et al. reported contradictory data of the molecular changes and reduced HSPG or SA content in PA nephrosis (87). They concluded that epithelial detachment led to the development of proteinuria, not the changes in GBM.

The crucial role of SD in ultrafiltration of blood shifted the focus onto SD molecules in the pathogenesis of PA nephrosis. Zonula occludens-1 (ZO-1), a protein of tight junctions, has been intensively studied (88-91). After PAN induction, ZO-1 appears to dislocate from SDs and has been found in *de novo*-formed occluding junction below SD (89, 92). An integral membrane glycoprotein maintaining the unique structure of podocytes, podoplanin, is downregulated in PAN (93). Takeda et al. reported that disconnection of a highly glycosylated CD34-related transmembrane protein, podocalyxin, from the actin cytoskeleton due to the loss of its negative charge led to the changes in foot processes (94)

3.2 Mercuric chloride ($HgCl_2$) induction

Mercuric chloride ($HgCl_2$) is classified as a nephrotoxin and proximal tubular cell damage is a major outcome after administration of $HgCl_2$ (17, 95, 96). $HgCl_2$ toxicity involves influences on sulphydryl groups, phospholipid membranes and lysosomes (97-99). In chronic

exposure, $HgCl_2$ is accumulated intracellularly in lysosomes of the kidney (97). In most cases $HgCl_2$ injury causes the depletion of glutathione that protects from oxidative renal damage (100, 101). Additionally, Nath et al. observed increased hydrogen peroxidase production (101, 102) that further leads to the progression of renal injury. Mercuric chloride affects the function of mitochondria in the kidney and in T-lymphocytes (101). The dosage of 5 mg/kg of $HgCl_2$ given to animals subcutaneously suppresses the rate of adenoside 5´-triphosphate (ATP) synthesis (95). $HgCl_2$ can also cause membranous nephropathy and anti-glomerular immune deposits (103). In T-lymphocytes, it induces apoptosis via mitochondrial malfunction (104).

3.3 Heymann nephritis

The authentic Heymann nephritis (HN) is an experimental renal disease in rats mimicking common MN in adults (2). Similarly to the human disease HN is classified as an autoimmune disease with production of subepithelial immune deposits. The formation of immune deposits on the podocyte cell surface can be considered a failure of endocytotic pathway (105). Activation of C5b-9/MAC occurs during the progress of the HN causing proteinuria (22, 105, 106). HN can be induced in two ways: passively and actively. In the active HN, rats are injected with a crude extract of proximal tubular BB antigen, Fx1A, whereas in the passive form of HN rats are injected with anti-Fx1A antibodies.

The antigen gp330, also called megalin, has been shown to be essential for the induction of HN (107). The 515 kDa protein gp330/megalin is located in invaginations of microvilli of the brush border of the convoluted proximal tubule. It forms antigen-antibody deposits at the clathrin-coated pits in GBM. The glycoprotein gp 330/megalin is related to the low-density lipoprotein receptors and links with a 44-kDa receptor-associated protein, RAP (22, 107)

Antibodies directed against lipid antigens that associate with immune deposit-forming gp30/megalin immune complexes activate complement C5b-9. MAC presumably causes

upregulation of NAPDH oxidoreductase enzyme complex in the glomerulus leading to the production of ROS, thus damaging podocytes. Lipid peroxidation is also observed in GBM matrix (105). Accordingly, proteinuria associated with HN can be reduced by treatment with probucol, an inhibitor of lipid peroxidation (105).

4. Molecular composition of the podocytes

The podocyte needs to be well equipped with functional and morphological molecules to maintain its highly organized morphology (Figure 3). The molecular architecture of podocyte foot processes, their location and postulated function is listed in Table 1. according to Kerjaschki et al. (108). Some of the main components of the foot processes are introduced more in detail below.

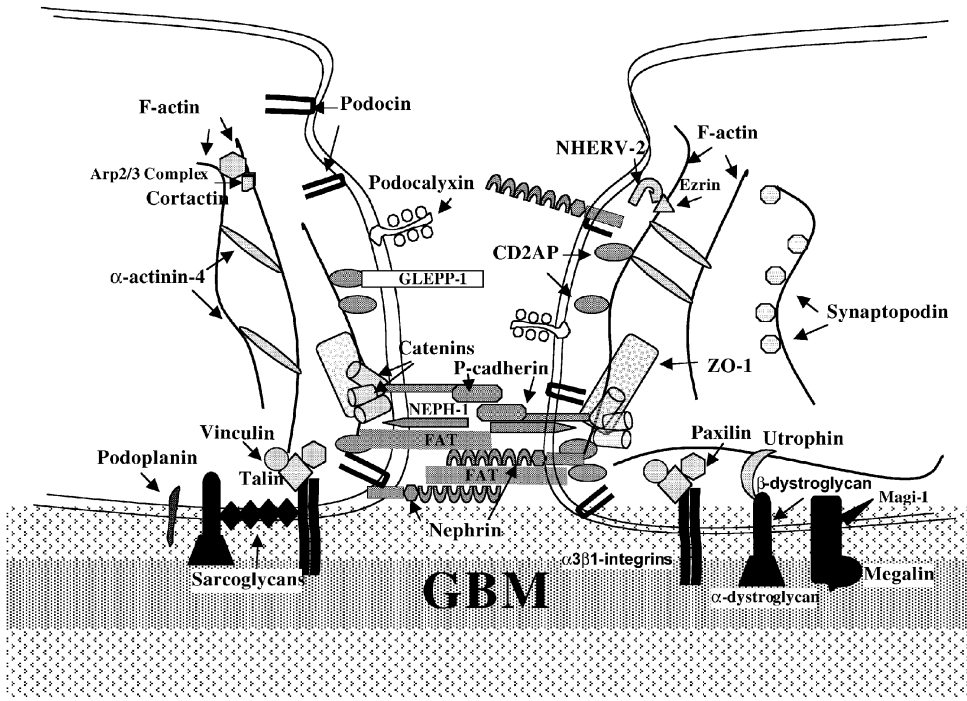

Figure 3. Molecular composition of podocyte foot processes. A shematic drawing of molecules located in different domains of the membrane of the foot processes.

4.1 Nephrin

Nephrin is a transmembrane protein encoded by *NPHS1*, and with 1241 amino acids, whereby the calculated molecular size of nephrin is 132.5 kDa in human. After post-translational modifications, such as glycosylation, the size of nephrin increases to about 185 kDa when detected by Western blotting. Nephrin belongs to the immunoglobulin-like superfamily of proteins and has typically eight extracellular immunoglobulin-like modules, a fibronectin type III motif, a transmembrane part, and an intracellular domain with tyrosine phosporylation sites (3). Nephrin is primarily located at SD (109-111). It is suggested that nephrin participates in cell-cell or cell-matrix adhesions. One hypothesis is that nephrin acts as a major functional component of the glomerular filtration barrier in SD. Further, it is proposed that nephrin binds to another nephrin of the opposite site podocyte, forming a ladder-like structure (111). Mutation in *NPHS1* leads to incomplete development of the kidney, lacking SDs (112). Simons et al. reported nephrin phosphorylation to be associated with lipid rafts, compartment of cell membranes enriched with glycoshingolipids, cholesterol, and signalling molecules (113). Additional proposed function for nephrin is involvement in cell signalling with podocin (114). In addition to the kidney, nephrin protein expression is found in human pancreas, newborn mouse cerebellum, and mouse testis (115-117).

4.2 CD2AP

CD2-associated protein (CD2AP) is an 80kDa soluble adapter protein expressed in a great variety of tissues (118, 119). CD2AP contains three SH-domains at the amino terminus that are needed in protein-protein interactions. This suggests a possible regulatory or signalling function for CD2AP. In the adult kidney it is located mainly in glomeruli and is also found in the proximal and distal tubuli, as well as the collecting duct (119). Acting as a link between adhesion proteins of the immunoglobulin superfamily and the cytoskeleton, CD2AP has numerous binding partners. It has been reported to be coexpressed with nephrin (120, 121)

and to bind actin (Lehtonen et al. in press). For the normal kidney organogenesis CD2AP has been shown to be crucial, especially in developing podocytes (120). It interacts with polycystin-2 gene and can be associated with type 2 polycystic kidney disease (119). CD2AP knockout mice develop nephrotic syndrome with proteinuria at the age of two weeks and they die at the age of six to seven weeks. Morphological changes in CD2AP knockout mice include fusion of the foot processes, mesangial deposits and proliferation similar to CNF (4).

4.3 Podocin

The *NPHS2* gene, which has been mapped to the long arm of chromosome 1, encodes a 42–kDa integral membrane protein called podocin (122). When mutated, it is responsible for an autosomal recessive steroid resistant nephrotic syndrome (5). Podocin belongs to the stomatin protein family, the members of which form homo-oligomers, and is solely expressed in developing and mature podocytes. A predicted structure of podocin is a hairpin-like molecule with a cytosolic N- and C-terminus. It functions as a link between the cytoskeleton and SD. Podocin has been suggested to interact with other proteins, e.g. α-actinin and nephrin (114, 123-125). It has been shown that podocin enhances the signalling events by nephrin (114). Recently it was reported that podocin is clusterred in lipid rafts in SD (125).

4.4 Alpha-actinin-4

Four isoforms of α–actinin-4 have been identified (126). These actin-associated proteins crosslink actin filaments into bundles. The gene, *ACTN4,* encodes a cytoplasmic protein, α–actinin-4 (126) Honda et al. found that α– actinin-4 was more concentrated in filopodia of the cell (126). This non-muscle isoform of actinin is essential for cell motility and cancer invasion. Furthermore, it plays a role in endocytosis (126, 128). In the glomerulus, α-actinin-4 indirectly anchors actin filaments of podocytes to GBM (129, 130). In *in vitro* analysis, a mutant α-actinin-4 adheres more intensively to filamentous actin than the wild type α-actinin-4 (127). Mutation in *ATCN4* gene produces renal defect known as familial focal segmental

glomerulosclerosis (127). α-actinin-4 has several interaction partners, including zyxin (131) and a cerebral transmembrane protein densin (132).

4.5 Integrins

The family of cell surface proteins called integrins is wide and versatile. These molecules share a common function of attaching cells to the extracellular matrix. Integrins form heterodimers consisting of two subunits, α and β (133, 134). There are several α and β subunits identified. The heterodimers cannot form in a random manner: certain α subunits require certain β subunits. Integrins are expressed in various tissues and have a variety of ligands (133, 134). In podocytes, especially α3β1 integrin is found at the soles of the processes. It attaches the cytoskeleton to GBM by binding laminin–1, collagen types I and IV, fibronectin or entactin/nidogen (135). The cytoplasmic site of β1 integrin has a binding site for a α– actinin-4 forming a link between actin cytoskeleton and integrins (129). Mice deficient (knockout) for α3 subunit fail to develop functionally or morphologically normal foot processes (136, 137).

4.6 Dystroglycans

Dystroglycans (DG) were first characterized in skeletal muscle cells associated with the dystrophin complex (138). DG function as a crucial link between the actin cytoskeleton and extracellular matrix (139). After post-translational modifications DG is cleaved into two forms: a peripheral membrane form α and a transmembrane form β. DG have various binding partners in the extracellular matrix, for example laminin-2,4 and agrin (140). They are involved in arenavirus infections by binding viruses directly, and are needed for entry of *Mycobacterium leprae* into Schwann cells (141). In non-muscular cells DG supports the assembly of the extracellular matrix, i.e. basal lamina, where they bind laminin-1, perlecan and agrin (140). DG are highly expressed in soles of the foot processes of glomerular

podocytes. DG knockout mice fail to develop Reichert´s membrane, extra-embryonic basement membrane, during early embryogenesis, and they die at embryonic day 6.5 (142). Due to this, chimaeric mice deficient for DG were constructed and they developed muscular dystrophy (143).

Table 1. The list of molecules linked to podocyte foot prosesses

molecule	location	type	function
NHERV-2	apical membrane domain	IC	link between podocalyxin and actin
Ezrin	apical membrane domain	IC	link between podocalyxin and actin
Glepp-1	apical membrane domain	TM	membrane phosphatase
Catenins	apical membrane domain	IC	associations with P-cadherin
P-cadherin	slit diaphragm domain	TM	component of slit diaphragm
FAT	slit diaphragm domain	TM	component of slit diaphragm
Neph-1	slit diaphragm domain	TM	component of slit diaphragm
Nephrin	slit diaphragm domain	TM	component of slit diaphragm
CD2AP	slit diaphragm domain	IC	association with nephrin
Podocin	slit diaphragm domain	IM	association with nephrin
ZO-1	slit diaphragm domain	TM	component of slit diaphragm
Dystroglycan	basal membrane domain	TM	receptor for extracellular matrix
$\alpha 3\beta 1$ integrin	basal membrane domain	TM	receptor for extracellular matrix
Vinculin	basal membrane domain	IC	link between cytoskeleton and integrin
Paxillin	basal membrane domain	IC	link between cytoskeleton and integrin
Talin	basal membrane domain	IC	link between cytoskeleton and integrin
gp330/megalin	basal membrane/ GBM	TM	endocytic receptor for lipoprotein
MAGI-1	basal membrane domain	IC	association with megalin
Utrophin	basal membrane domain	IC	link between DG and actin
Sarcoglycans	basal membrane domain	TM	link between DG and integrins
Podoplanin	cell membrane	IM	not known
α-Actinin-4	actin associated	IC	cross-link actin filaments
Cortactin	actin associated	IC	association with Arp2/3
Arp2/3	actin associated	IC	nucleate new branch of filament
Synaptopodin	actin associated	IC	association with actin microfilaments

IC intracellular protein; TM transmembrane protein; IM integral membrane protein

5. RNA processing

Multicellular organisms contain the same genome in every cell and need a special machinery to express a subset of genes in a cell type-specific and developmental stage-specific manner (144-146). mRNA delivers a message from the nucleus to the cytosol to be translated into proteins. Mature mRNA is not an exact copy of a specific gene, but has undergone a series of structural and chemical modifications, which are termed RNA processing (145). This processing takes place during transcription or afterwards as post-transcriptional modification. Some of the processing mechanisms, such as capping and polyadenylation, are examples of end modifications, whereas splicing is an example of internal modification (Figure 4.) (146). Multiple proteins, such as the spliceosome and polyadenylation enzymes, take part in the processing (144, 145).

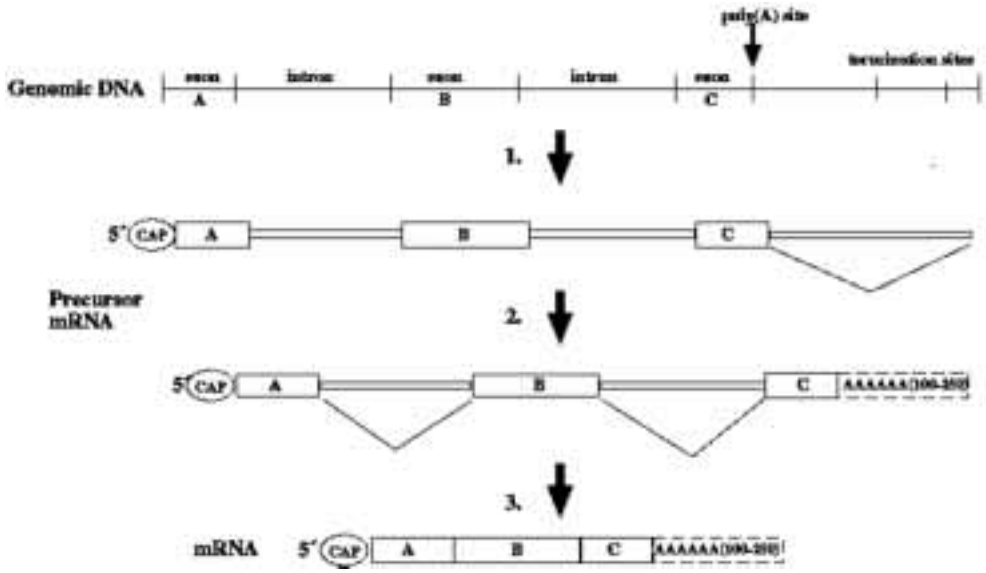

Figure 4. RNA processing. Step 1. The exact copy of genomic DNA is transcripted into precursor mRNA and ended by any of the termination sites. Immediately after transcription 7–methylguanosine (CAP) is added to 5´-end. Step 2. The excess of 3´end termination sites is removed and polyadelynate-tail (polyA-tail) is added to 3´end. Step 3. Introns are removed i.e. RNA is spliced**.**

5.1 Origin and processing of RNA splicing

Prokaryotic cells have a continuous genome, whereas in eukaryotic life forms the coding regions of the genes, exons, are usually intercepted by non-coding regions, called introns (144). During transcription, both exons and introns are copied into pre-mRNA. To remove introns, pre-mRNA is specifically spliced. There are four classes of introns classified according to the mechanisms of their splicing: self-splicing introns I-III and nuclear pre-mRNA introns (144). Self-splicing is autocatalytic and is usually found in mitochondrial and ribosomal RNA molecules (144) (146).

The splicing of nuclear pre-mRNA is a complex event catalysed by spliceosomes (147, 148). The spliceosomes contain five small nuclear ribonucleoproteins (snRNPs) and a great number of small nuclear RNA (snRNA) molecules. In addition to these proteins splicing needs an association with non-snRNP splicing factors, such as serine/arginine-rich (SR) proteins and the U2 auxiliary factor (U2AF) (147, 148).

The splicing sites are conserved sequences at the 5´-end and 3´-end (usually GU and AG, respectively) of the introns (144, 146). In the middle of an introns is a so-called branch point sequence (BPS) that is also needed in splicing. The splicing starts with 2´-hydroxyl group of adenylate residue at the branch point sequence attacking the phosphodiester bond linking the upstream exon (5´end) to the intron. A 2´, 5´-phosphodiester bond is formed between this adenylate residue and the 5´-end guanydylate, the donor site of the introns, and this intermediate structure formed is called lariat. Then the 3´-hydroxyl group attacks the phosphodiester bond between an intron and the downstream exon (3´end), so that upstream exon and downstrean exon are joined together. This releases the introns, which is subsequently degraded (Figure 5.) (144, 146).

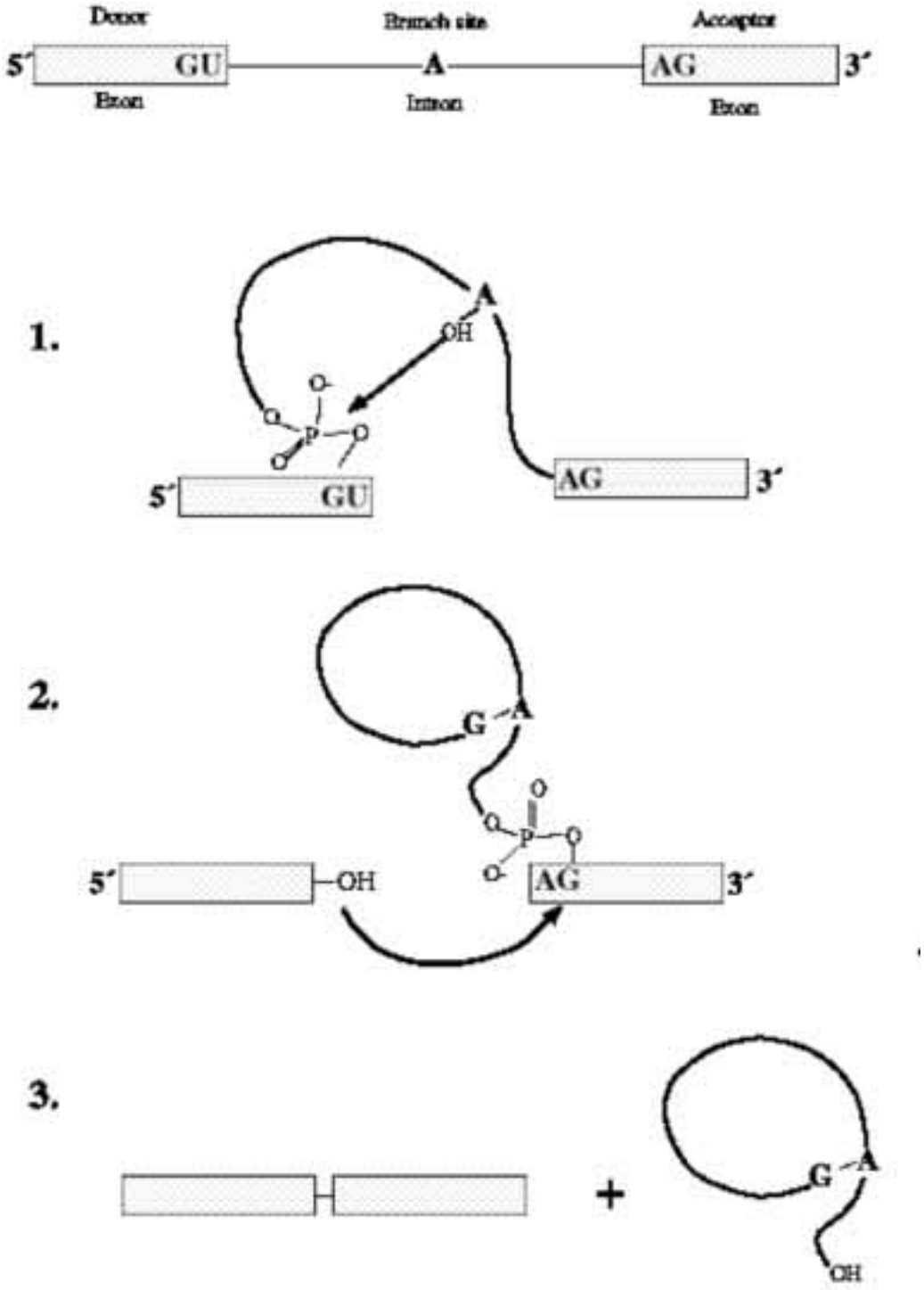

Figure 5. Splicing reaction. Step1. Attack of the 2-hydroxyl group of branch point adenylate. Step 2. Lariat formation through the transesterification reaction. Step 3. Ligation of exons and release of lariat. See details in text under the origin and processing of RNA.

5.2 Alternative splicing

In 1978 Gilbert *et al*. discovered that several mRNAs encoding multiple proteins with differing functions can be produced by combining exons differentially from a single gene (149). This significant gene function-modifying mechanism, still poorly known, is termed alternative RNA splicing. Alternative splicing plays a significant role in cell type-specific gene expression patterns, apoptosis, as well as in development and several other physiological conditions (150). Alternative RNA splicing has been found in viruses, invertebrates and vertebrates. Alternative splicing substantially increases genetic complexity, especially in higher eukaryotes (150). It has been studied mainly in the nervous and immunologic systems, where a vast diversity of proteins, particularly in the case of cell surface receptors, can be achieved. The human genome project has reduced the estimation of gene number down to 30000 (151). Recent studies have estimated that 42-57% of human genes are alternatively spliced (152). The diversity of protein isoforms cannot be explained solely by the expression of different members of multigene families. This emphasizes the significance of alternative splicing, in addition to RNA editing and post-transcriptional modification, as a producer of greater number of protein isoforms.

Alternative splicing is defined as a choice of splice sites (Figure 6). Splicing patterns can occur in several ways, e.g., the use alternative 5´splice sites, alternative 3´splice sites, optional exons, mutually exclusive exons and retained introns. The exclusion of 5´-exon(s) by alternative promoter usage switches sex in Drosophila (153). The exclusion of 3´-exon by alternative polyadenylation site usage produces calcitonin or calcitonin-gene-related peptide in mammalian cells (154). The exon skipping can influence rat brain axon outgrowth during the development (155). The mutual exon exclusion alters binding specifity of growth factors in prostate cancer (156). Alternative splicing can also introduce premature stop codons. Alterations in the splicing pattern can be a response to extra- and intracellular signals. It is

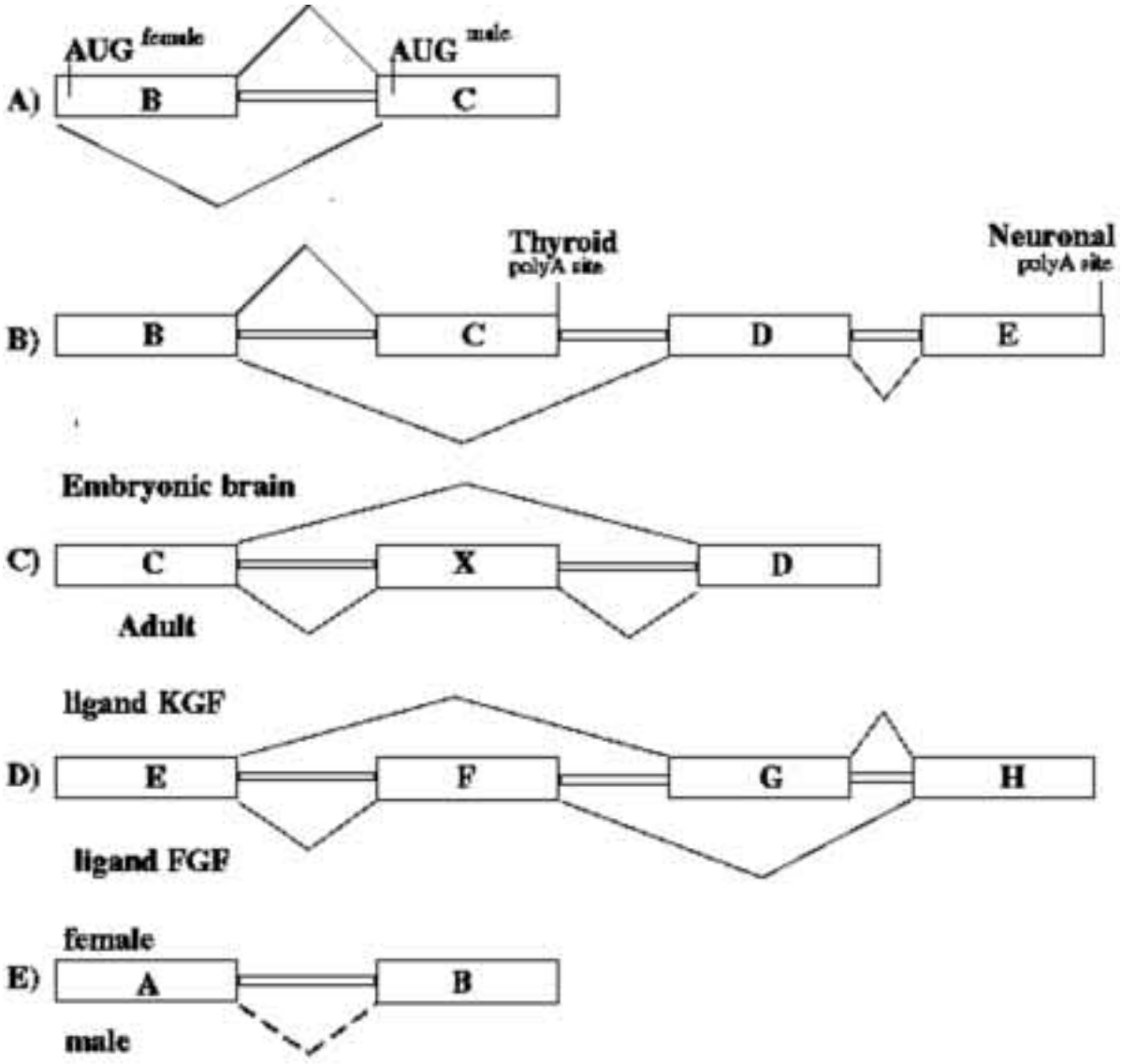

Figure 6. Alternative splicing. A) Alternative promoter usage changes sex orientation and behaviour in *Drosophila*. Upstream 5´initiation codon usage leads to female phenotype whereas downstream start codon usage leads to male character. B) Alternative polyadenylation site usage yields either thyroid calcitonin production or neuronal calcitonin-gene-related peptide production. C) Exon skipping represses neuronal outgrowth in rat embryonic brains. D) Mutual exclusion of exons changes the affinity of fibroblast growth factor receptor 2 to keratinocyte growth factor (KGF) or fibroblast growth factor (FGF). E) Retaining of intron. Female-specific retention of intron switches off the gene termed *male-specific-lethal* (msl-2) expression. (Modified from Smith CW and Valcarcel, 2000)

poorly known at present what the signals regulating splicing alterations or the factors utilized in alternative splicing are (150).

Below some examples of alternative splicing are described leading to increased protein diversity in different tissues. In the nervous system alternative splicing leads to differences in neurotransmission, protein localization, ion channel activation, protein phosphorylation and G protein coupling (157). *Drosophila* Dscam, a receptor of the nervous system, is a member of the immunoglobulin superfamily. Alternative splicing produces multiple isoforms of this protein and regulates its adhesion to other molecules (158). During the development of humoral immune system, alternative RNA splicing causes the switch of a membrane-bound antibody to a secreted form lacking the transmembrane domain.

Alternatively spliced molecules have been identified also in the kidney. The kidney development in mice shows the proportion-dependent different expression of splicing variants of Wilms´ tumour gene 1 (WT1). It produces a tumor suppressor zinc finger protein, but it is also important in embryonic development, and when mutated it leads to formation of nephroblastomas. The tightly controlled alternative splicing results in insertion or omission of the three amino acids lysine (K), threonine (T) and serine (S); the two isoforms have distinct functions (159).

Another example of alternative splicing during kidney development is the differential expression of RET receptor tyrosine kinase splicing variants (160). RET expression is at its highest in early gestation and regulates the ureteric branching in morphogenesis of the kidney. Skipping of exons encoding extracellular area results in a truncated or soluble form of RET, which can affect ligand binding (160). Soluble RET receptor can also participate in antiviral protection (161) as certain isoforms can function as type 1 interferon receptors.

Angiotensin II type 1 receptor is expressed in a great variety of tissues, including the kidney. Angiotensin II participates for instance, in fluid homeostasis, control of aldosterone

production and renal function. Angiotensin II type 1 receptor mRNA has four alternative splicing variants. These four variants vary in their exon content. Martin et al. showed that the expression and the affinity to ligand of the long and short splicing variant differs according to their location in tissues (162).

The study of the functions of multiple isoforms caused by alternative splicing has extended in recent years and is continuing. The determination of choice of splice site and the regulatory factors involved in alternative splicing still remain largely undiscovered.

AIMS OF THE STUDY

The major hallmark in the impairment of the glomerular filtration barrier is proteinuria. The molecular mechanisms of proteinuria are still unknown. Discovery of the *NPHS1* gene encoding nephrin that is mutated in CNF, provided a new tool to the research of kidney diseases. The aims of this study are listed here:

-to characterize the rat nephrin homologue;

-to study the regulation of the rat nephrin mRNA in experimental models of proteinuria of acute PA nephrosis and chronic HN;

-to study the regulation of alternatively spliced nephrin mRNA in PA nephrosis;

-to analyze the alterations in nephrin protein expression in experimental models;

-to explore the involvement of additional podocyte-associated molecules in PA nephrosis model;

MATERIALS AND METHODS

1. Animals

Young male or female rats of the Sprague-Dawley strain, weighing 250-280g, (Department of Bacteriology and Immunology, University of Helsinki) were used throughout the study. The rats were housed in controlled temperature and humidity. They were fed with standard rodent chow and had free access to tap water.

2. Antibodies

The primary monoclonal and polyclonal antibodies are presented in Table 1. The secondary antibodies are presented in Table 2.

Table 1. Primary antibodies used in the studies

Antibody	Antigen	Source	Dil.	Used in
extra-1 (030)	nephrin	rabbit polyclonal IgG, aa 780-772, 2,4 mg/ml	1:300	III
extra-2 (043)	nephrin	mouse monoclonal IgG, aa 765-772, 4,6 mg/ml	1:600	IV
PAM243 (intra)	nephrin	rabbit polyclonal peptide-specific serum, aa 1101-1126	1:50	I, II
extra-2 (7E9)	nephrin	mouse monoclonal IgM, aa 748-772, 4,6 mg/ml	1:50	V
anti-β1 integrin	β1 integrin	mouse monoclonal IgG, BD Transduction laboratories	1:2000	IV
anti-β-catenin	β-catenin	mouse monoclonal IgG, BD Transduction laboratories	1:1500	IV
anti-podocin	podocin	rabbit polyclonal, Dr.Corinne Antignac	1:2000	IV
anti-α-actinin-4	α-actinin-4	mouse monoclonal IgG Dr. Tesshi Yamada	1:600	IV
anti-α-dystroglycan	α–dystrogl.	mouse monoclonal IgG, Upstate Biotecnology	1:100	IV
R1774	CD2AP	rabbit polyclonal antiserum, aa 6-574	1:400	V
R211	CD2AP	rabbit polyclonal antiserum, aa 1-330	1:400	V
anti –CD2AP	CD2AP	rabbit polyclonal IgG, aa 350-369	1:5	V

Table 2. Secondary antibodies used in the studies

Antibody (Source)	Dil.	Used in
Fluoresceinisothiocyanate (FITC)-conjugated affinity purified goat anti-mouse IgG (Dako)	1:50	IV
FITC-conjugated affinity purified swine anti-rabbit IgG (Dako)	1:50	IV
FITC-conjugated affinity purified rat anti-rabbit IgG (Dako)	1:50	II
FITC-conjugated affinity purified rat anti-rabbit IgG (Boehringer-Mannheim)	1:50	I
Horse radish peroxidase (HRP)-conjugated affinity purified swine anti-rabbit IgG (Dako)	1:2000	III, IV
HRP-conjugated affinity purified rabbit anti-mouse IgG (Dako)	1:2000	IV
Rhodamine (TRITC)-conjugated aff. purified goat anti-rabbit IgG (Jackson Immunoresearch)	1:2000	V

3. Primers

The primers were used as presented in Table 3.

Table 3. Sequence-specific oligonucleotide primers used in the studies
Forward primer (For) , Reverse primer (Rev)

cDNA	Code	Sequence	Used in
human nephrin	NPHS-2606U	5´-CCAACATCGTTTTCACTTGG-3	I
	NPHS-3515L	5´-GGGAAGGCCATATCCTCA´-3´	I
rat nephrin	RN-S1	5´-CCACCTCAGCACCTCGAG-3´	I
	RN-AS1	5´-GAGACACGAGCTCGGGACC-3´	I
	RN-S2	5´-TCCAGGTCTCCGTCACTACC-3´	I
	RN-AS2	5´-GGCCATAGGCTCTCTCCACT-3´	I, III
	RN-S3	5´-AGCCTCTTGACCATCGCTAA	I, II, III
	RN-AS3	5´-CCCAGTCAGCGTGAAGGTAG-3´	I, II
	RN-S4	5´-CTGCTGCCTGTGCTCTTTGC-3´	I
rat nephrin	R-TM-2709U	5´-TAATGTGTCTGCGGCCCAG-3´	III
rat nephrin	R-TM-2783L	5´-TTGGTGTGGTCAGAGCCAAG-3´	III
rat α-nephrin	RN-alpha	5´-GTCCTCGCCTTCAGCACCTG	III
α-actinin-4	For	5´-TATCACGCGGCGAACCA-3´	IV
	Rev	5´-TCATCCTCCTGGGCCATGT-3´	IV
P–cadherin	For	5´-TCCATCATTGTGACAGACCAGAA-3´	IV
	Rev	5´-AGGCATTACTCCCTCCAGA ACA-3´	IV
α-dystroglycan	For	5´-GGACGCGAGGTGCCATT-3´	IV
	Rev	5´-CAGGAACCGTGGTACCAGCTT-3´	IV
podocin	For	5´-AATTCCTTGTGCAAACCACTATGA-3´	IV
	Rev	5´-CCAAGGCAACCTTTGCATCT-3´	IV
β1-integrin	For	5´-CTCTCCAGAAGGGTGGCTTTG-3´	IV
	Rev	5´-TCTCCTGTCCCATTCACTCC-3´	IV
CD2AP-1	For	5'-GAATGGGAGTGAACCTGCTC-3'	V
	Rev	5'-CAGTTCACCCTTCCACCAGC-3	V
CD2AP-2	For	5'-GAACTTTACACTGACGC-3´	V
	Rev	5'-ATGGTTGACTATATTGTGGAGTATGAC-3'	V

4. Methods

4.1 Animal experiments

4.1.1 PAN with modification with $HgCl_2$ and probucol (I-V)

Rats in experiment II (PA+$HgCl_2$+probucol) were given a single intraperitoneal injection of puromycin (PA 15 mg/100g, Sigma Chemicals, St. Louis, MO, USA) and/or mercuric chloride ($HgCl_2$ 0,5 mg/100g, Sigma Chemicals) with or without prior daily probucol (Sigma Chemicals) treatment for ten days before PA/$HgCl_2$ injection. Probucol was given in diet (2% wt/wt) mixed in the pellets and the consumption was recorded daily. The control group received 0.9% saline i.p. in equal volume at day 0 of puromycin injection. Urine was collected using metabolic cages at day 0, 3, 6 and 9. From every group three animals were sacrificed at day 3 and 10 as indicated in Table 1 in study II. Tissue samples for immunoelectron microscopy were also taken at day 7.

4.1.2 Induction of Heymann nephritis (II, V)

The modification of HN with NO synthase inhibitor (L-NAME) was induced with purified BB antigen (1 mg) according to the time schedule presented in Table 1 in study II and is previously described (163). Rats developed Heymann nephritis within 4 weeks.

4.2 Quantitation of urinary albumin (I-V)

To insure that the induction of nephritis was successful, urinary albumin was measured by nephelometry (Behring Nephelometer 100 analyzer, Behringwerke, Marburg, Germany) using rabbit anti-rat albumin antibodies (Cappel, Cochranville, PA, USA). All PA-treated and BB-induced animals developed albuminuria. The individual nephelometry results are presented in studies I-V.

4.3 Isolation of glomeruli of rat kidneys (I, IV, V)

The cortical kidney tissues of each experimental group were pooled together and the glomeruli were isolated by graded sieving as previously described (164). Briefly, cortical kidney tissue was finely minced and sieved sequentially through pores of 250, 150 and 105 µm of pore size. The purity of the glomerular preparation obtained was 92-96 % with minor tubular contamination.

4.4 RNA extraction (I-V)

Cortical kidney or glomerular RNA was isolated either immediately after sacrifice or from frozen pieces of tissue (50-150 mg) with the single-step acid guanidium thiocyanate-phenol-chloroform procedure using Trizol® reagent (Life Technologies, Gibco BRL, Paisley, Scotland) according to manufacturer´s instructions.

4.5 Cloning of rat nephrin cDNA (I)

The library screening for rat nephrin was performed with a rat glomerular cDNA library, using ZAP Express cDNA Gigapack II Cloning Kit and a PCR-amplified human probe (Stratagene, La Jolla, CA, USA) as described in detail in study I.

4.6 DNase treatment (II, III, IV)

For the removal of genomic DNA, the RNA was incubated with RNase free DNase I (Promega, Madison, WI, USA) for 30 min at 37 °C together with human placental RNase inhibitor (Promega).

4.7 Semiquantitative RT-PCR (I-V)

To compare different mRNA levels of nephrin, its splicing variant forms and other podocyte-associated molecules in experimental diseases, semiquantitative reverse transcriptase

polymerase chain reaction (RT-PCR) turned out to be most useful. For this, cDNA was first synthesized with the Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) enzyme (Promega) in the presence of oligo dT_{15}-primer (Boehringer Mannheim) and RNase inhibitor. cDNAs were diluted from 1:5 to 1:3125 to find the linearity range of PCR reactions. The amounts of cDNA were equalized according to the β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPHD) or cyclophilin levels. Sequence-specific oligonucleotide primers are presented in Table 3.

PCR amplifications were performed (total volume 25 µl) in the presence of 0.5 µM of each of the specific oligonucleotide primers, 0.2 mM dNTP (Finnzymes, Espoo, Finland), 10X PCR Buffer with 15 mM $MgCl_2$ (Perkin-Elmer, Cetus, Norwalk, CT) and 1U AmpliTaq polymerase (Perkin-Elmer). In the first cycle denaturation was for 3 min at 94 °C and thereafter for 45 seconds, annealing at 56 °C for 1 minute and extension at 72 °C 45 seconds.

PCR products were electrophoresed in 6% polyacrylamide gels and quantified with the NIH Image ® program as described previously (165).

4.8 Real-time PCR (III)

To confirm the results of semiquantitative RT-PCR, real-time PCR was performed. To detect the expression levels of nephrin we used a real-time fluorescence based PCR detection method (TaqMan technology®; PE Applied Biosystems, Foster City, CA, USA) (166, 167). In this method, a probe, with a reporter dye (FAM) and a quencher dye (TAMRA), attached to the 5´ and 3´ end, respectively, is used for the detection of PCR-product. During each extension cycle, the 5´ to 3´ exonuclease activity of Taq DNA polymerase cleaves the reporter dye from the quencher and a fluorescence signal is emitted. The instrumentation from PE Applied Biosystems (model 7700) was used for the detection of the fluorescence emitted.

4.9 Immunofluorescence microscopy (I, II, IV, V)

Kidneys dissected and snap-frozen in liquid nitrogen were embedded in Tissue-Tek® Compound (Sakura) and cut into cryosections of 3-6 µm and fixed. The fixative used was dependent on the primary antibody, either 3,5 % paraformaldehyde (PFA, Sigma Chemicals) for 10 minutes or acetone (Sigma Chemicals) for 10 minutes. The fixations were followed by repeated rinses in phosphate-buffered saline (PBS: 140 mM NaCl, 2,7 mM KCl, 1,5 mM KH_2PO_4, 8,2 mM Na_2HPO_4, pH 7,4) at room temperature for 15 minutes. Triton X-100 (Fluka BioChemica, GmbH, Buchs, Switzerland) at 0,1 % in PBS was added to the PFA fixative to enhance permeabilization. With acetone fixation, no detergent was necessary. To minimize unspecific binding of the antibodies, the cryosections were incubated in 10 % normal rat serum (NRS) or 10% fetal calf serum (FCS) at room temperature for 30 minutes followed by incubation with the primary antibodies diluted in 5-10 % NRS with 0.5% saponin in PBS at room temperarure for 1 h. The mounting medium consisted of 50 % glycerol in PBS (Immumount, Sigma). For fluorescence microscopy, an Olympus OX-5O microscope or a Zeiss Axiophot 2 microscope equipped with appropriate filter systems for FITC and TRITC-fluorescence was used.

4.10 Immunoelectron microscopy (II, V)

Immunoelectron microscopy was performed essentially by two alternative procedures. In procedure one, fixation was done for 6 h at 20 °C followed by embedding in Lowicryl K4M (Chemische Werke LOWI, Waldkraiburg, Germany). Ultrathin sections were blocked with 1% ovalbumin in PBS for 1h, followed by incubation with affinity purified rabbit anti-nephrin antibodies (50 µg/ml) and a goat anti-rabbit 10-nm gold conjugate (1:50) (Sigma). Sections were counterstained with lead citrate. For quantitations, the gold particles were counted from photographs, particularly for slit membrane/ plasmalemmal localization in the models studied (168). In procedure two, kidneys were perfused with Dulbecco's modified Eagle's medium,

followed by 4% PFA in 100 mM phosphate buffer, pH 7.4, for 15 min. Kidney cortex samples were then immersion fixed in 4% PFA for 45 min followed by 8% PFA for 15 min. Samples were cryoprotected and frozen in liquid nitrogen. Ultrathin cryosections (approximately 80 nm) were cut on a Leica Ultracut UCT microtome (equipped with a cryoattachment at -100°C), blocked with 10% FCS in PBS for 30 min, and incubated with anti-CD2AP antibodies at RT for 2 h. The primary antibodies were detected by incubation with goat anti-rabbit gold conjugate (5 nM; Sigma) at RT for 1 h. Sections were then stained with 2% neutral uranyl acetate for 20 min, adsorption-stained with 0.2% uranyl acetate, 0.2% methyl cellulose, 3.2% polyvinyl alcohol and examined with a JEOL 1200 EX-II electron microscope (169).

4.11 Western blotting and quantitative immunoblotting (II, IV)

For western blotting the glomeruli were lysed in RIPA buffer (150 mM NaCl, 1% Np-40; 0,5 % sodium deoxycholate; 0,1% sodium dodecyl sulphate (SDS), 50 mM Tris-Cl, pH 8, 0) with a glass homogenizer and the total protein concentration was measured with BCA Protein assay kit (Pierce, Rockford, IL). The urine samples were diluted in Laemmli buffer (300 Tris.Cl, 4% SDS, 20% glycerol, 0,05% bromphenolblue). The glomerulus lysates with adjusted concentrations were diluted 1:1 in Laemmli Buffer containing 1:20 of β-mercaptoethanol (FlukaChemie, Buchs, Germany) and boiled for 5 minutes. The samples were run on 8 % SDS-PAGE gels with the Protean Mini-gel electrophoresis facility apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The proteins were transferred onto 45-micron nitrocellulose membranes (Amersham LifeScience, Buckinghamshire, UK). The membranes were blocked with 3 % BSA in PBS overnight at + 4 °C, after which they were incubated with the respective primary antibodies at room temperature for one hour. This was followed by washes with 0,2 % Triton X-100-PBS. The peroxidase-conjugated secondary antibodies were diluted in 0,2 % Triton X-100-PBS to 1: 2000, and were incubated on the membranes at room temperature for one hour. Thereafter, the filters were washed thoroughly. The peroxidase

signal was enhanced using the ECL method (Amersham Pharmacia Biotech, Germany) and the images were developed on FUJI Super RX films (Fuji Photo Film Co, Ltd, Tokyo, Japan). The developed films were scanned with Biometra Gel Analyzer (BioDocAnalyze, Biometra Analytik GmbH, Göttingen, Germany) and analyzed by the NIH image analysis program (National Institutes of Health, Bethesda, MD, USA).

RESULTS

1. Characterization of the rat nephrin homologue (I)

Cloning of rat nephrin cDNA was essential for the further studies of renal diseases in experimental rat models. The cloning was carried out using a rat glomerular cDNA library, commercial cloning kit and a PCR-amplified probe hybridised with human nephrin. To retrieve the full-length cDNA, additional library screening was performed according to the mouse nephrin cDNA sequence with 5´ rat cDNA probe.

Rat homologue of nephrin showed 82% identity to human nephrin at cDNA level. In the translation into amino acid sequence the identity level was 90%, 99% and 83% in the extracellular, transmembrane and intracellular domains, respectively. The predicted amino acid sequence was 89% identical with the human sequence and its estimated protein mass was 134,7kDa. The proposed number of cysteine residues and predicted degree of glycosylation was almost identical to those of human.

In addition to the full-length nephrin cDNA, library screening yielded two splice variants, alpha and beta. The sequences of the cDNAs obtained were confirmed by sequencing with specific primers (Table 3.). The RT-PCR analysis revealed five amplified products. In addition to the full-length mRNA, α and β forms, two additional products; gamma (γ) and delta (δ) were detected. Upon verification the γ and δ forms were shown not to be in an open reading frame.

2. Nephrin mRNA levels in acute PA nephrosis (I, II, III)

To detect the expression level of rat full-length nephrin mRNA we used semiquantitative RT-PCR, as Northern blotting was not sensitive enough. Already after three days (early phase

changes) in all PA treated groups, nehprin mRNA levels showed a 40% downregulation (P=0.037), and after ten days (late phase changes) an 80% downregulation (P=0.027), as compared with the respective levels in the controls (Figure 2, study II). The verification of the semiquantitative RT-PCR was performed with real-time PCR. The results were compatible (Table 1, study III). The treatment with $HgCl_2$ led to a similar pattern with a rapid decrease of nephrin mRNA level at day 3, whereas pretreatment with probucol resulted in non-significant 1.4- and 1.35-fold increases at days 3 and 10, respectively (Figure 3, study II.). In the group combining PAN and $HgCl_2$ treatments, an even more pronounced drop in the nephrin mRNA level was observed at day 3.

Semiquantitation of mRNA with primers designed according to the α-nephrin sequence in PA-treated rats showed a significant reduction when compared with the control levels (Figure. 1 and 2, study III). The significant decrease was 40 % and 70 % on days 3 and 10 days, respectively. A similar pattern was seen in the full-length nephrin mRNA levels.

3. Alterations in the expression of nephrin protein in acute nephrosis (I, II)

The immunohistochemical stainings were performed with anti-peptide antibodies against the intracellular domain of nephrin. Nephrin-specific coarse linear reactivity was detected in normal glomeruli (Fig.1B, study I and Fig.4A, study II). In the PA-treated group the intensity was diminished along in the course of nephrosis, and the least intense and irregular signal was observed at day 10. Less marked changes in reactivity were also found for $HgCl_2$ treated kidneys (Figure 4 and Table 2, study II)

The quantitative immunoblotting results complemented the semiquantitative IF studies on nephrin. The protein expression was decreased 40% at day 3 and was more than 70% at day 10.

The confirmation of the expression and localization of nephrin in normal and PA glomeruli was performed by immunoelectron microscopy. Anti-intra-nephrin antibody with gold label bound specifically to SD area, but a few cold particles were also found at the apical membrane (Figure 5, study II). The counted gold particles revealed changes in localization of nephrin antigen after PA treatment. Relative distribution was 87% at the apical surface and 13% at SD after PA while in controls, the ratios were 27% and 73% at the apical surface and SD, respectively (Table 3, study II).

5. Screening of urinary nephrin after PA-treatment (III)

The urine samples of PA-treated rats were screened by immunoblotting. By using nephrin recognizing polyclonal antibodies, we found a reactive protein with a size of 166-172 kDa at day 6 when the first peak of proteinuria is detected and at day 10 during the highest peak of proteinuria, while in early phases no such signal in the urine could be detected. Since the detected protein appeared to be smaller than that found in glomeruli, it was proposed to be the α-splicing form.

6. Scanning of gene expression levels of other podocyte-associated molecules in PA nephrosis (IV, V)

The relative amount of mRNA of selected podocyte markers were analysed by semiquantitative RT-PCR. The specific primers designed according to the α-dystroglycan, β1-integrin, P-cadherin, α-actinin-4, podocin and CD2AP sequences are listed in Table 2. The most apparent changes were in α-actinin-4 and podocin mRNA levels. After 3 days of PA treatment, α-actinin-4 mRNA level was elevated 2-fold and continued up-regulated approximately 1.6-fold at day 10 compared to the controls (Figure 2, study IV). Podocin

expression was up-regulated 1.6-fold both days 3 and 10. Gene expression of α-dystroglycan, β1-integrin and P-cadherin was not considerably altered after PA-treatment.

Administration of PAN resulted in a 50% down-regulation of CD2AP already 3 days after the induction of nephrosis, and on day 10, CD2AP mRNA was down-regulated being 40% of that in the controls (Figure 1, study V).

7. Effect of PA treatment on relative protein content of other podocyte-associated molecules (IV, V)

Quantitative Western blotting achieved by protein concentration determination prior to blotting enabled more accurate analysis of particular proteins in PA nephrosis. The quantitations are presented in figure 3 in study IV. The most evident up-regulation was that of β1-integrin. The 2-fold rise in protein amount was observed both at early and later phases of the disease. The level of podocin dropped to 50 % in 3 days and continued to fall, being only 10% of control levels after 10 days, which turned out to be similar to that of nephrin. β-catenin was down-regulated about 50 % at day 3 but recovered back to control levels at day 10. No considerable changes were seen in α-actinin-4 or α-dystroglycan.

Immunohistochemical analysis supported the results achieved by quantitative Western blotting. Podocin specific reactivity was less intensive in 10-day PAN rat kidney section when compared to control (Figure 5, study IV). β1 integrin staining was more intense at later phase sections than in controls.

In the control rat glomerulus, CD2AP staining was intense along the basal poles of the podocytes (Figure 2A, study V). In the acute PA rat model of nephrosis this localization changed. In the 10-day samples of the PA group, the immunoreactivity for CD2AP was reduced as compared to the normal kidney. In PAN (not shown) and PAN together with

mercuric chloride (Figure 2D, study V) models, some patchy staining was observed in addition to a weak, diffuse immunoreactivity seen all over the glomeruli. In the samples treated with mercuric chloride alone, CD2AP signal was very weak and diffuse (not shown).

Double immunofluorescence analysis of normal rat kidneys, using a rabbit CD2AP antibody and a mouse nephrin antibody directed against the extracellular domain of nephrin, revealed basal staining of the glomerular epithelial cells with both antibodies (Figure 3A-C, study V). CD2AP staining was, however, more continuous as compared to the patchy staining of nephrin. In acute PA nephrosis and its modifications (Figure 3J-L, study V), the glomerular signals for both CD2AP (Figure 3J, study V) and nephrin (Figure 3K, study V) were distinctly reduced. Both staining patterns included occasional patchy, partially overlapping signals of more distinct fluorescence.

Immunoelectron microscopy regularly revealed CD2AP-specific labeling in the podocyte foot processes of control glomeruli (Figure 4A-C, study V). Gold particles were frequently seen in the vicinity of the slit diaphragm areas but also elsewhere in the foot process cytoplasm.

In 3-day PAN samples (Figure 4D-E, study V), representing early developing nephrosis, the podocytes started to show loss of foot processes, apical displacement of slit diaphragms (Figure 4E, V), and formation of occludin-type junctions (Figure 4E, study V). The intensity of the CD2AP signal in the modified foot processes appeared similar to that in the controls. Gold particles were seen both close to the basal cell membrane and elsewhere in the cytoplasm. Labeling was often, but not constantly, seen in the vicinity of occludin-type junctions (Figure 4E, study V) and preserved slit diaphragms.

In the 10-day PA nephrosis samples (Figure 4F-G, study V), the podocytes showed fully developed effacement including practically complete loss of foot processes and SD, presence of occludin junctions, and frequent detachment of podocytes from GBM. The intensity of the CD2AP signal in podocytes was dramatically diminished. The effaced foot processes almost

completely lacked gold particles, and only few particles were found elsewhere in the cytoplasm (Figure 4F, Study V). However, in accordance with the immunofluorescence results, some CD2AP signal was detected in the effaced foot processes on rare occasions (Figure 4G, study V). The remaining label for CD2AP was regularly found in close association with the disorganized actin cytoskeleton, and occasionally the gold particles were associated with discernible microfilaments (Figure 4G, study V).

8. Nephrin and CD2AP in Heymann nephritis (II, V)

After 12 weeks of BB antigen induction in HN semiquantitative RT-PCR results with nephrin primers did not show any significant changes in mRNA expression levels.

In IF the nephrin protein showed only slight changes in HN when compared to controls (Figure 4B and table 2, study II)

The distribution of gold particles was detected by immuno EM with nephrin antibody. The results showed equal distribution with controls (Table 3, study II)

The more significant changes were seen in the expression of the CD2AP protein. In IF the localization of CD2AP changed in HN. The signal for CD2AP in glomeruli was strong, but a large fraction of the protein appeared in a punctate fashion (Figure 2B, study V). In HN with L-NAME, a nitric oxide synthase inhibitor, however, the staining pattern was totally different: CD2AP immunoreactivity decorated the cells aligning the dilated glomerular urinary spaces as clear lines (Figure 2C, study V).

In double IF analysis the level of glomerular signal for CD2AP in HN glomeruli was similar to that in controls. The staining pattern of CD2AP included punctate aggregates (Figure 3D, study V), which often located in close proximity to similar aggregates of the nephrin signal (Figure 3E, study V). If the chronic nephrosis was induced with the combination of BB

antigen and L-NAME (Figure 3G-I, study V), the glomerular distribution of CD2AP was largely linear (Figure 3G, study V), whereas the expression and organization of nephrin were more severely disturbed (Figure 3H, study V).

DISCUSSION

This thesis collects together data obtained by studying the regulation of podocyte-associated molecules in proteinuric disease models in rats. Studies with animal models have been instrumental in providing data about the pathophysiology of proteinuria and rat models have been shown to be particularly useful in the study of renal diseases and have been used routinely from the mid 1950´s. The animal homologues of human molecules are essential for utilizing experimental models effectively.

1. Expression and localization of nephrin in tissues (I)

The human *NPHS1* gene, located in chromosomal area 19q13.1, encodes a transmembrane protein nephrin and, when mutated it causes CNF (3). Our characterization of the rat nephrin cDNA showed 82% identity with human nephrin cDNA and was shown to contain an open reading frame of 3705 bp. Cloning of the rat nephrin homologue also revealed distinct mRNA splicing: α– and β-variants. The alternatively spliced α-form of nephrin lacks the whole transmembrane area and part of the intracellular domain. The same variant is found in human nephrin, with an exact exclusion of the exon 24 encoding only the transmembrane domain (109). The expression of alternatively spliced nephrin and their possible significance in proteinuric conditions are discussed below. Additionally, RT-PCR analysis revealed rat nephrin mRNA expression in the spleen.

Comparison of the translated amino acid sequences of human and rat nephrin showed 89% homology over the extracellular, intracellular, and transmembrane domains. Translation revealed highly conserved cysteine residues, signal sequences and ten putative N-glycosylation sites. This proves that the structure of rat nephrin is closely related to that of the respective human molecule (170). The estimated molecular mass of rat nephrin protein is 134

kDa, however, in immunoblotting, a double band of approximately 185-200 kDa was seen. This can be explained by two different transcripts and post-translational modifications (170). Two other groups have also published the characterization of rat nephrin and confirmed the findings of the observed homology with human nephrin (170, 171).

It is of interest that we have cloned the mouse nephrin (110), a finding that was later confirmed by Putaala et al. (171). The isolated mouse nephrin RNA was the size of 4.1 kb and was predicted to contain 1242 amino acids. Cloning of mouse nephrin revealed 93 % homology compared to rat nephrin, and 82 % to human. The main difference between human and mouse nephrin cDNA is that mouse nephrin contains 30 exons instead of the 29 exons in human nephrin (171).

In the glomerulus of normal kidneys nephrin is primarily localized in the slit diaphragm (109-111, 171) In addition, nephrin is always found in the apical membrane of the foot processes of podocytes (109). First studies on the nephrin protein suggested that it is kidney-specific (3) but it has subsequently been found elsewhere, too. Palmén et al. reported both nephrin mRNA and protein expression in the insulin producing ß-cells in the adult human pancreas (115). Putaala et al. reported mRNA expression of nephrin in the mouse embryo hindbrain, spinal cord and cerebellar radial glial cells (116, 171). Furthermore, nephrin has also been reported in the Sertoli cells of testis (117). In addition to these findings we have found nephrin expression in rat lymphoid cells and tissues at both mRNA and protein levels (Åström et al., unpublished). Possible functions for nephrin judged by its structure and expression pattern as well as interactions with other glomerular molecules are discussed below.

2. Function of nephrin in SD

Localization of nephrin in SD suggests that it is a major structural component involved in the composition of the ultrafiltration barrier of the glomerulus. In addition, due to its molecular

structure with the repetitive Ig-like domains extending outwards from the plasma membrane and expression in the course of kidney development, nephrin is likely to participate in cell adhesion (3, 112) and signalling (113, 114). It has been proposed that nephrin, according to its structure, binds to another nephrin molecule of the adjacent podocyte in a homophilic fashion (111, 172). However, this proposal has not been verified.

Nephrin has been shown to be a crucial element in the development of glomerular podocytes in human (112), rat (170) and mouse (116). During normal glomerulogenesis, nephrin mRNA appears first in the late S-shaped bodies of the cells in vascular clefts (112). In addition to development of glomeruli, *in situ* studies on whole mouse embryos revealed nephrin mRNA expression in the hindbrain and spinal cord. Immunohistochemical analysis of nephrin protein implies nephrin also to have a role in brain, at least certain regions. However, mice with inactivated *NPHS1* gene did not show any morphological changes in the cerebellum (116, 171).

Findings from the pancreatic and lymphoid cells suggest that nephrin not only participates in the maintenance of SD in the glomerular filtration barrier, but may also play a role in islet cell function in the pancreas (115), and perhaps in the antigen presentation in the immunologic system (Åström et al., unpublished).

Association and co-localization studies with nephrin and other podocyte molecules have shown that nephrin interacts with many other proteins. It has been shown that nephrin interacts with CD2AP (4, 120), originally cloned from T cells. CD2AP is also expressed in podocytes, especially in SD and is crucial for normal kidney function (4). The interaction with nephrin occurs at the cytoplasmic carboxy-terminal domain of CD2AP (121). It has been suggested that nephrin is anchored to the cytoskeleton via this domain (121). Accordingly, nephrin/CD2AP complex provides part of the structural integrity of SD.

Podocin encoded by *NPHS2* is an integral membrane protein expressed in podocytes. Mutation in *NPHS2* causes autosomal recessive steroid-resistant nephrotic syndrome (5). Similarly to nephrin, podocin appears to accumulate in oligomeric forms in lipid rafts (113, 125). Oligomerized podocin then interacts with nephrin and CD2AP and is needed for the functional assembly of SD (125). Huber et al. showed that nephrin with its several potential tyrosine phosphorylation sites is also a signalling molecule. The interaction of its cytoplasmic tail with podocin enhances signalling of nephrin (114).

3. Rat nephrin is characteristically regulated in renal diseases (II)

The identification of *NPHS1*, mutated in CNF, opened a new way to approach molecular mechanisms of glomerular ultrafiltration. Using an experimental PA nephrosis model to study the alterations of rat nephrin, we found a 60% reduction in the early phase and a remarkable 80 % down-regulation of nephrin mRNA in the late phase of PA induction. This finding shows that nephrin mRNA is regulated before the highest peak of proteinuria. On the EM level the distribution of nephrin protein was also altered in PA nephrosis model: nephrin escaped from the slit area towards the apical membrane of foot processes after PA treatment. Another group subsequently also reported reduced on expression of nephrin mRNA in PA nephrosis (170). They found a 51.2% reduction in mRNA already after two hours but in IF they did not see any changes. After 9 days of PA induction the intensity of nephrin protein was significantly decreased in IF. These results get support from findings in human kidney diseases. For instance, in human acquired nephrotic syndromes nephrin mRNA is down-regulated and protein redistributed (173-175). In the present study, after $HgCl_2$, induction over 40% drop in expression of nephrin mRNA level was seen even in the absence of significant proteinuria. This early phase response can be due to lipid peroxidation seen both in $HgCl_2$ and PA induction.

In our model of HN, no significant changes in the expression of nephrin were observed. This can be due to the late time of collection of samples, after 12 weeks of induction. However, Benigni et al., as well as Yuan et al. reported reduction in nephrin expression in passive HN (176, 177). Thus, more detailed study of nephrin expression in chronic HN at different time points would be informative.

Several other studies also present evidence on involvement of nephrin in experimental renal diseases. Administration of a monoclonal antibody, 5-1-6 that binds to SD, results in transient massive proteinuria in rat (178). The antibody is postulated to recognize the extracellular domain of rat nephrin (179). Furthermore, expression of the antigen located in SD changes in proteinuric diseases (180) and in 5-1-6 nephropathy nephrin expressions is decreased (170, 181).

In the streptozotocin model of experimental diabetic nephropathy, modulation of nephrin mRNA and protein expression is seen. At early time points of diabetic nephropathy rats, i.e. four to eight weeks, nephrin mRNA is up-regulated and simultaneously free nephrin leaks into urine (182). In another study, in spontaneously hypertensive streptozotocin diabetic rats, nephrin mRNA expression was down-regulated and immunohistochemistry for nephrin showed depletion after 18 to 24 weeks of induction (183). These results suggest that nephrin is crucial for the permselectivity of glomerulus and is regulated in proteinuric conditions.

4. Splicing variant α-nephrin mRNA in proteinuric condition (I, II)

Alternative splicing produces a variety of protein isoforms *in vivo*. It can occur tissue-specifically, at different developmental stages, and disease conditions, but little is known about the regulatory mechanisms of alternative splicing.

We have found that both in man (109) and in the rat nephrin is characteristically spliced. In addition to the full-length nephrin mRNA, we described the splicing variants α and β in the

rat. α-nephrin mRNA lacks the exon encoding transmembrane domain and most probably produces a truncated and soluble form of the protein. The detailed study of α-nephrin mRNA in PAN showed similar reduction as full-length mRNA. Simultaneously, we found nephrin protein from PAN urine samples during the first and second peaks of proteinuria. Whether the nephrin found in the urine is the product of the α-variant mRNA that produces a soluble form of nephrin, or due to changes in glycosylation remains to be studied in detail. The α-form may possibly act as a regulator of full-length nephrin in diseased kidneys. An example of such regulation is the soluble interleukin-6 receptor, which has been shown to function as an antagonist, blocking or reducing the potency of the ligand. The soluble forms of receptors can show even higher affinity to ligands than membrane-bound isoforms (184). To determine the use of soluble nephrin as a predictive marker in proteinuric conditions is still under study.

5. Molecular architecture of podocytes (IV, V)

The plasma membrane of the foot processes of podocytes can be divided into three domains: basal domain, apical domain and slit diaphragm that is in between those two domains (Figure 2). This architecture is maintained by specific arrangement of podocyte molecules (108). Well-organized cytoskeleton with its associated proteins and interaction between proteins located in different domains of the podocyte cell membrane maintain the specific morphology of foot processes. The PA model of acute nephrosis was chosen for the analysis of alterations in the content of structural and functional molecules. Previously, only few reports have been published concerning the adjustment of podocyte-associated molecules in PA nephrosis (130, 185).

Here, the results indicate that the podocyte-associated molecules, located in different domains of the cell, are regulated distinctly but these changes may take place in a well-coordinated way. The defect in the molecular composition of slit diaphragm is seen in proteinuric

conditions. Besides nephrin, many molecules have been found to localize in SD. They include podocin (5), CD2AP (121), FAT (186), α, β, γ -catenins, P-cadherin, and ZO-1 (91).

The importance of nephrin in nephrotic disorders has been discussed above. On the other hand, podocin might also have an important function. Podocin links plasmamembrane proteins to the cytoskeleton (5). Additionally, podocin has been suggested to enhance nephrin signal transduction (114). In our study, the most significant changes obtained were in the reduction of podocin and nephrin proteins. PA-treatment increased the podocin mRNA level 1.6-fold, but at the same time the protein level decreased to 50% and 10% at days 3 and 10, respectively. Thus, the podocin protein levels followed patterns seen in the regulation of nephrin protein

CD2AP, another essential molecule for the normal kidney function, is expressed in podocytes, especially in SD (4). Accordingly, CD2AP-deficient mice develop kidney disease resembling nephrotic syndrome. CD2AP interacts with CD2 (118), polycystin-2 (119), nephrin (121) and recently it has been shown to interact with actin (Lehtonen, in press). In our study CD2AP showed alterations at mRNA level similar to its interacting partner nephrin. The IF studies paralleled well the mRNA semiquantitations; when the intensity of CD2AP staining was decreased also the mRNA expression pattern was reduced. Additionally, in immuno EM obliterated foot processes were obvious in later phases of the nephrosis.

Receptors for extracellular matrix proteins in the basal domain of foot processes, including dystroglycans and integrins, have an essential role in podocyte cell-matrix adhesion (135, 187). α3β1 integrin is the main form of integrins expressed in podocytes, and it participates in stabilizing podocyte foot processes. Our findings showed a remarkable 2-fold increase in β1 integrin at protein level after PA treatment, both in early and later phase.

α-actinin is a member of cytoskeleton-binding proteins and associates with various other proteins, linking for example the cytoplasmic domain of β1 integrin to the cytoskeleton (129).

The isoform α-actinin-4, encoded by *ATCN4*, is expressed in podocytes, especially in foot processes (127). Our results did not reveal any changes in α-actinin-4 protein quantities, but its mRNA level raised 2-fold at the early phase of PA nephrosis. Smoyer et al. reported changes in α-actinin before day 3 in PA nephrosis but did not see alterations thereafter (130). By adapting rapidly to a direct impact imposed upon the podocyte, α-actinin-4 plays a significant role in the maintance of a well-organised cytoskeleton.

Together these findings suggest that podocyte proteins are regulated differentially in nephrotic glomeruli according to the structure and location in the plasma membrane. Especially, certain SD molecules, such as nephrin, podocin and CD2AP, are regulated before the highest peak of proteinuria. These central molecules serve as an integrated composition of glomerular filtration barrier and disturbances in their expression eventually leads to proteinuria.

SUMMARY

The slit diaphragm (SD) that works as a final sieve of ultrafiltration is a complex assembly of many molecules. This assembly maintains the filtration barrier whose perturbation manifests as proteinuria. The mechanisms for this remain still largely unknown. Experimental animal models are extensively used to provide information concerning proteinuric diseases. The discoveries of nephrin and findings thereafter have pointed a new direction in studying the composition of the filtration machinery.

This thesis combines data that have been acquired using the experimental kidney disease models of the rat focusing on the regulation of expression of podocyte-associated molecules. First, it can be concluded that PA nephrosis is suitable for analyzing acute glomerular injury and molecular mechanisms of ultrafiltration within the kidney. Second, a clear correlation between the regulation of key molecules in SD, such as nephrin, podocin and CD2AP, and the manifestation of proteinuria are observed. Alterations of these proteins were seen already before the peak of proteinuria in the experimental acute model of nephrosis.

Understanding of the role of alternative splicing leading to multiple isoforms of expressed proteins is greatly expanding. Third conclusion from the present series of studies is that nephrin is characteriscally spliced in the rat. The alternatively spliced nephrin mRNA, α nephrin yields proposedly a truncated and soluble form of nephrin. It is possible that α-nephrin has a controlling role in the pathogenesis of proteinuria. Due to similar splicing characteristics in human, soluble nephrin may be valuable as a prognostic indicator even before the disease is clinically evident.

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