BASEMENT MEMBRANE COMPOSITION OF *DAG1* NULL CHIMAERIC MICE KIDNEYS

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May, 2002

A thesis submitted to The Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

TABLE OF CONTENTS

ABSTRACT

The growth of an organism involves the proliferation and migration of cells within an extracellular matrix. As a cell surface receptor, the *Dag1* gene product dystroglycan links the intracellular cytoskeleton to the extracellular basement membrane in many cells. Thought to act as a structural protein dystroglycan may also participate in signal transduction. This study aims to better understand the role of dystroglycan during kidney morphogenesis. I hypothesised that a lack of dystroglycan in the precursor cells of the kidney could lead to altered kidney growth. Chimaeric mice deficient in dystroglycan were generated to test this hypothesis. A total of 38 chimaeras had genetic contribution and histological analysis performed on their kidneys. Of the chimaeras analysed, only four demonstrated altered kidney morphology. Further histological, immunohistochemical and biochemical studies established whether a link existed between this morphology and a deficiency in dystroglycan. Normal laminar architecture and nephrotic structures of the kidneys suggest that normal kidney organogenesis occurred in the absence of dystroglycan. The pattern and expression level of basement membrane components suggests that normal basement membrane formation also occured in the absence of dystroglycan. Biochemical analysis revealed that although dystroglycan protein levels correlate with the genetic contribution of the chimaeric kidney, it does not correlate with the altered morphology. Ureter blockage causing hydronephrosis can explain the morphology observed. A deficientcy of dystroglycan in the ureter may in turn have caused this blockage. These findings suggest that dystroglycan is not necessary for kidney organogenesis, since kidney development occurred normally in all 38 chimaeric animals irrespective of genetic contribution.

RÉSUMÉ

La croissance d'un organisme dépend sur la prolifération et la migration des cellules à travers une matrice extra-céllulaire. Le récepteur céllulaire dystroglycan, produit du gène *Dag1*, relie le cytosquelette intra-céllulaire à la membrane basale extra-céllulaire. Principalment considéré comme étant une protéine structurale elle pourrait aussi participer à la transduction de signaux céllulaires. Mieux comprendre le rôle du dystroglycan dans la morphogenese du rein est Le but de cette étude. L'hypothèse qu'une déficience en dystroglycan dans les céllules précurseurs du rein mènerait a une croissance anormale du rein a été testée avec des souris chimères. La contribution génétique et une analyse histologique a été fait sur les reins de 38 chimères. Quatre ont démontré une morphology rénale anormale. Histologie, immunohistochimie et biochimie ont déterminer si un lien entre la déficience du dystroglycan et cette morphologie existait. Une cytoarchitecture normale de tous les reins suggère qu'un dévélopment normale ce produit dans l'absence du dystroglycan. La localisation et le niveau d'expression normale de molecules composant la membrane basale suggère que l'absence du dystroglycan n'empêche pas sa formation. L'analyse biochimique a révélé que même si les niveaux de protéines du dystroglycan étaient reliées avec la contribution génétique des reins chimères, elles n'étaient pas relier avec la morphologie observé. Cette morphologie pourrait être une conséquence d'un blockage de l'ureter causant une hydronephrose. Ce blockage pourrait cependant être due à une déficience du dystroglycan dans ce tissue. Ces résultats suggères que le dystroglycan n'est pas nécessaire pour le dévelopment du rein, puisqu'un dévelopment normale c'est produit quelque soit la contribution génétique.

ACKNOWLEDGMENTS

First and foremost, I must thank my supervisor Dr. Salvatore Carbonetto whose invaluable guidance and knowledge I should have sought more often during my first steps into the world of research. I thank Dr. Hakima Moukhles for having advised me during the establishment of my project and for her generosity of practical ideas. Thanks to Dr. Patrice Côté for showing me the ropes. To the present members of Dr. Carbonetto's laboratory: Dr. Joseph Babity, Youngen Zhang, Ramin Raouf and Mathieu Tremblay, I thank them for their helpful scientific and technical advice. The suggestions from Dr. Andrey Cybulsky and Dr. Chantale Bernard were also much appreciated. The guidance from Dr. John Silvius also helped greatly. Thank you to my advisory committee members Dr. Mike Ferns and Dr. Joe Dent for their guidance. Thank you to Naima Bachnou and Lydia Malynowski for their technical expertise. Last but not least, I thank those who have unconditionally supported me in all of my endeavors: my family.

ABBREVIATIONS

aa	amino acid
BM	basement membrane
Ch	chimaera
DG	dystroglycan
DGC	dystrophin-glycoprotein complex
E8	elastase fragment number 8 (referring to laminin)
E6.5	embryonic day 6.5 (referring to development)
ECM	extracellular matrix
EHS	engelberth-Holm-Swarm
ES	dmbryonic Stem Cell
GAG	glycosaminoglycans
GBM	glomerular basement membrane
HS	heparan sulphate
HSPG	heparan sulphate proteoglycan
mRNA	messenger ribonucleic acid
MCNS	human minimal change nephrotic syndrome
MM	mesangial matrix
NC1	non collagenous domain 1
PAMS	periodic acid methylamine silver
SDS	sodium dodecylsulphate
TBM	tubular basement membrane

INTRODUCTION

How cells adhere to each other and to their extracellular matrices (ECM) underlies essential cellular processes such as survival, migration, differentiation and maintenance of the differentiated state. The dynamic reciprocity between a cell and its extracellular environment is critical to its phenotypic fate. This study will focus on a widely expressed ECM receptor called dystroglycan which has been implicated in basement membrane (BM) formation, synaptogenesis and epithelial development (190). The aim of this thesis is to further investigate the role of dystroglycan in kidney development and function especially in epithelial cells, where binding to the BM is essential in the maintenance of a polarised state and function.

1. BASEMENT MEMBRANES

BMs consist of thin layers of highly organised extracellular matrices located at the epithelial/mesenchymal interface of most tissues. BMs also function as an envelope for muscle fibres, peripheral nerve fibres and fat cells. BMs have both mechanical and biological functions. For example, while forming an anchoring exo-skeleton for cells, BMs can act as a functional and selective barrier providing a micro-environment separate from the extracellular space. The molecular composition of a BM influences its chemical interactions, providing specificity and the dynamics necessary to dictate the quantity and timing of many cellular events (29, 169). These events mediated by the BM coupled with its capacity to continuously remodel, itself are essential during the development of an organism to allow such processes as cell proliferation, migration, morphogenesis and tissue maintenance. For example, morphogenesis can be induced by diffusible morphogens such as netrins (203). These molecules bind to the ECM in a gradient and attract or repel cells by interacting with cellular receptors to initiate signal transduction pathways within the cell (203). The process of cell migration itself often

entails cell adhesion to the ECM (31).

The characterisation of the Engelbreth-Holm-Swarm (EHS) mouse tumour which produces large amounts of extracellular matrix advanced the investigation of the molecular organisation of the BM(82). The major and ubiquitous families of BM components are type IV collagens, laminins, nidogen and heparan sulfate proteoglycans (HSPG) (83). The structure of BMs is based on the presence of two intertwining networks. A structural collagen type IV network is connected to a laminin network via linkages made by perlecan and nidogen. Heparan sulphates are trapped within this complex. In addition, a variety of more specialised molecules may be localised at key regions of BMs, such as acetylcholinesterase at the neuromuscular junction. Although only a few families of ECM molecules compose BMs, a high level of chemical complexity is achieved by the presence of multiple isoforms whose expression is spatio-temporally regulated. This diversity and complexity allows a continuum of structures that endow the BM with many different specialised functions (29).

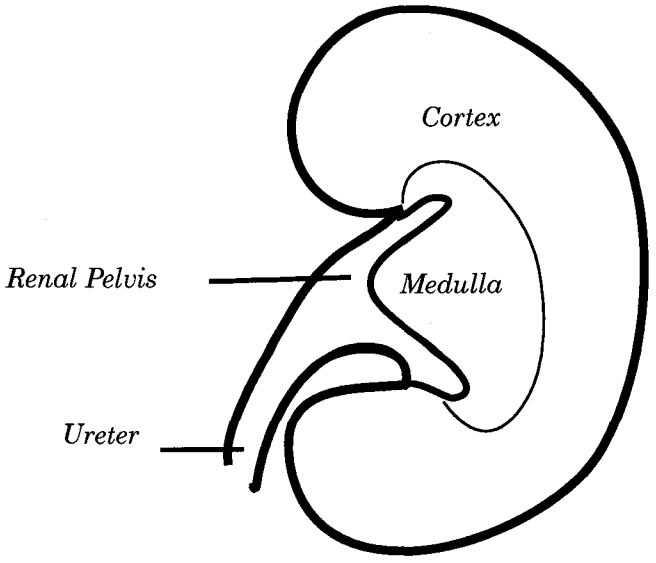
Epithelia completely cover all the free surfaces of an organism by forming contiguous sheets of cells connected by lateral cell-cell adhesion. Epithelia exhibit an apical-basal polarity in which the Golgi apparatus is partitioned to the apical cytoplasm and the basal cell surface resides on the BM (114). BM components and their receptors have been implicated in the regulation of epithelial morphogenesis. The connection between an epithelial cell and its BM may prevent apoptosis and maintenance of a differentiated state (130, 131). In the kidney, the epithelial cells form the nephrons and each epithelial cells rests on a continuous BM. Different anatomical/functional compartments of the nephron contain molecularly distinct BMs supporting a correlation of BM composition and function (121).

2. KIDNEY STRUCTURE

Mammalian kidneys are located on either side of the vertebral column against the posterior abdominal wall. The main function of the kidney includes regulation of the composition and volume of body fluids. The kidney carries out these functions by continuously filtering the blood supply, modifying the composition of the filtrate and excreting any waste fluid (urine). Located on the concave surface of each kidney is a longitudinal slit called the hilus, through which the renal artery and nerves enter and the renal vein, lymphatics and ureter leave. In unipapillate rodents, at the level of the hilus, is the pelvis that acts as a chamber for urine as it passed out of the collecting ducts and travels towards the ureter (fig.1a).

The parenchyma composing the kidney can be divided into two layers: the cortex forming the outer layer and the medulla forming the inner layer(fig.1a). Found spanning these different layers are the functional units of the kidney, the nephrons. Mice kidneys contain 10 000- 20 000 nephrons that consist of ramified networks of epithelial tubes that transport ultrafiltrate. The nephron forms a tube composed of epithelial cells which differ in structure and function along the length of the tube. Different sections of the tube function to reabsorb and secrete waste molecules from or into the plasma ultra-filtrate to ultimately produce urine. The nephron is the part of the epithelial renal collecting system, from glomerulus to distal convoluted tubule, which has metanephric mesenchymal embryonic origins. The remainder of the epithelial system has its embryonic origins from the uretic bud (174, 175).

A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Renal Pelvis
Medulla
Ureter

B

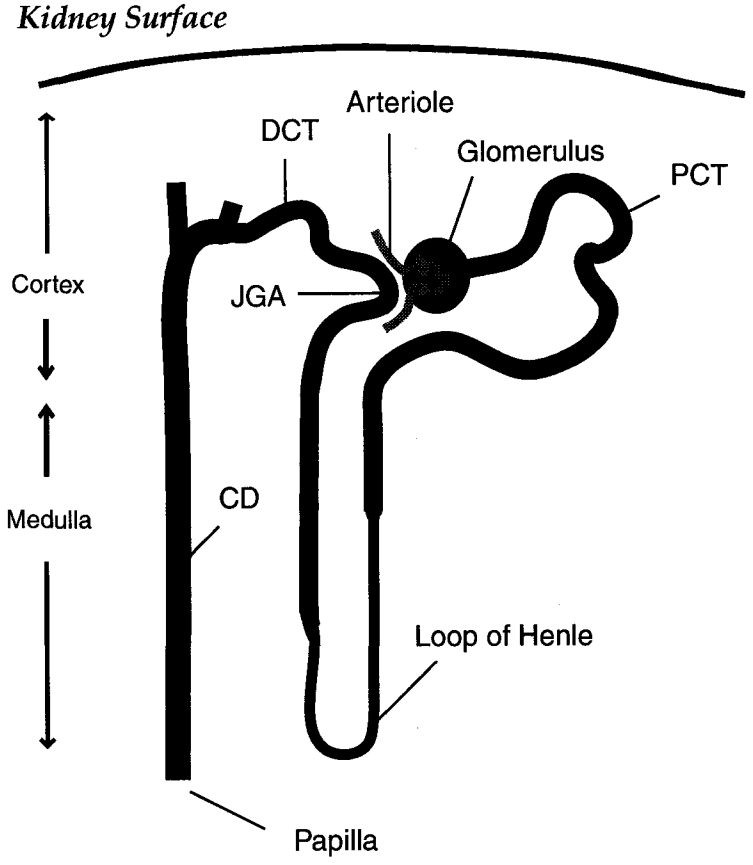

Kidney Surface
Arteriole
DCT
Glomerulus
PCT
Cortex
JGA
CD
Medulla
Loop of Henle
Papilla

Figure 1. **Anatomy of the unipapillate rodent kidney.** **A.** Gross anatomy of the kidney. The two layers of the kidney parenchyma are the (outer) cortex and the (inner) medulla. Urine produced by the kidney is released at the papilla into the renal pelvic space that is continuous with the ureter lumen. **B.** The different segments of the nephron. The nephron is the functional unit of the kidney. Filtration of the blood occurs at the glomerulus, ultra-filtrate then travels through the proximal convoluted tubule (PCT), the loop of Henlé and passes the juxta-glomerular apparatus (JGA) through the distal convoluted tubule. The modified ultra-filtrate of many nephrons is received by a collecting duct (CD) and ejected into the renal pelvis at the papilla. (Drawings not to scale)

The glomerulus is the beginning of the renal collecting system where the first urinary filtrate enters Bowman's capsule. The fluid continues to flow through the proximal convoluted tubule which then sends a straight, thick descending limb radially toward the renal medulla that is continuous with the thinner tubule of the loop of Henlé. The loop of Henlé extends for a variable length with those originating from juxtamedullary glomeruli reaching the depths of the medulla before making a hairpin turn and returning toward the glomerulus. In its ascending course the loop of Henlé thickens, becoming the distal convoluted tubule. After passing its glomerulus between the afferent and efferent arterioles to form the juxta-glomerular apparatus, the distal convoluted tubule becomes continuous with the collecting tubules which coalesce to form a collecting duct. Urine is ejected from the collecting ducts into the renal pelvis from which urine is directed toward the ureter (fig.1b). Though composed solely of epithelia, the nephron is functionally segmented due to the different protein expression patterns of the cells (174, 175).

The filtration of blood occurs in specialised units called glomeruli. A glomerulus is comprised of glomerular capillary loops enveloped by the interdigitating foot processes (pedicels) of the visceral epithelial cells(podocytes). Podocytes also envelop the mesangial matrix (MM) which contains mesengial cells and functions to support the capillary loops. Separating the podocytes from the MM and the endothelial cells is a continuous BM. At the podocyte/endothelial interface, this basement membrane is called the glomerular basement membrane (GBM) and is essential for filtration (174, 175). The glomerular filtration barrier consists of three layers: the innermost fenestrated vascular endothelium, the GBM and the podocyte cell layer facing the urinary space. The flow of the glomerular filtrate is thought to follow an extracellular route, passing through the endothelial fenestrae, then across the GBM and finally through the slit

diaphragm present between the inter-digitating pedicels of the podocytes. The filtration effect across the glomerular filtration barrier is dependent mainly on the size of molecules, but also depends on their charge. Molecules equal or greater than albumin are excluded from the filtrate. Neutral and cationic molecules are repelled less strongly than anionic molecules of equal size. Though the GBM serves as a pre-filter of larger plasma proteins, the slit diaphragm is thought to function as the decisive ultra-filter (49).

3. METANEPHRIC DEVELOPMENT

Development of the mouse renal system starts at embryonic day 7.5 (E7.5), of its twenty-one day gestation, when a subpopulation of mesenchymal cells form an epithelial tube, the Wolffian duct. The duct extends rostrocaudally and induces the formation of epithelial tubules in the adjacent nephrogenic mesenchymes. Three embryonic kidneys are formed in consecutive order: the pronephros, at about E8; the mesonephros at E9.5-E11; and the metanephros, which starts to form at about E11. Only the metanephros persists throughout the life of the organism forming the definitive functional kidney after birth.

Metanephric development begins when a small epithelial bud, the ureteric bud, emerges from the Wolfiann duct and grows dorsally to invade the metanephric mesenchyme (fig.2a). Two interdependent processes become essential for further development, branching morphogenesis and mesenchyme to epithelium transformation. Branching morphogenesis is the generation of complex branched tubular networks constructed of an epithelial monolayer of cells wrapped into a tubular structure (118). Mesenchyme to epithelium transformation, as the name implies, is the differentiation of mesenchyme into a polarised epithelia which, during nephrogenesis, occurs at the tips of the branching uretic bud. By maturity, branching morphogenesis will have contributed

to the collecting duct tree and ureter, and the mesenchyme to epithelium transformations will have contributed to the excretory nephrons.

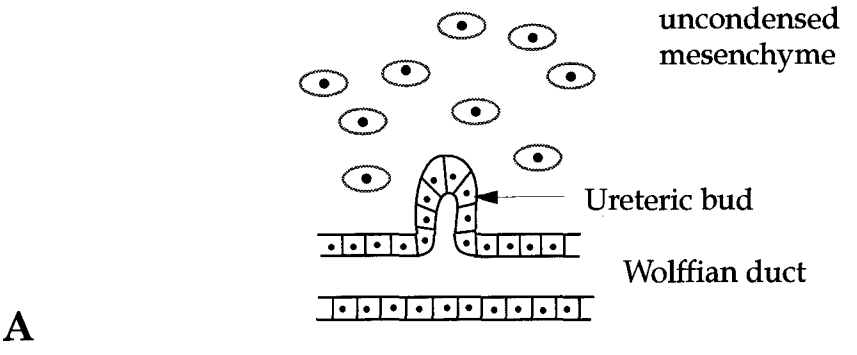
That nephric epithelium is derived from mesenchyme rather than ingrowing epithelium of the Wolffian duct was confirmed by *in vitro* experiments of Clifford Grobstein's in the 1950's. Grobstein demonstrated that embryonic kidney rudiments could be excised and grown in culture, recapitulating development *in vivo*, except that the explants were avascular. Two tissues, the metanephric mesenchyme and the ureteric bud epithelia, were found to be necessary and sufficient for *in vitro* kidney formation. Surgical ablation and genetic deletion of the ureteric bud also prevented epithelialisation of the metanephric mesenchyme (122, 123). The ease of the *in vitro* methods established by Grobstein has made the kidney a model system for epithelial morphogenesis.

Growth and branching of the ureteric bud is induced by signals from the metanephric mesenchyme. Reciprocally, signals from the ureteric bud trigger differentiation of the mesenchyme, into distinct cell types. A subpopulation of mesenchymal cells differentiates into epithelial tubules by a complex pattern of differentiation involving elongation, segmentation, convolution and fusion, to finally form the mature nephron. Epithelialisation starts with the formation of mesenchymal condensates that subsequently are transformed via renal vesicles, comma-shaped bodies and S-shaped bodies into the polarised epithelia of the nephron (fig.2b). This nephron then establishes a connection between its distal tubule and the portion of the uretic bud derivative that is itself maturing into a collecting duct. The cycle of growth and branching of the ureter, formation of epithelial tubules from mesenchymal cells, and fusion of the two structures is then repeated many times generating the complex network of nephrons of the final kidney. Each mesenchymal condensate, induced by the tip of a growing uteric branch, gives rise to a single nephron (fig.2c). Thus, the branching

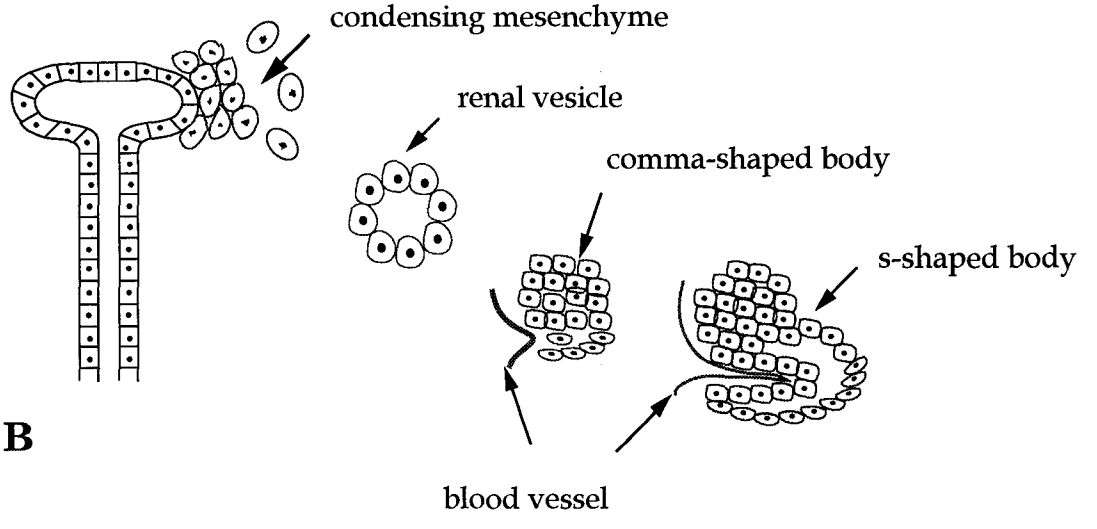
pattern of the uretic epithelium largely dictates the basic histoarchitecture of the kidney (117).

Blood vessel development is an integral part of glomerular morphogenesis. Visceral and parietal epithelia are detectable at the S-shaped stage. Development of the glomerulus begins by the invasion of endothelial cells into the glomerular cleft, a space between the lower and middle limb of the late S-shaped tubule. Lining the lower limb of the S-shaped tubule are the visceral epithelial cells that differentiate into the glomerular podocytes. As the podocytes mature, their basal surfaces develop into foot processes. During the development of the glomerulus the specialised GBM is assembled. *In vivo*, vascular components of the kidney may develop from metanephric mesenchymal cells *in situ* (vasculogenesis) or may migrate into the system from precursors outside the kidney (angiogenesis) (17).

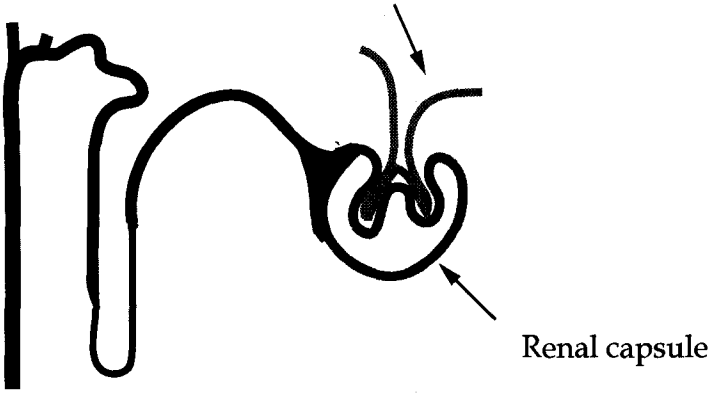
When mesenchymal cells begin to transform into an epithelium they aggregate and begin to synthesize a new basal lamina. This transformation involves a change in the type of ECM molecules and receptors expressed (115, 116, 101). Alterations in the type of BM produced during development has been reported. Molecules selectively found at the tips of the growing ureter tree would be involved either in the induction of the ureteric branching or the differentiation of the mesenchyme (93). Cell contact between inducer cells and mesenchyme are important for induction, but they do not exclude induction mediated by diffusion or by ECM components. *In vivo*, the ureteric cells deposit a BM. However, small gaps in the membrane do exist and direct cell-cell contacts between the ureter and mesenchyme may occur. The response to induction is


uncondensed
mesenchyme
Ureteric bud
Wolffian duct

A


condensing mesenchyme
renal vesicle
comma-shaped body
s-shaped body
blood vessel

B


Renal capsule

C

Figure 2. **Stages of metanephric development**. **A**. Development of the metanephric kidney begins by the epithelial budding of the Wolffian duct, toward metanephric mesenchymal cells, called the ureteric bud. **B**. As the ureteric bud grows and undergoes branching morphogenesis, mesenchymal cells condense at each of its tips. The condensed cells aggregate to form renal vesicles. These vesicles continue to grow and differentiate into a comma-shaped body (when blood vessels begin to invade) and eventually into an s-shaped body. **C**. By the end of nephrogenesis, tubule elongation and cellular differentiation will have occurred. The distal portion of the developing epithelial tubule will have fused with the differentiating ureteric bud and the glomerulus at the proximal end will be fully functional.

an increase in cell adhesion, therefore expression of adhesion proteins at the cell surface. The formation of a BM is an essential feature of development of epithelial sheets. BM material accumulates around the condensates that are not seen in the cells located in the central part of the condensate. Immunofluorescence has shown that BM materials contain laminin, type IV collagen and proteoglycans, the major constituents of most BM (94, 95, 96, 101)

4. Extracellular Matrix Components of the Kidney

a. CollagenIV

The non-fibrillar collagen IV group of proteins are involved in the formation of protein sheets that surround tissues (200). They are part of a large and diverse family of proteins, which are the main extracellular structural proteins of all multicellular organisms (63). Members of the collagen family all contain one or more collagen triple helix domain which arises from the intertwining of three helical polypeptides to form a superhelix, called a protomer. Every collagen protomer is therefore composed of three polypeptides, each individually forming a left-handed helix in which every third residue forms the centre of the superhelix. The three helices entwine producing a right-handed superhelix. The amino acid sequences of triple helical domains are thus characterised by the repetition of triplets Gly-X-Y within each polypeptide, where X and Y can be any amino acid. It is the presence of a glycine at every central third position, which enables the assembly of three helices into a triple helical structure. The X and Y amino acid side chains point outwards of the helice, characterising the chemical surface of the helice. Even though one third of these positions are occupied by imino acids which stabilise the structure, it offers an exceptional potential for lateral interactions, particularly with other triple helical domains (27, 199).

Type IV collagen includes six genetically distinct polypeptides named α1(IV) through α6(IV) (202, 50). These isoforms, therefore have the potential to organise themselves into unique networks providing specificity to different BMs. The type IV collagen protomer is composed of three α chains that bind through associations among their non-collagenous (NC1) globular domains followed by the folding of the collagen domain through covalent and non-covalent interactions. The type IV collagen protomer is divided into three different domains: an N-terminal 7S domain, a middle collagen domain interrupted by short non-triple helical sequences and a C-terminal NC1 globular domain. The type IV collagen protomer is formed during passage through the endoplasmic reticulum and the Golgi apparatus. Protomers are then secreted into the extracellular space where they are not processed by proteases but self-assemble into network-like structures. The assembly process requires lateral interactions and disulphide bonding between triple helices at two different locations. The first occurs at the 7S domain, where four triple helical domains from four different collagen IV protomers assemble head to head (via 7S:7S interactions) to form spider-like tetramers. The second is the tail to tail (via NC1:NC1 interactions) binding of Type IV molecules via the globular NC1 domains to produce linear dimers. The complex, branching network is formed by assembly of dimers and tetramers strengthened by lateral association and partial winding of molecules together. It is this fine network which contributes to the sheet-like structure of BM to help entrap large associated molecules such as the laminin-nidogen complex and the heparan sulfate proteoglycans (27, 200,63).

Interactions of all six chains of type IV collagen were identified by protein biochemistry techniques using bovine testis (201). The studies showed the occurrence of two different populations of type IV collagens. One population as in the EHS tumour contains only α1 and α2 chains, the other contains α1, α3-α6(IV). These findings support the idea that α3-α6(IV) play unique roles in specialised BM reflected in their more

restricted tissue distribution whereas the [α1(IV)$_2$ α2(IV)] has a more general function (50).

The distinct subtypes of type IV collagen, differing in chain composition, have been described and are reported as having different locations in normal human kidney tissue. The α1(IV) and α2(IV) chains are located mainly in the MM and in the vascular and tubular basement membrane (TBM). Ultrastructual studies showed that the α1 chain is distributed mainly along the endothelial side of glomerular basement membrane and MM. The α3 and α4 chains are detectable throughout the GBM but not MM (28). There is a lack of α6(IV) in GBM, however in Bowman's capsule BM α5(IV) and α6(IV) are present in association with α1(IV) and α2(IV) (50).

The embryonic GBM contains [α1(IV)$_2$ α2(IV)] collagen, but postnatally this is replaced by adult collagen IV trimers containing [α3:α4:α5] chains. Because of its higher cysteine content, the adult form contains more inter molecular disulphide crosslinks, which are thought to provide strength to the GBM. When the adult collagen IV network is absent or dysfunctional it is replaced by the most abundant but less crosslinked, type collagen IV [α1(IV)$_2$ α2(IV)]. This is seen in Alport's syndrome, an X-linked human condition resulting in severe kidney dysfunction due to mutations in the gene encoding the α3(IV), α4(IV) or α5(IV) chain (152,153,27). A mouse model for Alport's syndrome exists with a lack of the collagen α3(IV) expression. In these mice fetal collagen α1(IV) and α2(IV) chains persist in the adult GBM. Multilamination and splitting of the GBM is observed ultrastructurally prior to detectable renal functional defects that lead to end-stage renal failure and death at fourteen weeks of age. The glomerular filtration decreases over time and the integrity of glomerular structures is compromised, suggesting that collagens play essentially a structural role (168).

b. LAMININ

The laminin family of glycoproteins dramatically influence cell attachment, spreading, growth and differentiation. Laminins form the second principal network contributing to the structure of BMs. Laminins are heterotrimeric protein complexes formed by the covalent bonding of one polypeptide from each of the α, β, γ laminin subunit families. Each subunit is a family of multiple members encoded by individual genes (106). Thus many variant laminin trimers with specialised function may potentially be formed depending on spatio-temporal differential subunit gene expression. . The archetype form of laminins (αβγ) is a cruciform structure, where each short arm is formed by a single chain and the longer arm is the entwined C-terminus of all three chains to form a triple coiled α-helice, reminiscent of the collagen protomer. Laminin-1 (α1β1γ1) was the first member isolated from the murine EHS (30), there are now fifteen different laminins postulated in mammals resulting from five α, three β and two γ chains (170).

Localised, complex patterns of laminin chain expression have been described for the developing kidney. When kidney mesenchyme is induced, expression of β1, and γ1 chain mRNA increases. *In situ* hybridisation of whole kidneys has confirmed that the laminin α1 chain mRNA is expressed locally in those areas where new tubules form (93) The appearance of the laminin α1 polypeptide correlates with the onset of basement membrane formation in a polarised fashion at early stages of cell development (97). The α1 laminin chain is transiently expressed in the BM of podocytes and largely absent from GBM at later stages (99). The α2 chain mRNA is expressed only by mesenchyme(104). Laminin-8 (α4β1γ1), which is absent in the adult, is expressed by the nascent epithelial basement membrane of the renal vesicle shortly after the mesenchyme to epithelium

transition. Laminin-9 ($\alpha4\beta2\gamma1$), also absent in the adult, is present in the immature GBM. The $\alpha4$ chain gradually disappears with maturation (103).

The mature kidney nephron shows distinct regional expression of laminins within its BM (102, 103). Laminin-1, which is expressed at all stages, in the mature kidney is expressed in the cortex by proximal TBMs and in the medulla by the loop of Henlé BMs (165). Laminin-2 ($\alpha2\beta1\gamma1$), is located in the MM and a subset of TBMs (165, 100). Laminin-10 ($\alpha5\beta1\gamma1$) in the mature and adult kidney is found throughout the length of all tubular and collecting duct BMs (103, 165). Laminin-11 ($\alpha5\beta2\gamma1$), the only trimer shown to be important for proper renal function (51), is expressed only in the GBM and arteriolar BM (103, 165, 93).

Laminins can self-assemble into networks and cooperatively bind cell receptors, which act as nucleation sites contributing to the BM (62, 63). Two such types of receptors are integrins and dystroglycan. The mapping of regions within laminin that interact with such receptors has often utilised fragments of laminin prepared by enzymatic treatment of laminin 1. Two such fragments are elastase fragment 3 (E3), which is composed of the C-terminus of $\alpha1$ subunit containing the last two globular domains, and E8 which is composed of the C-terminus of all three chains excluding the two globular domains of $\alpha1$ laminin chain (126). Inhibition of development has been achieved with the antiserum against the E8 and E3 fragments, but not with antiserum against the central parts of laminin (97). The E8 fragment of laminin can bind $\alpha6\beta1$ integrin (98) and the E3 fragment can bind α-dystroglycan. This suggests that laminin-1 is an autocrine stimulator of kidney tubule development by binding integrins and dystroglycan.

Although no human kidney disease has be linked to any of the laminin genes, targeted mutations of different laminin genes in mice has lead to a greater understanding of the importance of certain laminins to kidney development and function. For example, disruption of the laminin β2 chain has been shown to result in abnormal development of neuromuscular junctions and of kidneys (51). Functionally the β2 knock-out mice show massive proteinuria which inhibits their growth rate (51). Normally the expression of β2 begins in the mature kidney concurrent with a decrease of β1, which was expressed during early development. Presumably as a compensatory effect, the laminin β2 knock-out mice maintain the expression of β1 in the mature kidney. The laminin β2 knock-out mice demonstrated an ultrastructurally intact BM in the kidney including the GBM, the BM involved in the filtration of blood. Though a structurally normal GBM is present the pedicels encircling the capillaries are fused ultrastructurally. The defects resemble those seen in human minimal change nephrotic syndrome (MCNS) which also show decreased dystroglycan expression, suggesting that both defects result from alterations in the same podocyte differentiation regulatory pathway (19).

The targeted mutations of other laminin chains are have either no effect on kidney development or function, or are embryonic lethal. Disruption of the γ1 chain, the most ubiquitously expressed laminin subunit and one of the earliest expressed laminin subunits, is an embryonic lethal. Mice with targeted laminin α5 chain gene lack both laminin 10 and 11, die *in utero* at E14 to E17 and show defects in neural tube closure, limb malformation and maturation of placenta (166). In laminin α5 chain null animals a renal phenotype arises consisting of small or absent kidneys and aberrant glomerugenesis due to an absence of GBMs. The podocytes fail to organise and the interaction between the GBM and endothelial cells is compromised resulting in

avascular glomeruli (209). Mice with targeted deletion of the laminin α2 chain show no effect in kidney formation or function. These animals show muscular dystrophic phenotypes demonstrating the importance of this laminin in skeletal muscle development and function (164).

c. NIDOGEN-1

Nidogen-1, also called entactin-1, found ubiquitously in BMs is considered to function as a crucial link between collagen IV and laminin networks allowing increased stability of all BMs (109, 119, 162). Nidogen was identified from two independent sources, from a basement membrane-secreting cell line (157) and from the ECM of EHS tumour where nidogen co-purifies with laminin-1 (158). Nidogen-1 comprises three globular domains (G1-G3). G1 and G2 are separated by a flexible link, whereas G2 and G3 are separated by a rod-like segment. The C-terminal globule (G3) binds non-covalently to domain III of the laminin γ1 chain (112). Domain G2 associates with collagen IV and the protein core of perlecan (60,160). These interaction suggest that nidogen 1 is a linker between the collagen IV and laminin networks of BMs. Nidogen 1 is highly susceptible to protease degradation and its destruction may be the initial step in the breakdown of the basement membrane during tissue remodeling (161). A second isoform has been found and others may later be discovered (159). In the kidney nidogen-1 is found in GBM, TBM and Bowman's capsule BM (56,57).

Antibodies that block the interaction between laminin-1 and nidogen have been shown to perturb branching epithelial morphogenesis of embryonic lung and kidney (111). In the presence of the antibody only half of the expected number of tubules form in kidney explants (111). *In situ* hybridisation studies suggest that nidogen during embryogenesis is produced by mesenchyme rather than epithelium in embryonic organs (110). Nidogen-1 production by mesenchymal cells is believed to promote epithelial

differentiation by promoting BM formation.

A human disease has not been linked to the nidogen gene. Moreover, nidogen 1 deficient mice are viable and their tissues form normal basement membranes. Although nidogen 2 antibody staining is increased in certain basement membranes such as muscle cells, organs like the kidney shows no compensation by this family member (163). These result suggest the role played by nidogen in laminin assembly may not be structural.

d. HEPARAN SULPHATE PROTEOGLYCANS

The negative charge of HSPG imparted by the carbohydrate chains is thought to be especially important to the charge-selective, ultra-filtration properties of GBM (80-81). As the name implies proteoglycans are proteins whose molecular weight is largely due to high levels of glycosylation. This involves glycosaminoglycan side chains such as heparan sulphates (HS), which are linear polysaccharides, with specific biochemical modifications notably the addition of sulfate groups. Any binding to proteoglycans can be via their protein core or their HS chains. Composition of basement membrane will therefore depend on the expression of the core protein of the proteoglycan as well as the composition and structure of the HS chains.

The regulation of the glycosylation dictates the final structure of proteoglycans. Although only one of many different types of carbohydrate moieties, recent studies suggest HS as an important cell regulator with specific domains important in binding proteins. Culture systems suggest HS proteoglycans are trafficked rapidly to cell surfaces offering rapid functional changes to BM (59). The majority (>80%) of proteoglycans in the mature GBM are associated with HS rather than with other GAG (chondroitin sulfate, dermatan sulfate or keratan sulfate) (84). An insertional mutation in the gene for the HS 2-sulfotransferase (an enzyme which adds sulfate groups to

iduronic and gluronic acid) produces renal agenesis indicating a necessity of HS for kidney development (172).

The first extracellular matrix HSPG isolated, characterised and cloned from mouse EHS was perlecan, named after its beads-on-a-string-like appearance in rotary shadowing electron microscopy (76,77,78,61,85). The sequenced perlecan core protein in the mouse is 396 kDa (120). Perlecan's core protein has five functional domains with three GAG chains linked to the N-terminus (179). The core protein of human perlecan, 467 kDa, is composed of five domains. The amino-terminal domain I contains three heparan sulfate (HS) chains; domain II has four LDL receptor-like repeats; domain III contains repeats resembling those in the short arm of laminin; domain IV has Ig-like repeats similar to those in NCAM, and domain V contains repeats similar to those in laminin α1 chain and EGF motifs. Such a domain structure suggests that the core protein carries multifunctional properties (182).

The integration of large proteoglycans such as perlecan into BM is dependent on both core protein and GAG structures. The perlecan core protein binds collagen IV and nidogen-1. The nidogen G2 domain was shown to interact with the core protein, but not the HS chains (180). The perlecan HS chains interact with laminins and other ECM proteins (109,180). The core protein is also involved in a self assembly process (181). Within BMs perlecan can have functions other than simply structural ones. The GAG chains at the N-terminus have been shown to bind basic FGF-2 and to promote mitogenic and angiogenic activities (183).

In the developing kidney, perlecan expression is initiated as early as the vesicle stage (177). Immunoelectron microscopy shows that perlecan is exclusively present on the endothelial side of the GBM and distributed in a non-homogeneous manner

throughout this BM (75). However, the majority of perlecan immunoreactivity is observed in Bowman's capsule BM and the MM(75, 179).

When perlecan expression is abolished in homozygous knockout mice no abnormalities are observed before embryonic day 10. However, between E10 and E12 the majority of embryos die due to BM rupture of the heart, at a time when the intraventricular blood pressure is though to increase significantly. Although BMs can form in the absence of perlecan, its presence is essential for maintenance of the mechanical and functional integrity of BM. The few embryos that survive this critical stage die perinatally with severe defects of the brain and the skeletal system (184). Identified mutations in perlecan in humans are associated with the human Schwartz-Jampel Syndrome, a chondrodystrophic syndrome (abnormal development of cartilage) (176).

Agrin is an other important proteoglycan (66) which was initially isolated from *Torpedo* electric organ basal lamina (67). The agrin protein core has a mass of 212 kDa in humans (79). The primary structure of agrin is composed of multiple modules. The N-terminal begins with a globular laminin-binding domain followed by nine follistatin-like protease inhibitor domains; two laminin-like epidermal growth factor (EGF) repeats; two serine/threonine-rich domains; a Sperm protein, enterokinase and agrin (SEA) module; four EGF repeats and three domains sharing homology with globules of the laminin α chains (79,85) Agrin contains two regions particularly favourable for HS attachment, both located in the central part of the molecule (86).

A number of isoforms of agrin exist. There are two distinct 5' N-terminus isoforms. Long NH_2-terminal agrin (LN) has 150 amino acids prior to the common core protein, whereas the short NH2-terminal form (SN) has 49 amino acids prior to the

common core protein. SN and LN airguns are likely to be transcribed from distinct promoters. LN agrin is an extracellular protein whereas SN agrin is a type II transmembrane protein (69,70). Three alternative tissue specific splicing sites (designated X, Y and Z) near the 3' end of the agrin generate multiple isoforms that either contain or lack short segments within these sites. Inclusion of a four amino acid (aa) at a site called Y in mammals is required for agrin to binding to heparin. Inclusion of 8, 11, or 19 aa segments at a nearby site called Z in mammals is required for agrin to induce postsynaptic differentiation at the neuromuscular junction (65, 71,72, 73, 74).

Agrin is expressed in a wide array of tissues with particularly high levels in kidney and lung (79). SN agrin is largely confined to the nervous system whereas LN agrin is broadly distributed in neural and non-neural tissue (70). Although found in all renal BMs, agrin is a major component of the GBM indicating that it may play a role in renal ultrafiltration and cell matrix interaction. Immunoelectron microscopy has shown agrin to have a linear distribution along the GBM being present throughout its width (75). The N-terminal of LN agrin binds laminin at a central region in the long arm of the laminin cruciform allowing agrin to contribute to the molecular architecture of the GBM (86).

Abnormal kidney organogenesis or protein expression levels have not been reported in any agrin mouse mutant. These mutants include animals null for LN-agrin (70), z-agrin and a hypomorph for all other agrin forms (68) as well as agrin isoform specific mutants lacking a Z or a Y insert (65). Consistent with the work in mice, the agrin gene has not been directly linked to any human kidney diseases. However, agrin may be involved in the pathogenesis of diabetic glolerulopathy and MCNS, two diseases that produce proteinuria. The agrin core protein is down regulated in diabetic glomerulopathy, whereas in MCNS there is alteration of the HS chains of the core

protein (87, 88, 89).

5. Extracellular Matrix Receptors in the Kidney

a. Integrins

Integrins are a superfamily of cell surface receptors, that mediate cell-matrix and cell-cell adhesion. The first integrin to be discovered bound to fibronectin (125). In mammals more than 20 integrin heterodimers are expressed, each composed of two transmembrane glycoproteins, α and β chain. Integrins are cell membrane receptors involved in cell signaling pathways necessary for cell differentiation and survival (154, 155). Integrins are found in a wide range of cells including epithelia, muscle, stromal, and mesenchymal cells as well as cells of the nervous system and immune system (156).

There is widespread expression of integrins during kidney development and in adult kidneys. During the initial stages of development the uretic bud epithelia express α6β1 and smaller amounts of α3β1, whereas α8β1 is expressed on the metanephric mesenchyme at sites where it is in contact with uretic bud (143). The α6 subunit also appears during conversion of mesenchyme to epithelium. The α6 integrin is expressed by the cells of the condensing mesenchyme but not by uninduced mesenchyme becoming enriched on the basal side (99). By the S-shaped tubule stage different segments of the nephron already express distinct integrins.

Each section of the nephron has a distinct set of integrin receptors. In the glomerulus the α3β1-integrin is the major ECM receptor expressed by podocytes along the GBM. Integrins expressed on Bowman's capusle include α1β1, α3β1, α6β1 and αvβ3. The α1β1-integrin is the most prominent type expressed on mesangial cells which also express to a lesser amount α2β1, α3β1,and α6β1. The α6β1 appears to be the major

laminin receptor expressed by proximal tubule epithelial cells. The α2β1, α3β1 and α6β1-integrins are all expressed in the epithelia of distal tubules and collecting ducts (146-8).

Antibodies against α6 subunit have been shown to interfere with mouse kidney tubule development *in vitro* (99). However, there is an absence of a renal phenotype in the α6 integrin null mouse(146), in contrast to the from *in vitro* studies evidence, which suggests it is not critical for development. The majority of mice null for α8-integrin subunit do not develop kidneys. The uretic bud never expands into the mesenchyme which suggests a role of this subunit in the production of induction signals by the mesenchyme (143).

The α3β1-integrin, expressed by podocytes, binds laminin-5, -10 and -11 (151). It is probably the laminin-10 or -11 which is its ligand in the GBM since the laminin α5 chain has been colocalised with this integrin receptor (150). Targeted mutation of the α3-integrin gene has several defects in kidney development (144). Podocytes are unable to assemble mature foot processes even though they remain adherent to the GBM. The GBM itself is fragmented, there is at times failure of fusion of the epithelial and endothelial derived components. There are fewer capillary loops in each glomerulus and those present have larger diameters. The kidneys also have fewer collecting ducts within the papillary region of the medulla. These phenotypes are suggestive of a decrease in branching morphogenesis or an increase in apoptosis (144). No human kidney diseases have been directly related to mutations in the integrin genes however altered expression levels of integrins is often associated with kidney pathologies (149)

b. DYSTROGLYCAN

The first identification of dystroglycan was the discovery of a 120 kDa glycoprotein laminin receptor, called cranin, in the membranes of rodent NG 108-15 neural hybrid cells and chick brain membranes (8, 167). Soon thereafter, it was found that dystroglycan was part of a complex of proteins, termed the dystrophin-glycoprotein complex (DGC), which anchors dystrophin to the sarcolemma of skeletal muscle (1,9). Conserved in mammals, dystroglycan is the product of a single gene, *(Dag1)* located on chromosome 9 in mice (2,3) and (DAG1) located on chromosome 3 band p21 in humans(4). The DAG1/*Dag1* gene product is actually a precursor protein of 97 kDa which is post-translationally cleaved into two fragments, the N-terminal and C-terminal regions giving rise to the α and β-dystroglycan subunits respectively (5). Post-translational processing of both subunits, produces α and β subunits of 120-200 kDa and 43 kDa respectively. It is this transmembrane heterodimeric complex of α and β dystroglycan subunits that has been shown to be the central component of the DGC by forming a link between the extracellular matrix and the actin cytoskeleton. This connection is possible since α-dystroglycan is an extracellular peripheral membrane protein which binds non-covalently to the ectodomain of the transmembrane protein β-dystroglycan. The different molecular weights of α-dystroglycan's are due to spatio-temporal differential levels of glycosylation which may reflect distinct functional states of the receptor (6,7).

The assembly and function of BM requires cell surface receptors. Dystroglycan is such a receptor which has been demonstrated to bind to laminin α1 and α2 chains (1,8,9,10, 107, 108) as well as the proteoglycans agrin and perlecan (11, 12, 13). Dystroglycan null mice are embryonic lethal, at approximately E 6.5, due to disruption of Reichert's membrane, one of the first basement membranes formed during murine development (14). Dystroglycan has also been suggested to be required for the

formation of a basement membranes in embryoid bodies (15) further demonstrating a role for dystroglycan in the assembly of ECM proteins.

Dystroglycan is expressed in a broad array of embryonic and adult mammalian tissues. In the mouse at E13, dystroglycan has been localised to the epithelia of the choroid plexus, lung, gut, urogenital and tooth systems as well as skeletal muscle and cardiomyocytes. In the adult mouse dystroglycans are localised to: skeletal muscle sarcolemma; epithelial cells of the digestive tract, kidney and lung; spleen, pancreas, liver, trachea, mammary glands, testis, choroid plexus (14), uterus (3), skin, Schwann cell outer membrane and in neurons of both the central and peripheral nervous system. Dystroglycan has also recently been localised to rat thyroid tissue (64). This tissue distribution of dystroglycans suggests a role as a widespread cell surface receptor involved in linking cells structurally and functionally to BM, particularly epithelial cells (16).

The monoclonal antibody IIH6 is known to block the binding of α-dystroglycan to laminin-1 and agrin, and perturbs development of the epithelium in kidney organ culture, suggesting that dystroglycan acts as a receptor for BM components during epithelial morphogenesis (113). Localisation of dystroglycan in the developing kidney suggests a function both in the branching of the uretic bud and in the mesenchyme to epithelial differentiation. During the initial stages of kidney morphogenesis (E12), dystroglycan is expressed in the condensed mesenchyme and ureter epithelium. By E14 dystroglycan is locally expressed in comma-shaped bodies where cells are differentiating into polarised epithelial cells. At later stages dystroglycan is also expressed in s-shaped bodies which give rise to the epithelium of Bowman's capsule and elongating tubules. During the *in vitro* culture of metanephric mesenchyme, mRNA of the α1 chain of laminin is increased in expression concurrently with dystroglycan (18). Dystroglycan

and laminin 1 colocalise in the BM of the ureter and developing tubules of the kidney. This suggests that dystroglycan is an epithelial receptor for laminin-1 in the developing kidney. In adult tissue the dystroglycan complex has been more specifically localised to the basolateral membrane of tubular epithelia and to the visceral epithelia of the glomerulus(also called podocytes). These podocytes are integral components of the glomerular basement membrane (GBM) which selectively filters blood.

Whether alignment of GBM matrix components, which dictate the filtration properties, is achieved by self-assembly or is actively regulated by podocytes, is not known. However, adhesion of podocytes to the GBM is critically associated with proper filtration through the glomerular capillary wall since proteinuria is invariably associated with flattening of foot processes and occasionally also detachment of podocytes. Adhesion molecules that have been localised to the podocyte membrane in contact with the GBM include $\alpha 3\beta 1$ integrin and dystroglycan. Dystroglycan is misexpressed in Minimal Change Nephrosis and Adriamycin Nephropathology and is mislocalised in Focal Segmental Glomerulosclerosis (13,19). These are considered 'podocyte diseases' mainly because their principal pathologic feature is extensive flattening of foot processes accompanied by nephrotic-range proteinuria. The severe derangement of podocyte foot processed raises the possibility that the interaction of extracellular matrix dystroglycan could be involved in the maintenance of the differentiated state of these cells.

DYSTROPHIN - GLYCOPROTEIN COMPLEX

The DGC is a large oligomeric complex that bridges the sarcolemma connecting the ECM to the actin cytoskeleton (1,189,190, 105). The core skeletal muscle DGC is composed of intracellular dystrophin, the sarcoglycans (α-, β-, γ-, ∂-SG)(193), dystroglycan (α and β), sarcospan (188) and the syntrophins (194). A number of extracellular and intracellular proteins associate with the DGC. Intracellularly nitric

oxide synthase (191), dystrobrevin (192), rapsyn (195) and Grb 2 (196) have all been shown to interact with the cytoplasmic DGC. The association of nitric oxide synthase and Grb1 suggests the DGC may initiate signal transduction pathways. The purpose of these pathways has yet to be determined. Extracellularly, laminin-2 (107, 108, 9), perlecan (198), biglycan (197) and agrin (11, 12, 13) have been shown to interact with the DGC via α-dystroglycan

The DGC and its associated proteins are not restricted to skeletal muscle expression. There are diverse localisations of different DGC complex members in different cell types. The DGC members present in kidney epithelial cells exclude the transmembrane sarcoglycans and sarcospans and full-length dystrophin but, include cytoplasmic utrophin, dystrophin isoforms Dp 71 and Dp 140 (embryonically) (58), dystrobrevin and syntrophins (20,21). Mutations in different members of the DGC are known to produce varying forms of muscular dystrophy, though none have yet to be associated to renal anomalies (186, 187). Further characterisation of the epithelial specific complex is needed to gain knowledge about its functions in cells and more precisely the role of dystroglycan.

DAG1 -/- CHIMAERAS

A complete *Dag1* knock-out mice is embryonic lethal at E6.5 (14). Alternative genetic methods were therefore required to study DG function. To circumvent the bottleneck of embryonic lethality Dr. Carbonetto's laboratory chose to study chimaeric animals composed of wildtype cells and *Dag1-/-* cells (26). The use of chimaeras allowed complete development of the embryo and survival of newborn animals and analysis of defects in mosaic tissues. This approach, utilised by Dr. Patrice Coté, indicated that these mice could serve as models of muscular dystrophy. The major phenotype of the chimaeras (occurring in 10% of live births after blastocysts injection)

with high *Dag1*-/- percentage cell contributions was a progressive muscular dystrophy of the hindlimbs (26). The phenotype begins as an increase in mass of the hindlimb muscles followed by a decrease in hindlimb flexibility. The inflexibility of the hindlimbs leads to a restricted penguin-like gait. Kyphosis also develops in these animals probably due to degeneration of thoracic muscles.

Muscular dystrophy was not however, the only phenotype developed by *Dag1*-/- chimaeras. This is not surprising due to the wide spread expression of dystroglycan coupled to the fact that the totipotent ES have the potential to populate any cell lineage within an embryo. The eyes and kidneys are two other organs in which altered development was repeatedly seen. Eye variances included malformations and cataracts. A single kidney morphological anomaly was seen in four out of sixty five dissected chimaeras. Whether this phenotype was a consequence of a lack of dystroglycan during organogenesis was further investigated in this work.

AIMS AND PROPOSAL

The *in vivo* localisation of dystroglycan in kidney epithelial cells during development and the inhibition of kidney formation *ex vivo* by an anti-alpha dystroglycan blocking antibody (18,22) suggests a role for dystroglycan both in branching morphogenesis and mesenchymal to epithelial differentiation during kidney organogenesis. To test *in vivo*, whether dystroglycan has a role in kidney development and whether this role is in BM formation, *Dag1* -/- chimaeric animals, which rescue the embryonic lethality of *Dag1* -/- knockouts (14), will be used to generate kidneys deficient in dystroglycan. Such kidneys, deficient in dystroglycan may lead to abnormal basement membrane formation resulting in perturbed kidney organogenesis. Biochemical, histological and immunohistochemical detection methods will be used to analyse gross morphology of chimaeric kidney and the finer composition of the basement membrane.

MATERIALS AND METHODS

Generation of dystroglycan deficient chimaeric mice. Due to the early embryonic lethality of homozygous dystroglycan null mice (14) alternative systems lacking dystroglycan expression were necessary. The use of the precursor to the knockout mouse, the chimaeric mouse, allows the production of viable mice with random localised disruptions of *Dag1*. As previously, chimaeric mice were produced by injecting double knockout *Dag1* R1 embryonic stem (ES) cells (clone 3H1, passage 13) into C57Bl/6J blastocysts (26). The male R1 ES cells were isolated from blastocytes resulting from a cross of two 129 strains of mice (129X1/SvJ x 129S1/Sv+-p+$^{tyr\text{-}c}Mgf^{Sl\text{-}J}$)(52). The blastocysts injected with ES cells null for Dag1 were implanted into foster mothers and developed to full term. Injections were performed by a transgenic facility run by Dr. A. Peterson at the Royal Victoria Hospital. All other 129X1/SvJ and C57Bl/6J mice used through out the experiments were purchased from Jackson Laboratory.

Dissection and tissue collection. Weaned chimaeras were transferred from the Royal Victoria Hospital to the Montreal General Hospital animal facilities. Observations of the chimaera behaviours were noted throughout their lifespan. Once a chimaera developed a dystrophic phenotype, which include a waddling gait, enlarged hindlimbs and decreased flexibility of hindlimbs, they were anaesthetised and tissues dissected. Prior to dissection mice were anaesthetised using non-lethal doses of xylazine (2mg/ml, Bayer) and ketamine hydrochloride (15 mg/ml, Ayerst). Photographs were taken of the chimaeric animals and their organs during the dissection. For histology organs were either fixed in 4% paraformaldehyde at 4°C for 48 hours and embedded in JB-4 plastic embedding media (Electron Microscopy Science) or flash frozen in the cryopreserver O.C.T. (Sakura) using nitrogen cooled 2-methylbutane (171). The flash frozen samples were stored at -80°C for future experiments. The production of chimaeras spanned a few years therefore much of the kidney samples taken dissected prior to my arrival in the

laboratory by Dr. Patrice Côté and Dr. Hakima Moukhles.

Assessment of DAG-1 null ES cell contribution to chimaeric kidneys. Fresh frozen kidney samples were homogenised in a protein extraction buffer containing 1% Triton X-100 (25 mM Tris, 25 mM Glycine, 150 mM NaCl and protease inhibitor cocktail [Roche], pH 7.5). Homogenate protein concentrations were determined using the Dc protein assay (Biorad). The sample homogenates for chimaera 19, 21, 26, 70 and their respective age matched C57Bl/6J and 129X1/SvJ controls were used for glucose phosphate isomerase isozyme 1 (E.C. 5.3.1.9) (GPI1) analysis and immunoblotting. All other samples were solely used for the GPI1 assay. GPI-1 is a cytoplasmic enzyme expressed in all somatic tissues where it functions as a dimer (24, 25). Mice have two alleles, $Gpi1^a$ and $Gpi1^b$, which produce electrophoretically distinguishable isozymes. The use of the GPI1 assay in mouse chimaeric tissue analysis is based on the fact that different inbred strains of mice are homozygous for either Gpi1 alleles (23). The *Dag1* -/- chimaeras are a mosaic of *Dag1* +/+ C57BL/6J cells that express $Gpi1^b$, and *Dag1*-/- 129 cells that express $Gpi1^a$. The ratio between GPI-1 A and GPI-1B protein levels therefore reflects the ratio between *Dag1*+/+ and *Dag1*-/- cells within that organ. GPI isoforms were separated electrophoretically and visualised according to a procedure modified from Peterson et. al. (23). Electrophoresis using cellulose acetate plates (Helena Laboratories) was performed on 1µg of protein per sample to separate the GPI1AA and GPI1BB isoforms (AA and BB since the enzyme functions as a dimer), the slow and fast cathodally migrating isozymes respectively. Electrophoresis was performed initially for 26 min. at 100 volts (14 volts/cm) followed by 1 hr at 200 volts (28 volts/cm). To visualise the enzymes (23, 25), a 1% agarose solution containing glucose-6-phosphate dehydrogenase (50 units), D-fructose-6-phosphate (75 mg), nicotinamide dinucleotide phosphate (10 mg), phenazine methosulphate (1.8mg), nitroblue tetrazolium (10mg) and $MgCl_2$ (0.032M) was overlaid on the cellulose acetate plate and incubated at 37°C. All staining

reagents were purchased from Sigma. Once the agarose gel had set and the insoluble precipitate diformazan could be visualised, the gel was scanned using an AGFA ArcusII flatbed scanner. GPI1AA and GPI1BB band intensity was measured using NIH Image densitometry software. The relative intensity of the GPI1AA to GPI1BB band was taken to be the percentage contribution of *Dag1*-/- cells.

Histological Analysis of Chimeric Kidneys. Chimeric kidneys were examined histologically to qualitatively determine their gross morphology. Horizontal sections (1μm thick) of chimaeric, 129X1/SvJ and C57Bl/6J fixed kidney samples were stained using periodic acid methylamine silver to highlight the glomerular and tubular basement membranes (38). The haematoxylin and eosin counterstain highlighted cells and their nuclei. Embedding and staining were performed by the Montreal General Hospital Pathology Laboratory. Sections were observed and photographs were captured with Zeiss Axioskop microscope.

Western Blotting. Freshly dissected 1.5months old male C57Bl/6J tissues were homogenised using a dounce homogeniser in PBS containing a protease inhibitor cocktail (Roche). Homogenates were centrifuged at 16 000 xg for ten minutes. The pellet (P1) had its proteins extracted using a protein extraction buffer containing 1% Triton X-100 (25 mM Tris, 25 mM glycine, 150 mM NaCl and protease inhibitors, pH 7.4) at 4°C for fifteen minutes. The lysate was further spun at 16 000g for ten minutes. The detergent soluble supernatant (S2) and detergent insoluble pellet (P2) were kept. Protein concentration was determined using the Bradford Assay (Biorad).

Sodium dodecyl sulfate (SDS)-polyacrylamide (10%) gel electrophoresis was performed to separate proteins from tissue extracts. Electrophoresis was performed using a running buffer (0.2 M glycine, 0.025M Tris and 0.002M SDS). Separated proteins

were electrophoretically transferred onto Optitran nitrocellulose transfer and immunobilisation membranes (0.45μm, Schleicher and Schuell) in a transfer buffer (20% methanol, 0.2M glycine and 0.025M Tris). Following transfer membranes had non-protein bound sites blocked by incubating one hour at room temperature in 5% dried milk Tween-20 Tris-buffered saline, pH 7.5. β-Dystroglycan was visualised using a 1.5 hour primary incubation with rabbit polyclonal anti-β-dystroglycan antibody (92) followed by a 1 hr secondary incubation with a horseradish peroxidase linked donkey anti-rabbit antibody. Chemiluminescence was produced by activating the enzyme using the renaissance kit (NEN) and recorded on Kodak BioBlue film. Densiometry was performed on chimaeric and equivalent control samples using the densiometer SciScan 5000 (United States Biochemical). This enabled the quantification of β-dystroglycan protein expression levels.

Immunohistochemistry. Flash frozen kidneys from chimaeric and control (C57Bl/6J and 129X1/SvJ) of the same age and sex were chosen and serially sectioned (8μm) horizontally, using a Leica CM3050S cryostat. Mounted on each slide was a section from chimaeric tissue as well as each control tissue. This ensured that during later stages of the processing the tissues would be exposed to exactly the same conditions. Sections were stored at -20°C for up to five months. For immunohistochemistry, sections were thawed to room temperature, washed with PBS and blocked with 1% horse serum and 0.3% Triton X-100 PBS for one hour prior to immunostaining. All primary antibody incubations were 1.5 hours and secondary antibody incubations were 30 minutes. Sections were washed three times, 10 minutes per wash, after both primary and secondary antibody incubations. Sections were incubated with either rat monoclonal antibodies against perlecan domain 4 (Chemicon) or nidogen-1 (Chemicon) or rabbit antisera against rat β1 integrin (90), laminin 1 (α1β1γ1) (91), agrin C-terminal half (gift from Dr. M. Ferns, McGill University) or murine collagen IV (Chemicon). Slides were

mounted in immunofluore mounting medium (ICN biomedicals). Fluorescence was visualised using a Zeiss Axioskop and recorded with Kodak Select series Elite chrome 400 colour film.

RESULTS

Solubilisation of β-dystroglycan from murine kidney tissue. To effectively determine and quantify the level of β-dystroglycan expression in chimaeric kidneys, it was essential to determine its extractability within this tissue. Published protocols rely on extraction of β-dystroglycan with non-ionic detergents such as Triton X-100 (26). However, comparison of 1% Triton X-100 (4°C) soluble fractions from a variety of wild type C57Bl/6J mouse organs and tissues demonstrated that, unlike the majority of tissues, β-dystroglycan expressed in kidney is not extracted by this detergent. This can be seen by the lack of β-dystroglycan in the kidneys soluble fraction (fig.3a). Further investigation of all tissue fractions indicated that β-dystroglycan remained in the Triton insoluble fraction (fig.3b). This may reflect association of β-dystroglycan with the cytoskeleton of with distinct lipid domains (132). Regardless, subsequent determinations of protein expression levels during quantification of *Dag1* -/- cell contributions were therefore performed using whole tissue homogenates.

High *Dag1* -/- cell contribution does not directly correlate with an altered kidney morphology. Kidney morphology consisting of an enlarged pelvic cavity was found in a small number of chimaeric animals. A correlation between this kidney structure deviation and the level of *Dag1* -/- cell contribution was not found when comparing histological observations to cell contribution results.

The percentage cell contribution to chimaeric kidneys from the 129 mouse strain derived *Dag1* -/- cells was quantified by densiometric analysis of GPI1 assays which discriminates between the strain specific glucose phosphate isomerase isozymes. A total of 40 chimaeric mice between the ages of three months and twenty-two months had the *Dag1* -/- cell contribution to their kidneys determined by GPI1 assay. The cell contribution between left and right kidneys of a chimaera could vary by as much as

twenty percent which is within experimental error of the GPI1 genetic assay. An anomaly arose during these experiments. GPI1 analysis of Ch 19 indicated a great difference in cell contribution between the left (35%) and right (80%) kidneys. When Ch 19 right kidney was first dissected, all its tissue was utilised for experiments such as GPI1 analysis and histology by Dr. P. Coté. None remained for my analysis. A percentage cell contribution had been determined at the time of dissection for Ch 19 right kidney(Côté, P., unpublished), which I utilised for my work. Whether the different values obtained reflect asymmetrical distribution of *Dag1* null cells or differences in experimental technique cannot be determined. The percentage of C57BL/6J *Dag1*+/+ contributions to the chimaeric left and right kidneys are summarised in figures 4. A low wild type contribution indicates a high 129 *Dag1* -/- cell contribution to the chimaeric kidney.

Histological analysis was performed using periodic acid methylamine silver, to highlight basement membranes, with a haematoxylin and eosin counter stain to highlight cells and their nuclei. To help ensure that any anomalies found in chimaeric renal tissue were due to the lack of dystroglycan expression and not simply a strain related morphological difference, both age and sex matched 129X1/SvJ and C57Bl/6J wild type kidney tissues were examined. No significant macroscopic morphological difference between these two strains of mice was found. Thus indicating that any difference found in chimaeric kidneys should be related to a decrease in dystroglycan expression levels.

Identical gross morphological anomalies were found bilaterally in Ch 19 and 70 and unilaterally in Ch 21 and Ch 26 (right kidney). Each one of these kidneys showed an enlarged pelvic cavity, a thinner medullary layer and dilated cortical tubules. Further examination of Ch 19 (fig.5) and Ch 21 (fig.6) left kidneys at higher magnification

revealed the maintenance of normal intricate renal morphology including glomerular structure and juxta-glomerular junctions. The Ch 19 left kidney, as opposed to the higher *Dag1-/-* right kidney, was examined since the right kidney fresh frozen tissue was no longer available for later immunohistochemical analysis. The Ch 19 left kidney also showed a greater degree of morphological change than Ch 26 right kidney which had a 10% higher *Dag1-/-* cell contribution.

Over a third of the kidneys showing the altered gross morphology had high Dag1-/- cell contribution (fig. 7a): Ch 19 right kidney (80%) and Ch 21 left (80%) and right (80%) kidneys. The remaining kidneys: chimaera 19 left kidney (35%), Ch 26 right (45%) kidney and Ch 70 left (10%) and right (10%) kidneys showed altered morphology, but low *Dag1 -/-* cell contribution. Next, the expression level of β-dystroglycan in the chimaeric kidneys with altered morphology was determined in total organ homogenates (fig. 7b). Relative to expression in control kidney: Ch 21 left and right kidneys had no detectable levels of β-dystroglycan; Ch 26 right kidney had 2% β-dystroglycan expression levels; Ch 19 left kidney had 64% β-dystroglycan expression levels and both Ch 70 kidneys had 100% β-dystroglycan expression levels. Next, the correlation between the genetic contribution and actual protein expression levels was determined. Although the β-dystroglycan percentage expression levels were not expected to be identical to the cell percentage contributions, since not all cells within the kidney express β-dystroglycan, the protein expression levels did negatively correlate with the *Dag1 -/-* percentage contribution (fig. 8).

Basement membrane components are normally expressed in chimaeric kidneys. Basement membrane molecules and their receptors were probed semi-quantitatively by fluorescence immunohistochemistry to detect any differences in expression levels or altered localisation. In wild type tissue the patterns of protein localisation and

expression levels, reflected by the intensity of the fluorescence signal, were as follows. Collagen IV was expressed in all renal BMs at equal intensities. Laminin-1 was also detected in all renal BMs but with differential expression in the medulla, due to staining of only loop of Henlé BMs. Perlecan was detected in all areas of the kidney with a slight increase in GBMs. Nidogen expression was differential between the cortex and medulla, with higher intensities in the medullary BMs. Within the cortex nidogen expression in the GBMs was much greater than the TBMs. Agrin's expression pattern had like nidogen, a greater intensity in medullary BMs and cortical GBMs. The β1-integrin subunit was expressed equally in all areas. β-Dystroglycan expression shows differential expression within the kidney being found principally in the cortex with higher expression in GBMs than TBMs.

A 'mosaic analysis' of chimaeric glomeruli had been the principle aim of the immunohistochemistry experiments. In particular the aim had been to localise cells within a glomerulus, which did not express β-dystroglycan and to examine the neighbouring serial sections to probe for ECM molecules expressed by the same dystroglycan deficient cell. It became clear early on that such an analysis would be very difficult due to the intrinsic relationship of the cells forming the glomerulus, and the relatively low resolution of the light microscope, which was unable to localise the labeling to a specific cell type. Due to these constraints I sought glomeruli with complete lack of β-dystroglycan expression in all their cells and to probe the ECM composition of these glomeruli using neighbouring serial sections.

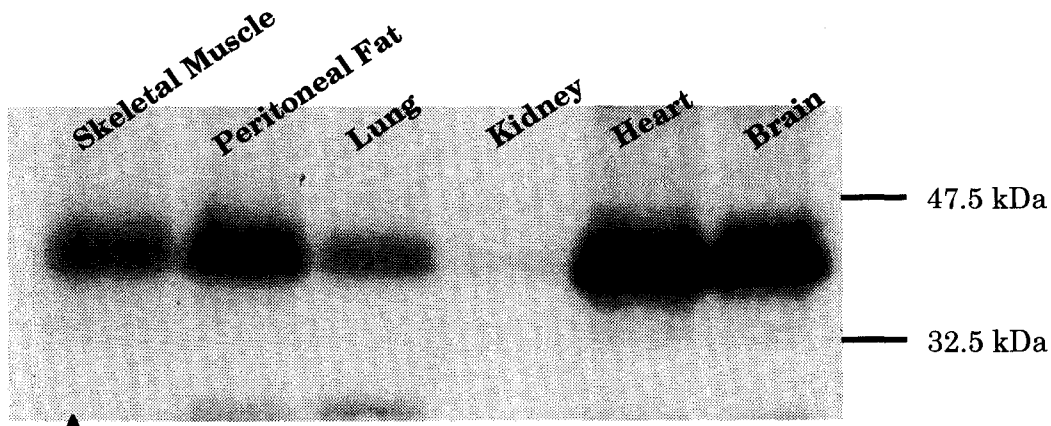
The overall results of the antigen probing of Ch 19 and Ch 21 left kidney sections did not reveal significant changes, relative to C57Bl/6J wild type tissue, in the localisation and expression levels of any proteins other than β-dystroglycan (fig. 9 and fig. 10). Although the western blot analysis of Ch 21 kidneys did not detect dystroglycan, immunohistochemically it was detected in Ch 21 kidneys. The Ch 21 left

kidney showed reduced levels of β-dystroglycan expression in glomeruli and practically no expression in cortical TBM (fig. 10 m, e). Ch 19's left kidney showed an overall decrease in β-dystroglycan, similar to Ch 21 (fig. 9 m, e). The tissue architecture of the Ch 19 was not well preserved which meant only a few glomeruli were preserved per section. A glomerulus completely lacking β-dystroglycan expression was not found in either chimaera. A glomerular analysis was however still performed on the β-dystroglycan deficient glomeruli Ch19 (fig. 9) and Ch 21 (fig. 10) left kidneys. Immunohistological labeling for laminin-1 (fig. 9 and 10, b), collagen IV (fig. 9 and 10, j), nidogen-1 (fig. 9 and 10, l), agrin (fig. 9 and 10,h) and the β1-integrin subunit (fig. 9 and 10, p) in these glomeruli did not reveal any differences in expression levels relative to the control. There was a very slight variation in perlecan levels with higher intensities in Ch 21 (fig. 10, d) and lower intensities in Ch 19 (fig. 9, d) relative to wildtype. Thus, a deficiency in dystroglycan during development does not appear to alter renal basement assembly or components.

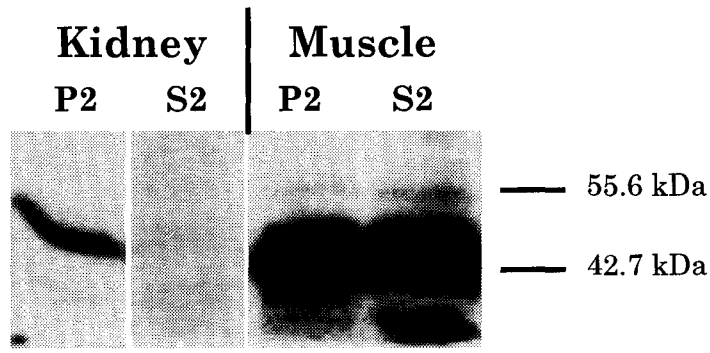
Morphologic anomaly due to secondary hydronephrosis. The analysis to date suggests the deficiency in dystroglycan is acting in a cell non-autonomous fashion rather than in a cell autonomous fashion. The validity of this idea was supported after consultation with renal pathologist, Dr. Louis R. Begin, Department of Pathology, University of Montreal. The pathologist performed a comparative histological analysis of kidney sections from Ch 19 and a zero percent *Dag1* -/- contributing littermate, Ch 20. The pathologist observed significant/severe parenchymal atrophy (namely decreased number and caliber of tubules in the medulla), with no evidence of infection or ancillary evidence of chronic pyelonephrosis. It was the pathologist's opinion that this atrophy was a consequence of secondary hydronephrosis (i.e., marked pyelocalyceal dilation), attributed putatively to obstruction at the level of the ureter or urethra. This was further supported by pediatric pathologist Dr. Chantale Bernard (Montreal Children's Hospital,

McGill University).

Hydronephrosis is a very striking pathology where the blocked urine will accumulate in the pelvis causing distention of the kidney walls (fig. 11a). This distention enlarges and discolours the kidney tissue, very evident features during any dissection. Prompted by this information I sought to confirm these observations. Dissection of one chimaera (Ch 70) revealed urine filled distended kidneys (fig 11c). Cross-section of Ch 70 kidney also showed atrophied parenchyma and enlarged pelvic space as for Ch 19 (fig. 4) and Ch 21 (fig. 5). The dissected kidneys from chimaera 19, 21 and 26 appeared normal until cross-sectioning when the enlarged pelvic cavity was revealed. Thus the kidney abnormalities appear not to result from differences in the kidney cortical and medullary parenchyma but possibly due to anomalies of the pelvic or ureter parenchyma.


Skeletal Muscle
Peritoneal Fat
Lung
Kidney
Heart
Brain
47.5 kDa
32.5 kDa

A


Kidney
Muscle
P2
S2
P2
S2
55.6 kDa
42.7 kDa

B

Figure 3. **Beta-dystroglycan expressed in murine renal tissue is not solubilised at 4°C by 1% Triton X-100.** **A**. Triton X-100 soluble tissue extracts (100 μg protein) from different C57BL/6J male organs were analysed for the presence of β-DG by immunoblotting using an anti-β-DG polyclonal antibody. β-DG was found in all extracts except those originating from the kidney. **B.** Upon solubilisation with 1% Triton X-100, renal β-DG remains in the detergent insoluble tissue fraction (P2) and none is extracted into the soluble fraction (S2). For comparison muscle tissue contains β-DG in both fractions. Protein loading differs between lanes: kidney P2, 62 μg; kidney S2 140 μg; muscle P2, 38 μg and muscle S2, 96 μg.

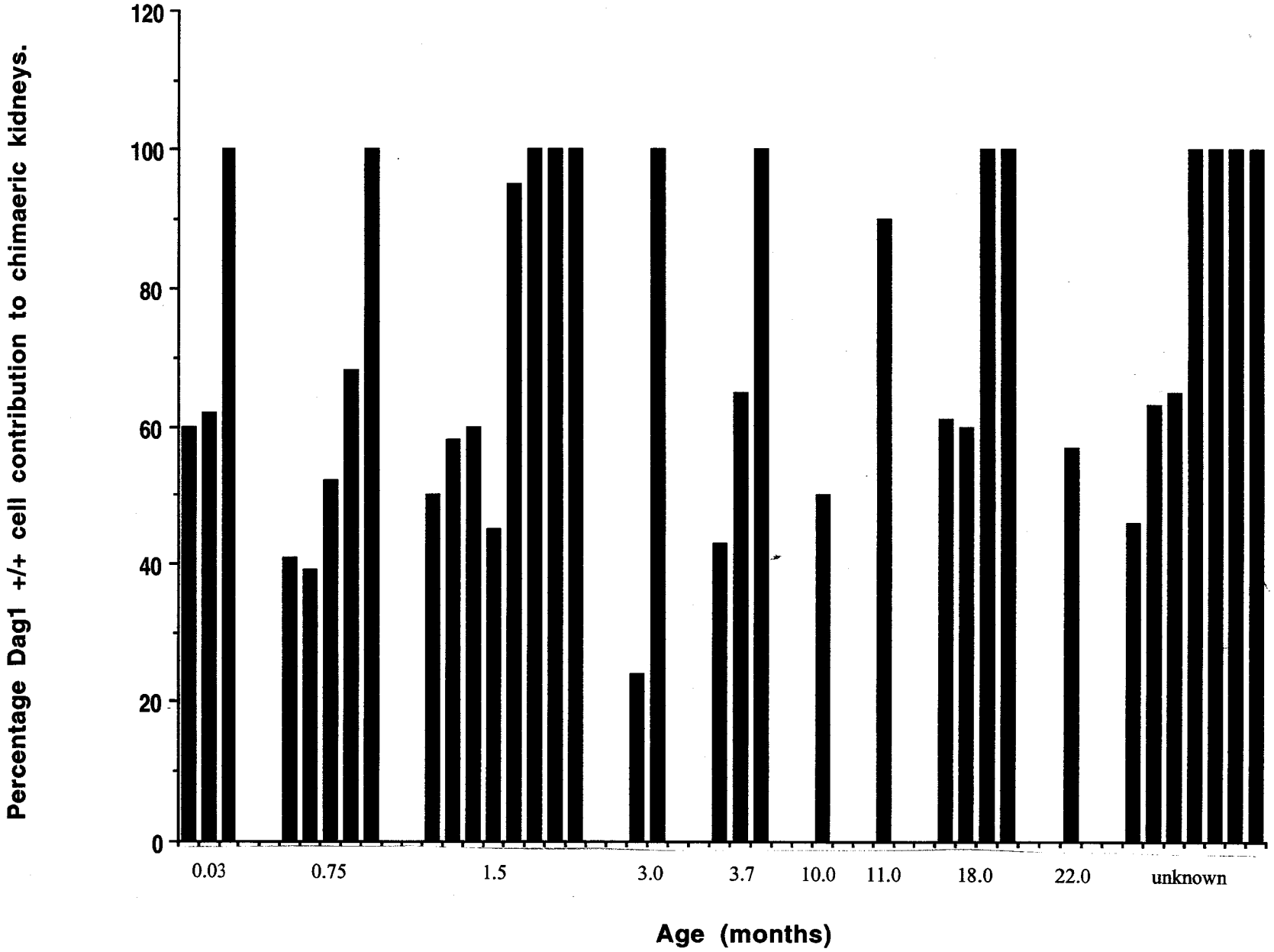

Percentage Dag1 +/+ cell contribution to chimaeric kidneys.
120
100
80
60
40
20
0
0.03
0.75
1.5
3.0
3.7
10.0
11.0
18.0
22.0
unknown
Age (months)

Figure 4. **Percentage R1 cell contribution to chimaeric kidneys.** A total of 64 left and right chimaeric kidneys had their percentage cell contribution quantified by GPI1 assay (methods). Sixty-three percent of the kidneys showed mosaicism ranging from 20-95 percent wild type contribution.

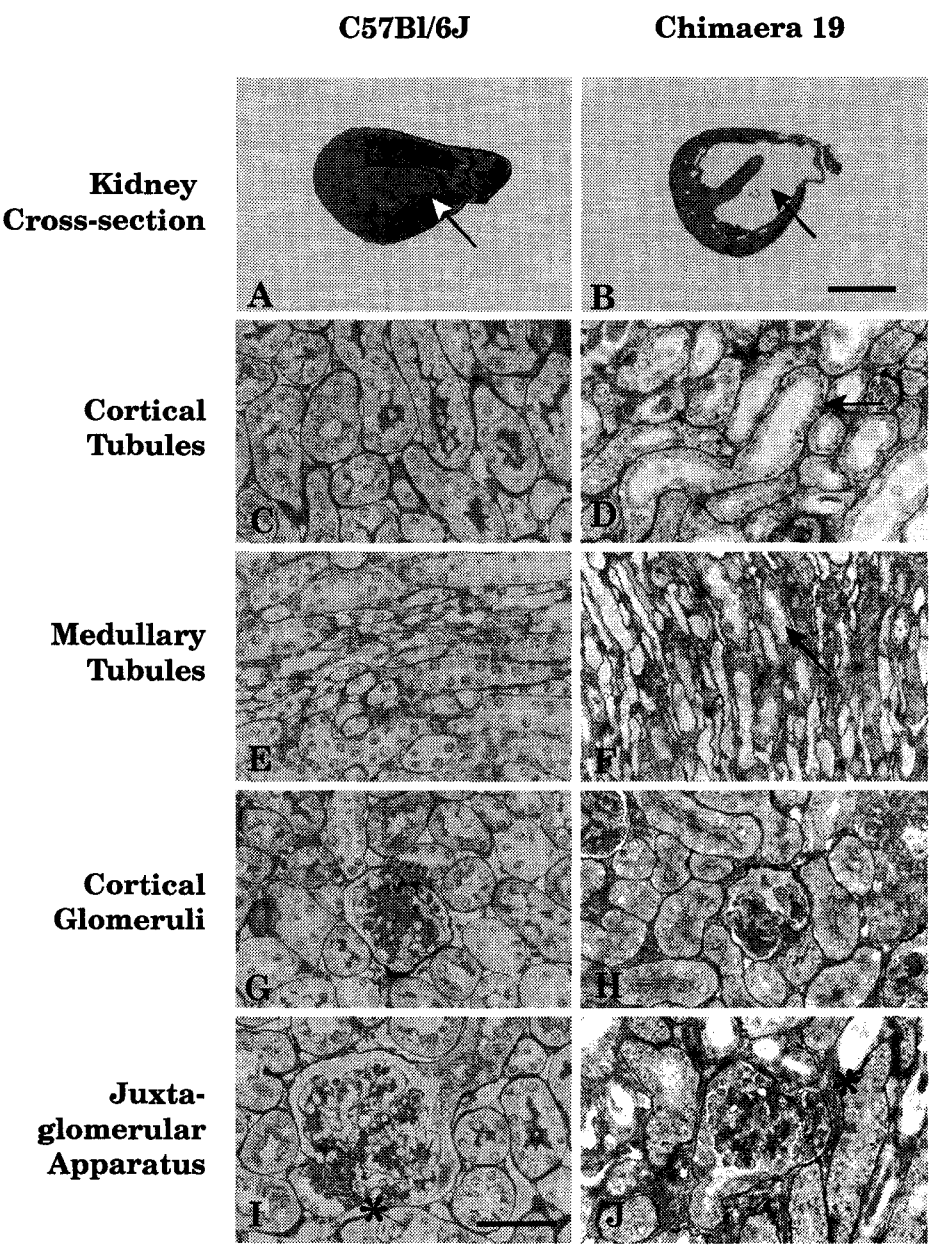

C57Bl/6J
Chimaera 19
Kidney Cross-section
Cortical Tubules
Medullary Tubules
Cortical Glomeruli
Juxta-glomerular Apparatus
A
B
C
D
E
F
G
H
I
J

Figure 5. **Histological analysis of Ch 19 left kidney.** Periodic acid methylamine silver staining revealed that the overall organisation of the organ in Ch 19 was normal relative to control. Segregation of cortical and medullary layers occurred, as well as the formation of intricate structures such as glomeruli (**G, H**) and juxta-glomerular junctions (asterisks) (**I, J**). Anomalies however, were seen in the morphology of the Ch 19 kidney relative to wild type C57Bl/6J. The kidney cross-section (**B**) shows a greatly enlarged pelvic cavity coinciding with an atrophied medulla compared to the control (**A**) (arrow points to pelvic space in **A** and **B**). At higher magnification (x126) dilation of cortical and medullar tubules is seen (**D, F**) relative to control (**E, G**) (arrow points to dilated tubule in **D** and **F**). Scale bar in A and B is 2 mm; Scale bar in C-K is 50μm.

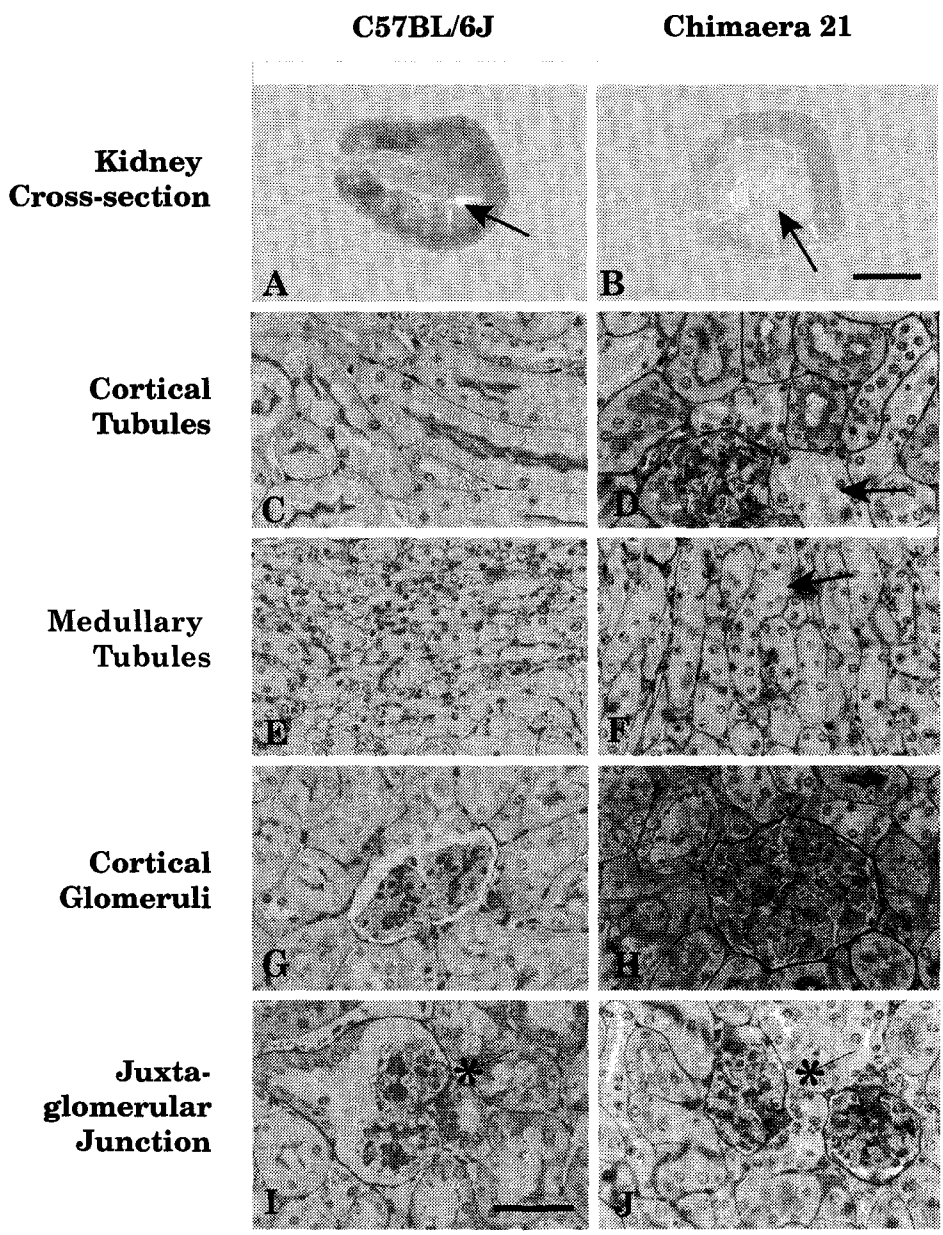
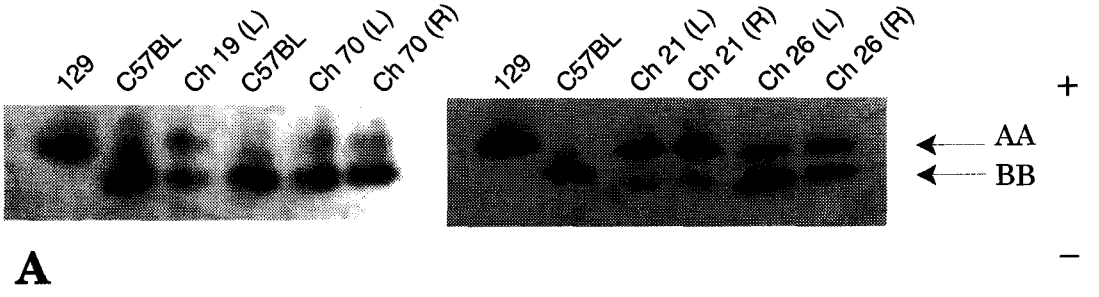
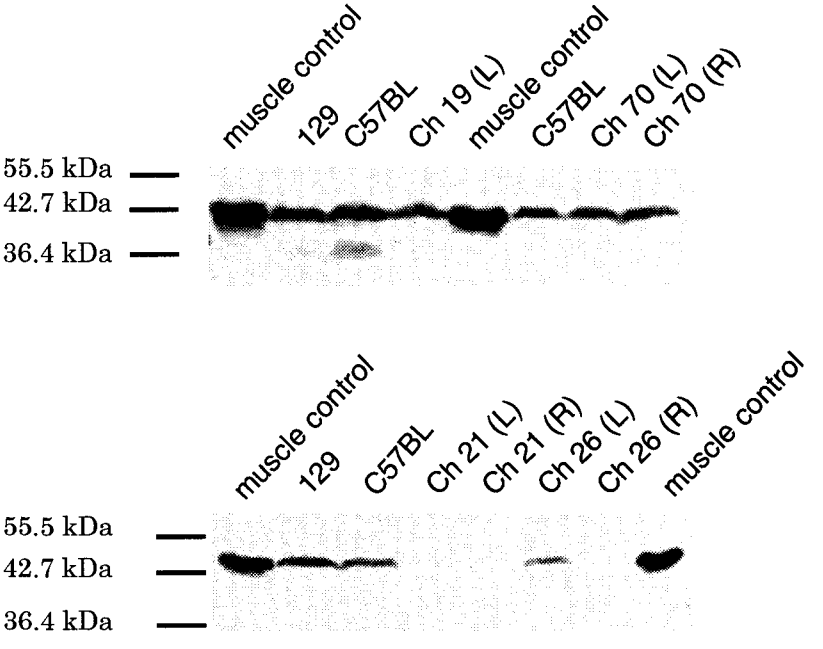

C57BL/6J
Chimaera 21
Kidney Cross-section
Cortical Tubules
Medullary Tubules
Cortical Glomeruli
Juxta-glomerular Junction
A
B
C
D
E
F
G
H
I
J

Figure 6. **Histological analysis of Ch 21 left kidney.** Periodic acid methylamine silver staining revealed that the overall organisation of the organ in Ch 21 was normal relative to control. Segregation of cortical and medullary layers occurred, as well as the formation of intricate structures such as glomeruli (**G, H**) and juxta-glomerular junctions (asterisks) (**I, J**). Anomalies however, were seen in the morphology of the Ch 21 kidney relative to wild type C57Bl/6J. The kidney cross-section (**B**) shows a greatly enlarged pelvic cavity coinciding with an atrophied medulla compared to the control (**A**) (arrow points to pelvic space in **A** and **B**). At higher magnification (x126) dilation of cortical and medullar tubules is seen (**D, F**) relative to control (**E, G**) (arrow points to dilated tubule in **D** and **F**). Scale bar in A and B is 2 mm; Scale bar in C-K is 50μm.


129
C57BL
Ch 19 (L)
C57BL
Ch 70 (L)
Ch 70 (R)
129
C57BL
Ch 21 (L)
Ch 21 (R)
Ch 26 (L)
Ch 26 (R)
+
AA
BB
−

A


muscle control
129
C57BL
Ch 19 (L)
muscle control
C57BL
Ch 70 (L)
Ch 70 (R)
55.5 kDa
42.7 kDa
36.4 kDa
muscle control
129
C57BL
Ch 21 (L)
Ch 21 (R)
Ch 26 (L)
Ch 26 (R)
muscle control
55.5 kDa
42.7 kDa
36.4 kDa

B

Figure 7. **Determination of *Dag1-/-* cell contribution of chimaeras 19,21, 26 and 70 kidneys. A.** The cellulose acetate plate of the GPI isozyme assay qualitatively shows the cathodal electrophoretic migration of the dimeric glucosephosphate isomeraseenzyme (+/- indicate electrophoresis potentials). There are two strain specific isozymes: GPI1AA (129 derived) and GPI1BB (C57BL derived) dimers. Quantification by densiometry indicated that *Dag1-/-* cells contributed: 35% to Ch 19 left kidney (Ch 19 L); 10% to both Ch 70 kidneys (Ch 70 L and R); 80% to both Ch 21 kidneys (Ch L and R) and 35% to Ch 26 left kidney (Ch 26L) and 45%to Ch 26 right kidney (Ch 26 R). **B.** Qualitative expression levels of β-DG from chimaeric renal tissue was revealed by immunoblotting using anti-β-DG polyclonal antibody. An increase in *Dag1 -/-* cell contribution was found to produce a decrease in the levels of β-DG expression. Both Ch 21 kidneys and Ch 26 right kidney had no detectable levels of β-DG; both Ch 19 left kidney and Ch 26 left kidney showed decreased levels of expression whereas both Ch 70 kidneys had normal levels of expression. Protein loading = 50μg; C= skeletal muscle control served as a positive control.

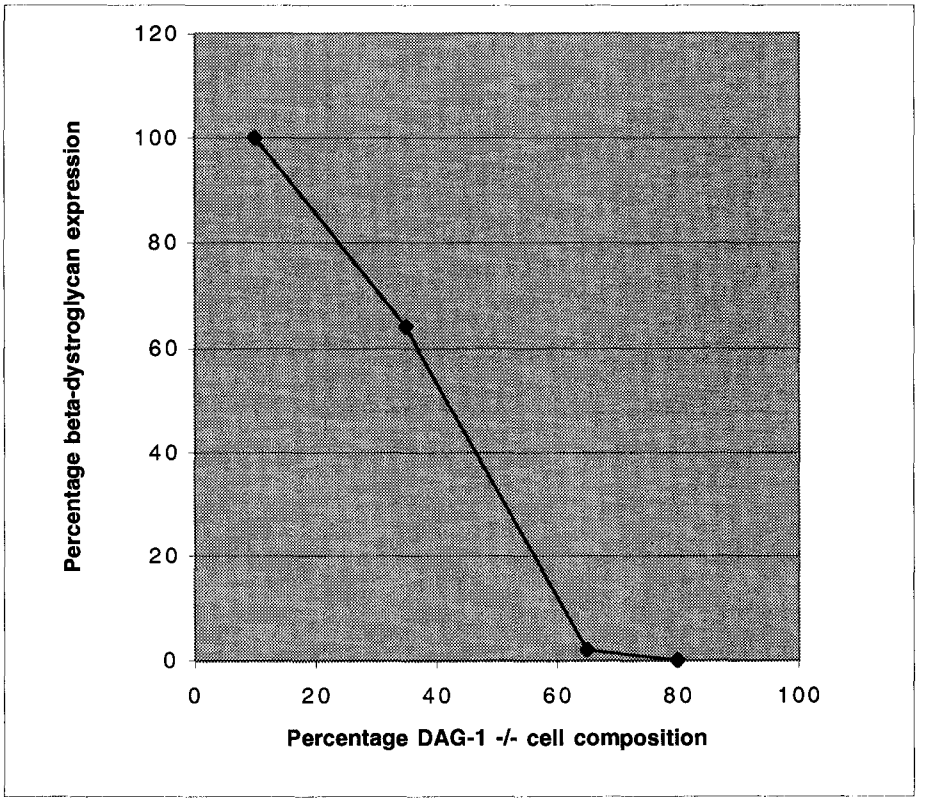
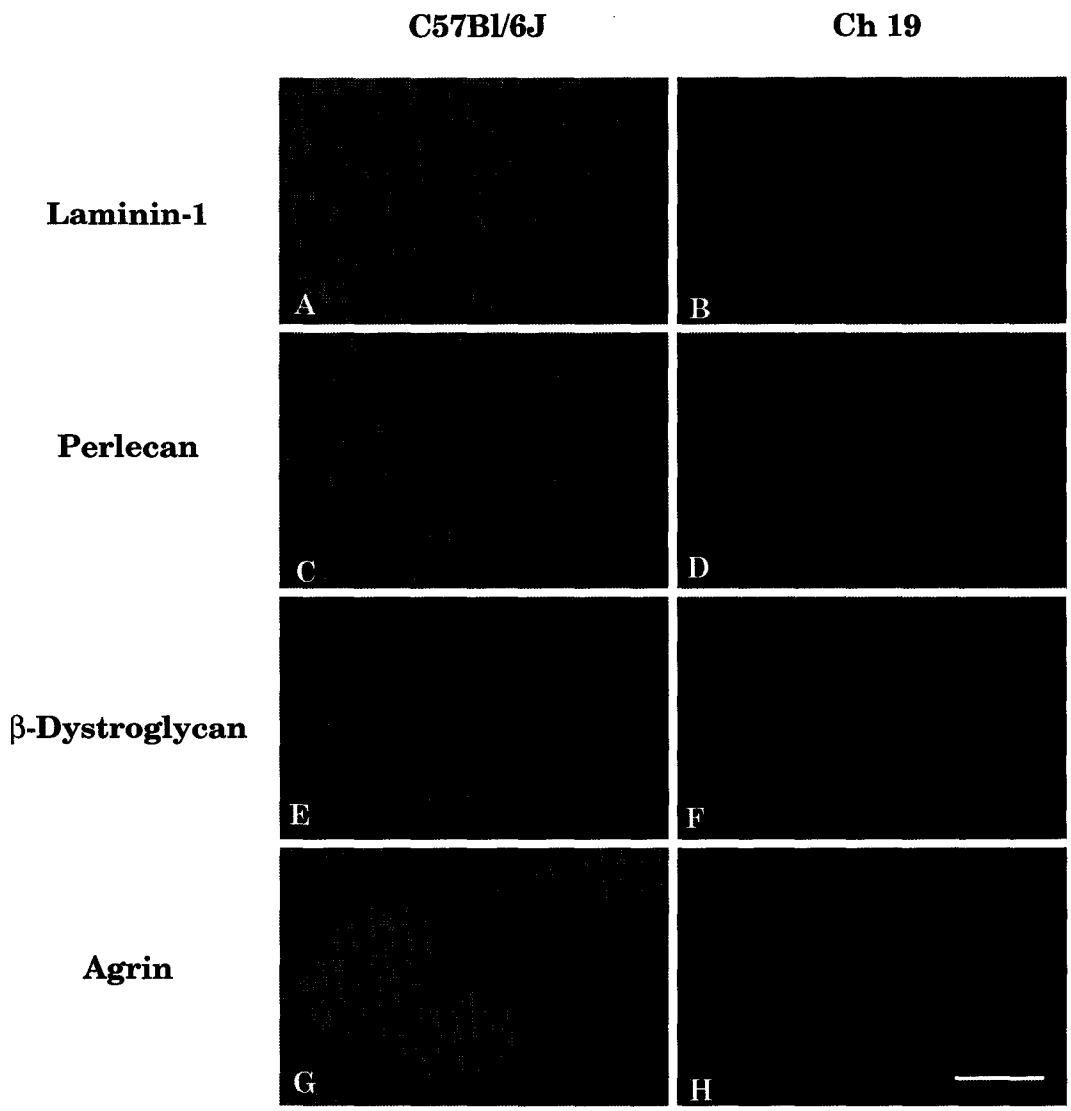

120
100
80
60
40
20
0
Percentage beta-dystroglycan expression
0
20
40
60
80
100
Percentage DAG-1 -/- cell composition

Figure 8. **Expression levels of beta-dystroglycan in chimaeric kidneys decreases with increased *Dag1-/-* cell contribution.** The β-dystroglycan expression levels of chimaeras 19, 21, 26 and 70 kidneys were quantified relative to control expression levels and compared to the percent cell composition of the same kidney.


C57Bl/6J
Ch 19
Laminin-1
Perlecan
β-Dystroglycan
Agrin
A
B
C
D
E
F
G
H

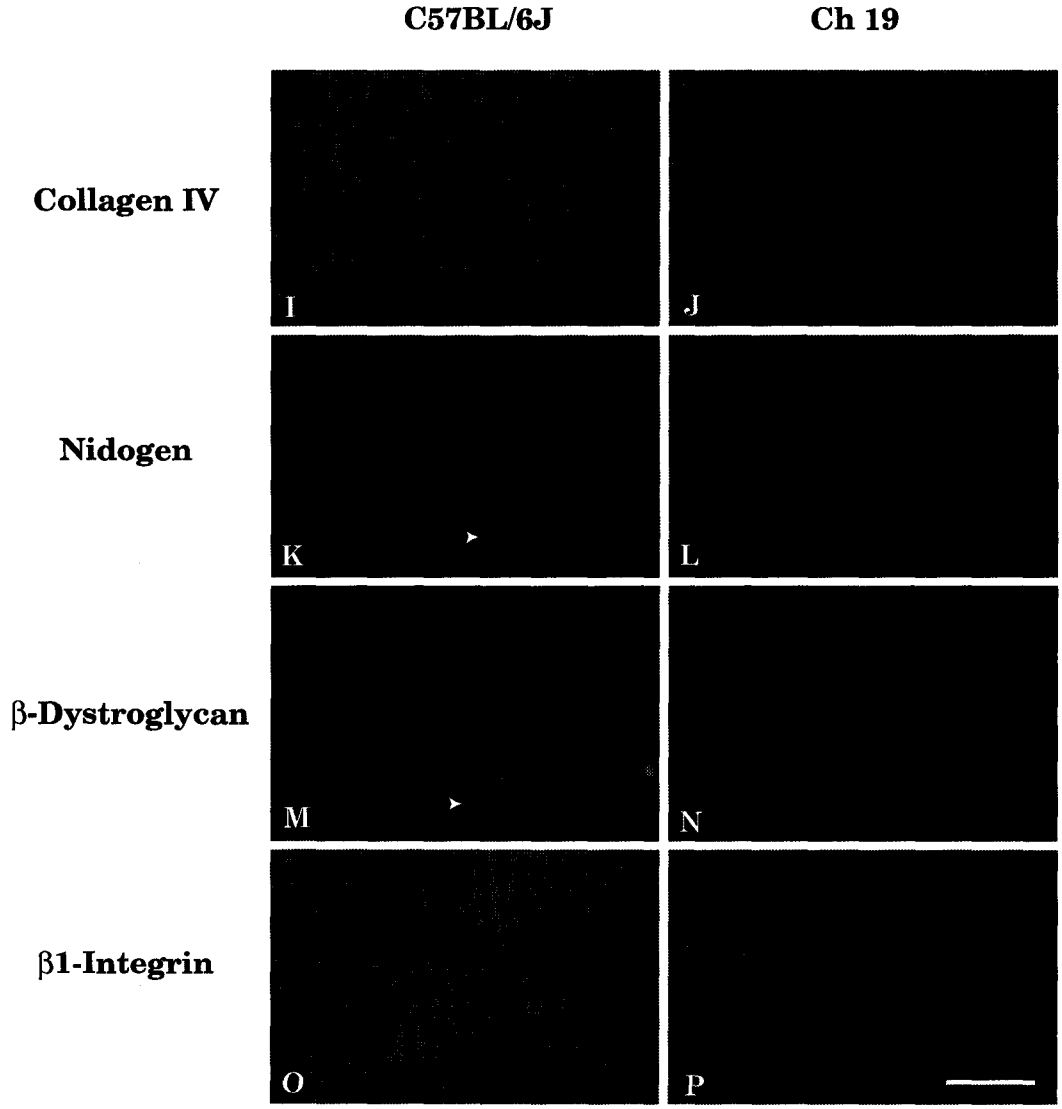
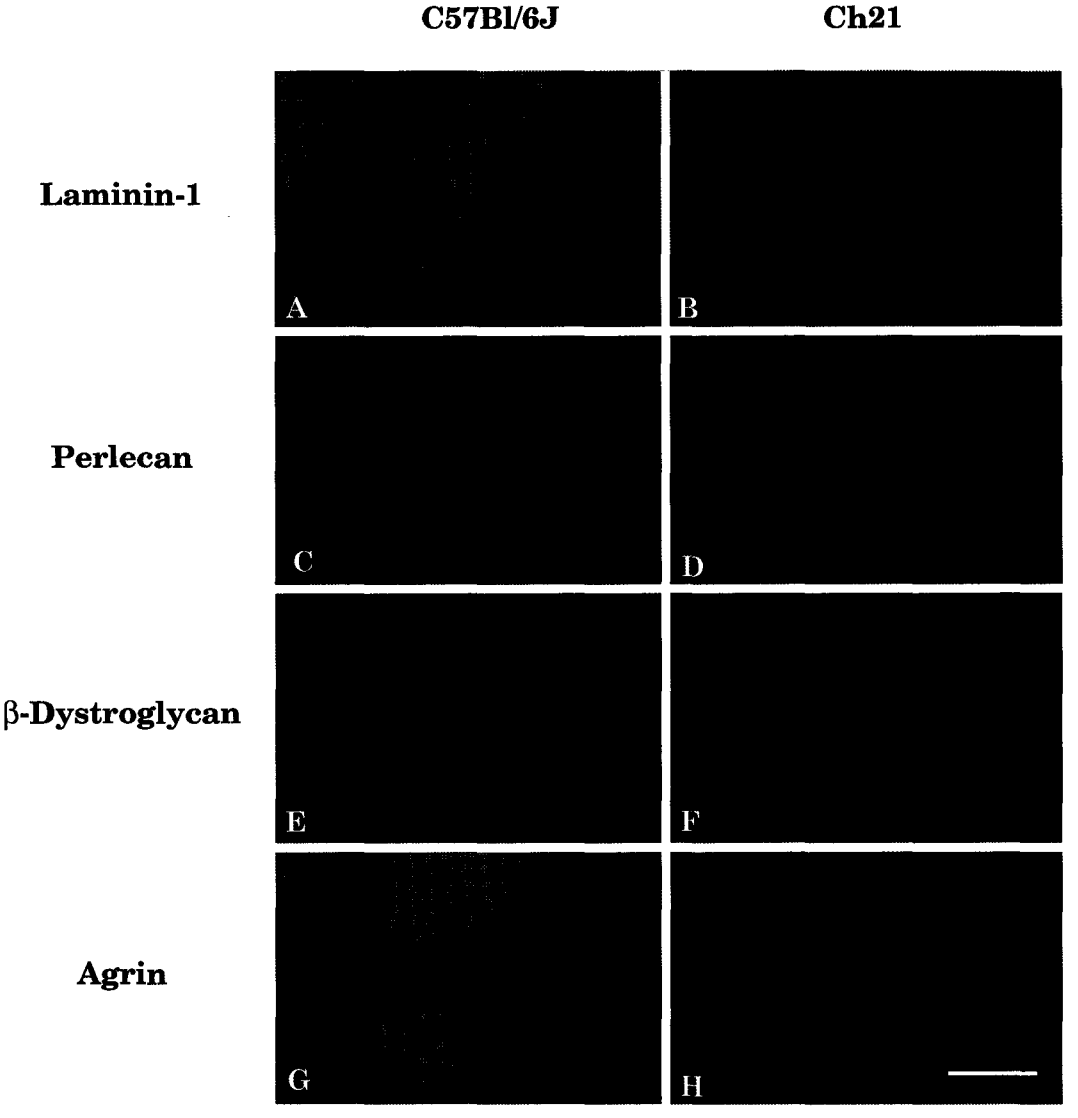

C57BL/6J
Ch 19
Collagen IV
Nidogen
β-Dystroglycan
β1-Integrin
I
J
K
L
M
N
O
P

Figure 9. **Immunohistochemistry of basement membrane components and receptors in Ch 19 left kidney cortex.** (*first page*) Laminin (A,B), perlecan (C,D), β-DG (E,F) and agrin (G,H) staining. Perlecan and β-DG are co-stainings of the same sections. (*second page*) Collagen IV (I, J), nidogen (K, L), β-DG (M, N) and β1-integrin(O, P) staining. Nidogen and β-DG are co-stainings of the same sections. In wild type tissue, laminin-1 (A), collagen (I) and the β1-integrin subunit (O) are equally expressed in all cortical BMs. Perlecan(C) was detected in all cortical BM with a slight decrease in GBMs. Nidogen (K) and Agrin (G) expression was differential within the cortex, with the GBMs having greater expression than the TBMs. β-Dystroglycan expression shows higher expression in GBMs than TBMs. Chimaera 19 tissue had only decreased levels of glomerular and TBM β-DG expression and a slight decrease of perlecan expression levels was found in Ch 19 left kidney relative to C57BL/6J kidney. The scale bar is 50μm; arrow points to blood vessels.


C57Bl/6J
Ch21
Laminin-1
Perlecan
β-Dystroglycan
Agrin
A
B
C
D
E
F
G
H

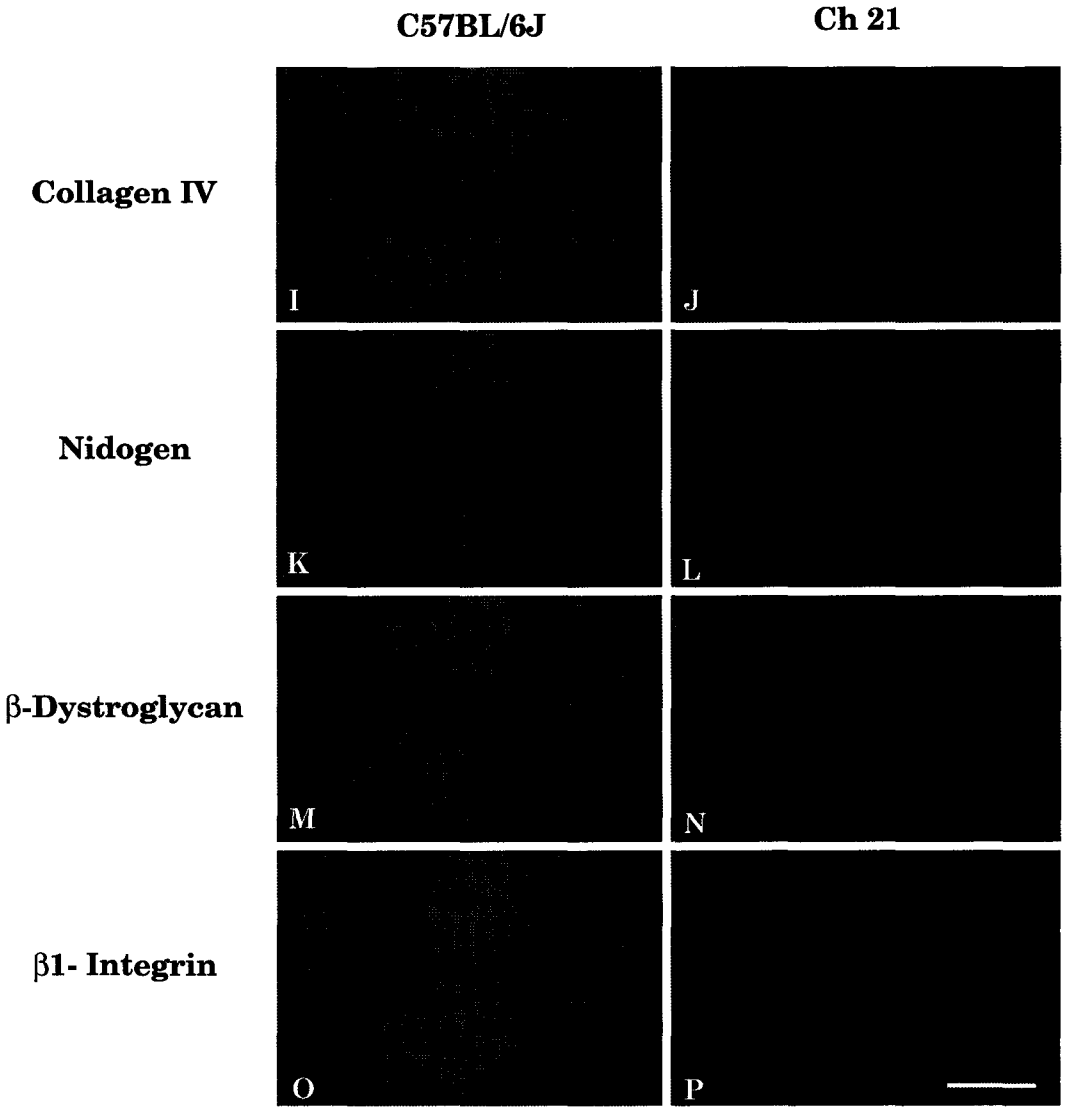
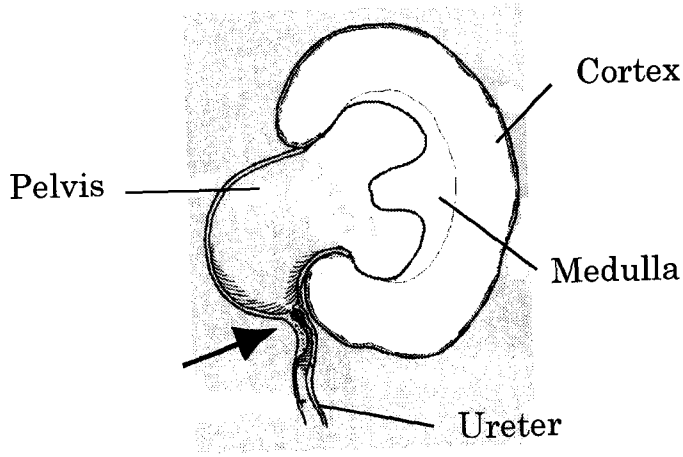

C57BL/6J
Ch 21
Collagen IV
Nidogen
β-Dystroglycan
β1- Integrin
I
J
K
L
M
N
O
P

Figure 10. **Immunofluorescence analysis of basement membrane components and receptors in Ch 21 left kidney cortex.** *(first page)* Laminin(A, B), perlecan(C, D), β-DG(E, F) and agrin(G, H) staining. Perlecan and β-DG are co-stainings of the same sections. *(second page)* Collagen IV(I, J), nidogen(K, L), β-DG(M, N) and β1-integrin(O, P) staining. Nidogen and β-DG are co-stainings of the same sections. Nidogen and β-DG are co-stainings of the same sections. In wild type tissue, laminin-1 (A), collagen (I) and the β1-integrin subunit (O) are equally expressed in all cortical BMs. Perlecan(C) was detected in all cortical BM with a slight increase in GBMs. Nidogen (K) and Agrin (G) expression was differential within the cortex, with the GBMs having greater expression than the TBMs. Only decreased levels of glomerular and TBM β-DG expression and a slight increase in the general perlecan expression was found in Ch 21 left kidney relative to C57BL/6J kidney. The scale bar is 50μm.



A

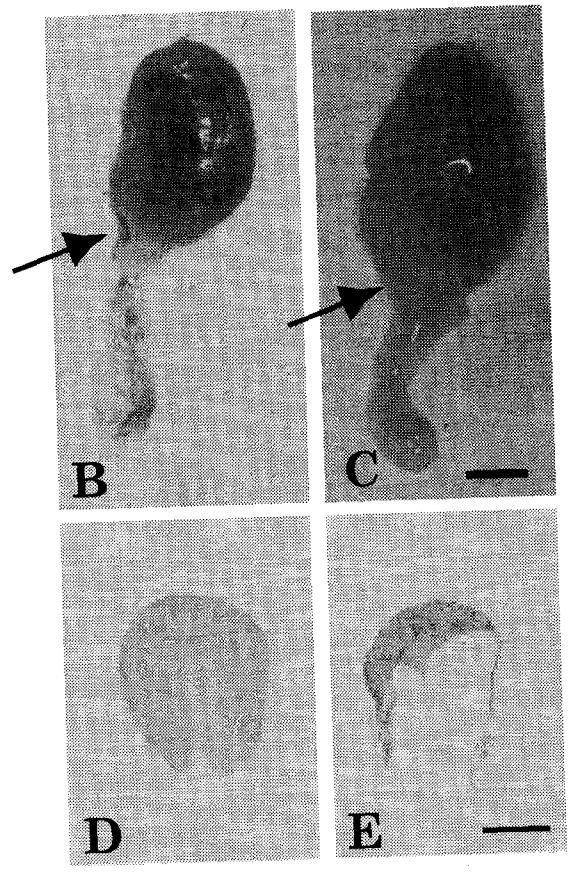


Figure 11. **Hydronephrosis of Chimaera 70 kidney.** **A**. Descriptive diagram of hydronephrosis in a unipapillate rodent kidney. **B.** Dorsal view of a freshly dissected right kidney and upper urinary tract of an age and sex matched 129X1/SvJ control mouse. **C**. Dorsal view of the freshly dissected right kidney and upper urinary tract from Ch 70. **D**. Periodic acid methylamine silver stained transverse section of control kidney. **E.** Periodic acid methylamine silver stained transverse section of Ch 70 right kidney. Ch 70 suffered secondary hydronephrosis due to obstruction of either the ureteropelvic junction or the upper ureter. The distension of Ch 70 kidney has caused discolouration of the kidney becoming a pale yellow relative to the control dark red kidney. The arrows in A, B and C point to the area of obstruction in Ch 70 and to the equivalent area in the control tissue and the descriptive diagram. Note that Ch 70 has an equal body weight as the control. Scale bar in B and C is 3 mm. The scale bar in D and E is 2 mm.

DISCUSSION

Dystroglycan is an ECM receptor expressed in many cell types (14, 3, 64, 16). Investigating dystroglycan in the different tissues is necessary to determine whether it has a general or cell specific function. Study of dystroglycan in epithelial cells during kidney organogenesis had been carried out by Ekblom and coworkers due to the ease of kidney organ culture (113). The *in vivo* localisation of dystroglycan coupled to the results of antibody blocking studies, suggest a role for dystroglycan during nephrogenesis. Also, the simultaneous expression of dystroglycan and the laminin α1 chain during mesenchymal condensation suggests participation of dystroglycan in basement membrane formation, and kidney development. In the mature kidney, dystroglycan is localised to the basolateral side of podocytes and cortical tubular epithelia allowing it to contribute to GBM and TBM formation and maintenance. Localisation studies however, cannot fully reveal dystroglycan's function and *ex vivo* studies such as the antibody blocking experiments do not always reflect *in vivo* processes.

I hypothesised that a lack of dystroglycan *in vivo* in kidney precursor cells, would lead to a misassembly of renal basement membranes, causing inhibition of developmental processes such as branching morphogenesis and mesenchymal to epithelium transformation. This inhibition of processes fundamental to growth and differentiation was predicted to produce kidney malformations. In fact, *Dag1* -/- chimaeras produced an abnormal kidney morphology in four of the 38 chimaeras generated. These malformed kidneys were found bilaterally in Ch 19 and 70 and unilaterally in Ch 21 and Ch 26. Histologically, I found that all kidneys showed correct laminar organisation of the cortex and medulla, with the presence of complex structures such as glomeruli, juxta-glomerular junctions and BM. Immunohistochemially, I found that the molecular composition of the resulting BM did not show significant variation of

components or integrin receptors.

The maintenance of fine nephritic structures in these chimeric kidneys may reflect a lack of function for the dystroglycan complex in epithelia formation during kidney organogenesis. However, it could equally reflect redundancies in the molecular program of kidney formation and maintenance. Histology showed that apparently normal basement membranes were present in the malformed kidneys, whether their assembly was correct was examined by immunohistochemistry. Molecularly, the general composition of the chimaeric kidney BMs was normal. The degree of significance of the slight variations in perlecan intensities (decreased in Ch 19 and increased in Ch 21) could not be quantified due to the semi-quantitative nature of immunofluorescence. Also, there may be compensation of certain BM components by the expression of normally absent isoforms. These experiments could not detect such fine changes due to the nature of the antibodies. Antibodies against all family members (collagen IV) and pan anti-bodies (perlecan, agrin) cannot detect changes is the proportion of different isoforms or family members expressed. Antibodies directed toward single family members (integrin, nidogen and laminin-1) could not detect the presence of other members. The developmental role of dystroglycan may be compensated by other ECM receptors such as the integrin or syndecan families. My results suggest that the β1-integrin subunit was not upregulated to compensate. However, compensation by the integrins may have been by the expression of different α-integrin subunits. Other ECM cell surface receptors may compensate such as synedecan-1 whose expression in the embryonic kidney is suggested to reflect involvement in maintenance of epithelial cell polarity (128, 129). Some forms of syndecan do bind to the E3 protease fragment of laminin-1, known to bind cell surface receptors (127).

If functional compensation had occurred in the *Dag1-/-* chimaeric kidneys why

was this not predicted by previous *ex vivo* experiments (113). The results of different *ex vivo* experiments using antibodies as inhibitors of protein/protein interaction suggested a chain of interactions necessary for kidney development. Antibodies interrupting laminin-1/nidogen-1 interactions, thereby removing the link between the laminin and collagen networks, inhibited branching morphogenesis (111). Both antibodies to the E3 and E8 laminin-1 domains indicated necessary interactions of laminin-1 to cell receptors (97). Antibodies specifically preventing laminin interactions with either α6-integrin subunit (99) or α-dystroglycan(113), inhibited kidney development indicating that both are laminin receptors. Yet, the α6-integrin subunit deficient mice do not demonstrate kidney malformations suggesting that other receptors can compensate for its function (145). Alternatively, the difference between *ex vivo* and *in vivo* experiments may arise due to the different dynamics of the experiments. During *ex vivo* experiments immediate compensation may not occur, and if it dord, it may not initiate the same compensatory pathways as *in vivo* experiments. In gene knockouts or genetic chimaeras, the lack of a gene prevents the expression of a protein at all stages of development. Detection of the lack of this protein occurs rather than disruptions of protein. This may trigger a compensatory sequence of gene expressions at an early stage to allow normal development. The occurrence of development in kidneys deficient in dystroglycan probably reflects this discrepancy where different *in vivo* compensatory gene expression pathways, whatever they may be, are allowed to develop.

Understanding the results obtained by histology and immunohistochemistry requires understanding of the chimaeric model. The ability to inactivate specific genes has demonstrated the extreme complexity of genetic determination in mammals. The chimaeric mice used in this study are a method of studying nondirected restriction of a genome alteration (20). The genome alteration is restricted to a limited number of cells that randomly populate the organism because ES cells can potentially contribute to any

cell in the embryo proper, the amnion and yolk sac mesoderm (90). When injecting mutant ES cells into a wildtype blastocyst the final location of differentiated mutant cells will be dependent on several factors including the initial location and number of mutant cells in the embryo and the capacity of mutant cells to differentiate into specific cell lineages. The location of precursor cells in an embryo, dictates to which mature tissue they will contribute. Each chimaera is therefore unique due to the random location of mutant cells after blastocyst injection (32).

The rescue of embryonic lethality is not the only advantage of chimaeras, they also allow deductions about cell lineage which requires successful cell proliferation, migration, and response to growth factors (55). When using gene targeted ES cells, chimaeras can provide information about the necessity of a protein in different cell lineages. The range of *Dag1* -/- cell contributions to the different chimaeric kidneys indicates that complete selective death of genetically *Dag1* -/- cells had not taken place either during organogenesis or in the mature kidney. This indicates that *Dag1*-/- cells could respond to the cues inducing kidney development. However, during kidney development wild type cells may have segregated to cell lineages which critically need the function of dystroglycan, whereas *Dag1* null cells were directed to cell lineages in which dystroglycan was less critical. This could explain why every glomerulus of the phenotypic chimaeric kidneys showed immunohistochemical staining of dystroglycan while TBM showed complete lack of dystroglycan. Since agrin is the major heparan sulfate proteoglycan of the GBM (79), the role of dystroglycan as an agrin receptor is likely to be more critical in kidneys.

Cell autonomy can also be deduced by the use of chimaeras (55). An organ is not a closed system. The multiple effects of innervation, endocrine system and vascularisation to name but a few, on the development and functioning of any organ is

complex. To solely correlate the morphology of a chimaeric organ to its cellular composition is to ignore the complexity of the organism and its development. Although extreme, absolute correlation of a phenotype to the lack of genetic expression of a protein would require spatio-temporal genotyping of the whole organism. Therefore, to be able to determine the source cause of the kidney phenotype it would be necessary to quantify the percentage contribution of *Dag1* -/- cells in every organ of the organism for each individual chimaera. However, since only a third of the morphologically different *Dag1*-/- chimaeric kidneys had high levels of *Dag1* -/- cell contributions, it suggests that its cause is cell non-autonomous.

The cell non-autonomous cause of the kidney phenotype was suggested by the pathologists Dr. Chantale Bernard and Dr. Louis R. Begin. It was their opinion that the enlarged pelvic cavity was indicative of a blocked ureter causing an accumulation of urine in the pelvic cavity. This accumulation could have compressed the medullary zone or induced either necrosis or apoptosis of medullary tubules. The accumulated fluid may also have caused the enlarged lumens of the cortical tubules (207). Since urine production begins during embryogenesis, a blocked urethra may have important developmental impacts (175).

Each ureter is the tubular extension of the renal collecting system, which travels caudally and medially to connect the kidney to the urinary bladder. The ureters and the collecting system extending to the renal papillae are lined by a transitional cell epithelium above a layer of connective tissue called the lamina propria. Together they form the mucosa, which lies in longitudinal folds when not distended by urine. The next layer is of smooth muscle, which in the ureter consists of longitudinally coursing muscle bundles and an outer layer of circular and oblique muscle. The urinary effluent does not passively drain but is actively propelled from the renal pelvis to the bladder by the

peristaltic action of the ureteral muscle. The ureteral caliber is not constant there are three distinct narrowings, the ureteropelvic junction, the crossing of the iliac vessels and the ureterovesicle junction (175, 176). The ureter receives both sympathetic and parasympathetic innervation. However, the exact role of the ureteral autonomic input is unclear. Normal ureteral peristalsis does not require outside autonomic input but rather originates and is propagated from intrinsic smooth muscle pacemaker sites located in the minor calyces of the renal collecting system in humans. The autonomic nervous system may exert some modulating effect on this process (175, 176).

However, hydronephrosis is simply a descriptive term referring to the presence of dilation of the pelvis and not to the etiology of that dilation (207). The etiologies leading to the hydronephrosis are numerous and each dictates the degree of manifestation of the hydronephrosis (206). Conditions which obstructs the urinary tract, and therefore urine flow, can ultimately lead to hydronephrosis. Conditions which disrupt the concentration of urine can also cause hydronephrosis.

The symptoms evoked by urinary tract obstruction vary considerably according to whether it is a unilateral or bilateral obstruction as well as the degree and timing of obstruction (174, 175, 207, 208). Clinically chronic obstructions are silent for long periods until symptoms of advancing renal failure occur. The advanced atrophy of dilated kidneys is related to the combination of the interrelated factors of increased tension in the wall of the kidney due to increased pressure from the accumulation of urine and the consequent prominent vasoconstriction. In concert these two lead to the marked reduction in renal blood flow that is responsible for parenchymal ischemic atrophy (174, 175, 207, 208). Some of the possible consequences of acute obstruction is the outpouring of macrophages and lymphocytes into the renal cortex as well as proliferation of the fibroblast like interstitial cells. During chronic obstruction tubular function declines

causing an inability to concentrate urine on release of obstruction. Defects in sodium, potassium and glucose reabsorption also arise. Due to reduced renal blood flow there is an overall shift from aerobic to anaerobic metabolism. Tubular epithelium in the medulla appears flattened and gradually atrophies and disappears leaving empty lacunae in the dense network of degenerating collagen fibrils. However, in cases of human UPJ obstruction over fifty percent of kidneys do not show great histological change (174, 175, 207, 208).

The causes of obstruction are either intrinsic or extrinsic to the kidney itself (207, 208). The cause of ureteropelvic junction obstruction in patients suffering intermittent hydronephrosis is not clear. In contrast to the regular smooth propagation of contractions over the pelvis to the ureter in normal kidneys, abnormalities in the triggering and propagation of impulses have been observed in hydronephrosis with ineffective peristalsis apparently associated with an infiltration of collagen and a considerable decrease in intracellular myofilaments. Normally contractions begin with high frequency in the fornixes and continue over the pelvic wall. In hydronephrosis the waves are delayed or extinguished at the UPJ. Obstruction may be due to mucosal valves at UPJ, vessels crossing the ureter or clockwise rotation of the pelvis with high ureteral origin are primary contributing factors or secondary to obstruction (207, 208). An adynamic segment high in the ureter can delay or abolish muscular contractility (44). Abnormalities of smooth muscle or collagen in the UPJ have been revealed by light and electron microscope (39). The abnormalities described include absent or deficient muscle at UPJ (40) or abnormal muscle orientation (41) and replacement of the muscle by collagen (42). Marked reduction of muscle innervation of UPJ (43) suggesting possible defective innervation. This lead to the idea that disruption of the coordinated motion of smooth muscle cells may

result in both mechanical and functional obstruction of the urinary flow (45,46,47,48).

DGC members are expressed in smooth muscle cells, including dystroglycan, utrophin, dystrophin, β, ε, δ-sarcoglycans and sarcospan (173). Lack of dystroglycans presence in ureter smooth muscle may disrupt the complex causing dysfunction of the smooth cell contributing to its blockage. The ureter of the chimaeras, had they been stored, could have been studied to locate any anomalies of smooth muscle, innervation or collagen expression. Confirmation of dystroglycan's expression in control tissue would also be required.

The hydronephrosis may also have been caused by an inability of the kidneys to concentrate urine. The renin-angiotensin system contributes to many homeostatic mechanisms including the maintenance of blood volume through the control of fluid retention by kidneys. Inhibition of the renin-angiotensinogen system in mice and rats has revealed striking renal histopathologies similar to those found in the *Dag1-/-* chimeric kidneys. Mice completely lacking angiotensinogen show papillary atrophy and generalised cortical thinning with focal areas of severe cortical atrophy (210, 211). Very similar renal pathologies are found in mice null for the angiotensin converting enzyme including lesions of the renal cortex and papillary atrophy (212). Inhibition of the angiotensin converting enzyme in neonatal rats leads to increased water consumption and urine volume. Consequently these animals develop irreversible renal abnormalities including cortical tubulointerstitial inflammation, papillary atrophy and pelvic dilation (213). Due to antidiuretic hormone deficiency Brattleboro rats are unable to concentrate urine and show an increased incidence of hyponephrotic lesions (214). Though an atrophied papilla and a dilated pelvis was the common feature of the chimeric animals the severe cortical lesions were never found. A lack of dystroglycan in tubular epithelial

cells may have contributed to the disruption of urine concentration mechanisms. Determining whether the etiology of the chimaeric hydronephrosis was due to urinary blockage or loss of urine concentrating mechanisms would require urine analysis prior to the dissectioning of the animals.

A kidney epithelial conditional knock-out, using such methods as Cre-lox recombination, would more precisely determine the function of dystroglycan in these cells (204). Placing spatial and temporal restrictions on the genome greatly facilitates functional analysis. Embryonic promoters restricted to the uretic bud could indicate dystroglycan's role in branching morphogenesis whereas promoters restricted to mesenchymal cells could elucidate dystroglycan's role in mesenchyme to epithelial differentiation. A promoter expressed in the adult podocytes could reveal dystroglycan's role in glomerular filtration (53,54,55).

The finding of differential extractability of β-dystroglycan from different tissues, suggest that β-dystroglycan is found within different molecular environments in different organs. Since whole organ homogenates were used for the solubilisation experiments, the results represent extraction from the different cells composing the organ. Differences in the members of the DGC complex in different cell types has previously been shown (7). It was therefore surprising to find that β-dystroglycan was completely insoluble in the kidney since dystroglycan is expressed in two different cell types: epithelial cells and smooth muscle cells of the vascular system, which express different DGC complexes. The lung is also composed of epithelia and vascular smooth muscle which express dystroglycan in both these cell types, yet has a different extractability than the kidney (7).

The biological significance of a protein's insolubility in Triton X-100 at 4°C has

not yet been determined (133). As the cytoskeleton is detergent insoluble most early work on detergent insolubility of cell membrane proteins and lipids was interpreted in terms of cytoskeletal attachment. More recently insolubility is proposed to reflect association of proteins within distinct lipid domains in the plasma membrane such as a 'rafts,' believed to be precursors of a caveolae (132). Lipid rafts are shingolipid and cholesterol rich subdomains of plasma membrane proposed to have functions in both cell signaling and membrane trafficking (142). Detergents can differentially solubilise membrane domains in different physical states (133). Lipid bilayers can exist in several physical states ranging from fluid liquid crystalline states to solid-like gel state (134). An intermediate state called the liquid ordered state is produced upon mixing of lipids and cholesterol and is representative of biological lipid rafts. The liquid ordered physical state is similar to the gel state, yet lateral diffusion is almost as rapid as the fluid state(141). Separate domains with different lipid and protein composition can exist in biological membranes therefore differential solubilities might reflect presence of a protein in a specific lipid domain such as a lipid raft(136). Lipids and proteins in the liquid ordered state are insoluble in excess non-ionic Triton X-100 unlike those in the fluid state(135).

Whether detergent resistant membranes represent the lipid rafts believed to exist in cell membranes prior to detergent treatment requires further analysis (133). The main limitation of these experiments is whether they reflect the distribution of molecules between different membrane domains prior to detergent addition. Triton X-100 solubilisation at 37°C yields little pelletable detergent-insoluble material, which is why all solubilisations are performed at 4°C. Unfortunately cooling membranes decreases the energy state of membranes increasing the total amount of lipid in the liquid ordered phase, causing an overestimation of the amount of material present in rafts at physiological temperatures (137,138). Also, the final distribution may not represent

distributions prior to detergent addition because molecules can exchange rapidly between liquid ordered domains and the fluid state (141).

Further experiments are required to explain β-dystroglycan inextractability in the kidney. Milder detergents such as n-octly β-D-glucopyranoside solubilise lipid rafts (140). Gradient centrifugation would distinguish low density lipid raft domains from other detergent-insoluble complexes (136). The presence of β-dystroglycan in lipid rafts would further support its function in cell signaling. In muscle, β- dystroglycan has been shown to interact with the muscle specific caveolin-3 that is part of a family of proteins involved in the formation of caveolae (139). Such an interaction may also occur within epithelial isoforms.

In conclusion, the results obtained in this thesis do not support the hypothesis that dystroglycan participates in either renal basement membrane formation or kidney development. The complete inextractability of β-dystroglycan from kidneys was unexpected. Further study may provide evidence linking dystroglycan to membrane lipid domains that function in cell signaling. With respect to cell lineage, the exclusion of *Dag1-/-* cells from glomeruli suggests a role for dystroglycan during glomerulogenesis. Localisation studies support this idea since dystroglycan is found on the basolateral surface of podocytes which rests on the GBM (18, 19). Also, GBMs contain a high concentration of agrin, the possibility that dystroglycan functions as an agrin receptor in the kidney podocytes merits further investigation.

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