Integrin alfa3beta1 and tetraspanin CD151 in particular in the skin and the kidney
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Summary, Discussion and Conclusions.
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Remark: the references mentioned in this section are listed in the reference list of chapter 1 (pages 19-29).

Background

Integrins are cell surface receptors that are conserved during evolution and expressed by all nucleated cells. A distinct group within the integrin family is formed by the laminin-binding integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$ and $\alpha 6\beta 4$. They share structural homologies, have laminin isoforms as their ligands, and the mRNAs for both their extracellular and their cytoplasmic domain of the $\alpha$-subunit are subject to alternative splicing. Initially the work presented in this thesis was focused on $\alpha 3\beta 1$ as the main integrin expressed in the glomerulus. When it became evident that integrins may form complexes with tetraspanins, the expression and function of this newly detected family of cell surface molecules was also studied. We soon determined that the integrin $\alpha 3\beta 1$ preferentially associates with the tetraspanin CD151 and vice versa. Furthermore, we showed that CD151 is colocalized with $\alpha 3\beta 1$, as well as with the other laminin-binding integrins. Therefore the attention widened from the kidney expressing $\alpha 3\beta 1$ in the glomerulus and $\alpha 6\beta 1$ in the tubules, to skin and muscle, mainly expressing the integrins $\alpha 6\beta 4$ and $\alpha 7\beta 1$, respectively.

Chapter 1 is an introduction on integrins, extracellular matrix molecules and tetraspanins with an emphasis on laminin-binding integrins, laminin isoforms and the tetraspanin CD151. The kidney and the skin, two of the tissues in which laminin-binding integrins are abundantly expressed, are described in more detail.

In chapter 2 the tissue distribution and some of the functional aspects of the A and B variants of the integrin $\alpha 3$ subunit are described. For this purpose, we prepared a panel of monoclonal antibodies specifically reacting with one of the cytoplasmic splice variants. One of these antibodies was able to discriminate between phosphorylated and non-phosphorylated $\alpha 3A$. $\alpha 3A$ is far more widely distributed than $\alpha 3B$, and is strongly expressed by glomerular cells, basal keratinocytes of the epidermis and endothelial cells of veins and arterioles. $\alpha 3B$ is only expressed by a subset of endothelia lining veins in the brain and the heart. Phosphorylation and ligand binding specificities of the two cytoplasmic splice variants were investigated by transfection experiments of the $\alpha 3$ variants in K562 cells that only express integrin $\alpha 5\beta 1$. The phosphorylation of $\alpha 3A$ and the increase of phosphorylation after PMA stimulation described by us, have recently been confirmed by the group of Martin Hemler. Although $\alpha 3B$ is not phosphorylated, there is no difference between $\alpha 3A$ and $\alpha 3B$ in the binding of the laminin isoforms, not even after stimulation by PMA or after stimulation with antibodies directed against the $\beta 1$ subunit. Although we could not show any difference in ligand binding after phosphorylation, the group of Hemler recently demonstrated by mutagenesis of the serine in the conserved QPSXXE motif in the cytoplasmic domain of $\alpha 3$ that phosphorylation does induce differences in the morphology and motility of a cell and in the subcellular localization of $\alpha 3\beta 1$ itself (Zhang et al., 2001b).

Chapter 3 contains a descriptive study on the expression of integrins and extracellular matrix components during nephrogenesis and in the adult human kidney. In vivo data were compared with in vitro data obtained by culture of rat glomerular visceral epithelial cells and mesangial cells. When mesenchymal to epithelial transformation is induced, laminin-1 (400, 220, 210 kDa, respectively) is deposited by adjacent cells and
becomes part of the primitive basement membrane. At the same time, the adjacent cells express \( \alpha 6 \beta 1 \), a receptor for laminin-1. Early in glomerulogenesis the laminin \( \alpha 1 \) chain is replaced by what we now call the laminin \( \alpha 5 \) chain (395 kDa) whereby laminin-10 is formed. Simultaneously, \( \alpha 3 \beta 1 \) is expressed by the primitive glomerular cells, while \( \alpha 6 \beta 1 \) remains expressed in cells which will become mature tubular epithelial cells. The laminin \( \beta 1 \) chain is replaced somewhat later in glomerulogenesis by the \( \beta 2 \) chain, whereby laminin-10 becomes laminin-11. This second switch in laminin chain composition is not accompanied by a change in integrin expression. During further development, the expression of the cytoplasmic variants \( \alpha 3 \alpha \) and \( \alpha 6 \beta \) remains the same.

Interestingly, while mesangial cells in vitro did, glomerular visceral epithelial cells in vitro did not produce nidogen. It was already known from the literature that mesenchymal cells, among which mesangial cells as well as endothelial cells, produce nidogen (Thomas and Dziadek, 1993). An essential contribution of the glomerular endothelial cells to the proper tertiary structure of the glomerular basement membrane (GBM) was therefore suspected (Aumailley et al., 1993). Although at the time it was generally thought that the podocytes contributed most to the production of GBM components, there are now indications that indeed the contribution of the endothelial cells, not tested at the time, is greater than previously thought (St John and Abrahamson, 2001).

We were the first to describe the expression of \( \alpha 8 \beta 1 \) by mesangial cells. Later \( \alpha 8 \beta 1 \) appeared to be not only a receptor for fibronectin present in the mesangium, but also a receptor for nephronectin. Nephronectin, an extracellular matrix molecule also present at sites where mesenchymal cells undergo epithelial transformation, is demonstrated to be an important factor in kidney organogenesis (Muller et al., 1997; Brandenberger et al., 2001).

In chapter 4 it is described that the tetraspanin CD151 is present in hemidesmosomes. Hemidesmosomes are distinct structures present at the interface between certain epithelia and their underlying basement membrane that function as cell attachment sites where the intermediate filaments are linked to the extracellular matrix. CD151 is the only tetraspanin present in hemidesmosomes and the only tetraspanin present at the dermo-epidermal junction. It forms complexes with \( \alpha 3 \beta 1 \) and \( \alpha 6 \beta 4 \), both expressed by the basal keratinocytes of the epidermis. By confocal laser-scanner microscopy (CLSM) it was determined that cultured \( \beta 4 \)-deficient PA-JEB keratinocytes that are not able to form hemidesmosomes, first produce laminin-5 to which the cells adhere by \( \alpha 3 \beta 1 \) associated to CD151. The then formed patches of laminin-5/\( \alpha 3 \beta 1 \)/CD151 complexes are referred to as pre-hemidesmosomal structures. During maturation in \( \beta 4 \) expressing cells, these pre-hemidesmosomal structures serve as nucleation sites for the attachment of CD151/\( \alpha 6 \beta 4 \) complexes. As expression of \( \alpha 6 \beta 4 \), probably bound to CD151 is a prerequisite for hemidesmosome formation, hemidesmosomes are also formed in PA-JEB cells when they are transfected with full-length \( \beta 4 \) cDNA. In untransfected PA-JEB cells \( \alpha 3 \beta 1 \)/CD151 is colocalized with vinculin as part of focal adhesions surrounding the hemidesmosomes. However, after transfection with \( \beta 4 \), \( \alpha 3 \beta 1 \), probably bound to CD151, is not only eliminated from the hemidesmosomes, but in addition the number of \( \alpha 3 \beta 1 \)/CD151 complexes that remain present as part of peri-hemidesmosomal focal adhesions is limited. The classical focal adhesions at the cell periphery do not contain any \( \alpha 3 \beta 1 \)/CD151 or \( \alpha 6 \beta 4 \)/CD151.

By the use of interleukin-2 receptor/\( \beta 4 \) chimeras it was demonstrated that the \( \beta 4 \) cytoplasmic tail by itself is not sufficient for recruiting CD151 into hemidesmosomes.
For CD151 recruitment, β4 needs to be associated with α6, confirming that integrins bind to tetraspanins via their α subunits. Recruitment of CD151 is needed for the enlargement and coherent spatial organization of hemidesmosomes as was shown by the relatively small hemidesmosomes formed in the absence of CD151. How CD151 is involved in the spatial organization of hemidesmosomes is still elusive, but as a tetraspanin, CD151 can function as a potential docking and connecting protein for both structural and signaling molecules needed for the stabilization and maturation of the hemidesmosomes.

How and to which effect CD151/α3β1 complexes, which are present in pre-hemidesmosomal structures, are replaced by CD151/α6β4 complexes present in matured hemidesmosomes is still subject to debate, but there are several possible explanations. It is possible that CD151 is present all along and that only the integrins associated with it are switched. However, as the affinity of CD151 for α3β1 is stronger than for α6β4, it is unlikely that CD151 bound to α3β1 in pre-hemidesmosomal structures would be replaced by the incoming α6β4 after being expressed. Furthermore, association of α3 and α6 integrins with CD151 is established early in biosynthesis (Yauch et al., 1998; Kazarov et al., 2002) and fits with the supposed role of CD151 in vesicle transport and its necessity for the expression of integrins at the cell surface. Another possibility is that the affinity of α6β4 for laminin-5 is stronger than that of α3β1, or even that the affinity of both receptors for laminin-5 is regulated from within the cell by inside-out signaling. Alternatively and supported by increasing evidence, the affinity of laminin-5 for its receptors can be altered. Such alteration of laminin-5 via processing or cleavage of the laminin-5 α3 and γ2 chains by metalloproteases could play a pivotal role in the adhesion and migration of keratinocytes as part of wound healing. During wound healing and keratinocyte migration, hemidesmosomes have to be disassembled and reassembled. Although speculative, it is therefore tempting to assume that the processing of laminin-5 will occur simultaneously with a switch from α3β1 which participates in dynamic functions such as cell migration and proliferation, to α6β4 which is present in the relatively static hemidesmosomes. Furthermore, the combined integrin α3/β4 and α6/β4 knock out studies described by DiPersio et al., suggest a contributing role of other laminin receptors present at the dermo-epidermal junction (DiPersio et al., 2000). The combined expression of the laminin-binding integrins together with laminin receptors such as dystroglycan and Lutheran blood group antigens as shown by us (see chapter 5) stresses the complexity of events concerning laminin binding that take place at the subcellular level.

As CD151 is already present in pre-hemidesmosomal structures, its function in the formation and spatial organization of hemidesmosomes is not easy to determine. In order to do so, CD151 expression has to be knocked out by means of conventional or conditional targeted mutagenesis or by means of anti-sense or RNA-inhibition techniques of which the latter are under development in our group. Dynamical studies with integrin receptors or tetraspanins bound to a fluorescent dye will probably give definite answers to questions concerning the sequence of events in the formation of hemidesmosomes and the migration of keratinocytes. Truly a challenging field for further investigation!

In chapter 5 the special association of CD151 with all the laminin-binding integrins is described, including the first description of the very strong and selective binding of CD151 to α7β1. Alternative splicing of mRNAs for cytoplasmic or extracellular domains or prevention of cleavage of the α subunit seemed to have no effect on the binding of the integrins to CD151. By the use of TS151R, a monoclonal antibody that recognizes
an epitope at the CD151 binding site for α3 (Serru et al., 1999; Yauch and Hemler, 2000) it was determined in vitro that the same CD151 binding site is also involved in the binding of the other laminin-binding integrins. In vivo results suggested that the strength of the interaction between CD151 and the laminin-binding integrins is subject to regulation, either from within the cell or by the cell’s environment.

At many sites there is extensive codistribution of the different kinds of laminin receptors i.e. integrins, dystroglycan and the Lutheran blood group antigens. Although some people would call the combined expression of different laminin receptors a good example of redundancy, I am convinced that the concept of redundancy in general is only invented to disguise the limited knowledge of a certain subject and will be contradicted when more detailed information becomes available. Although the molecules mentioned might share functions like the binding of laminin, this does not exclude additional properties such as e.g. in the fine-tuning of laminin binding. It was hypothesized that CD151, as a tetraspanin, would be a good candidate to connect the different laminin receptors. However, in cultured human GVECs and proximal tubular epithelial cells, no direct association between the CD151/α3β1 complexes and the other laminin receptors, dystroglycan and Lutheran blood group antigens, could be demonstrated.

Apart from cells that express both CD151 and one or more of the laminin-binding integrins, there are also cells in which the amount of CD151 is obviously greater than the amount of laminin-binding integrins, such as endothelial cells. In addition there are cells that seem to express CD151 in the absence of laminin-binding integrins, such as fibroblasts and cells from the K562 cell line. This is in agreement with the different functions suggested in the literature and again an indication that CD151 does not play one single role, but that its function can be diverse.

**Chapter 6** deals with the effect of blocking and non-blocking murine monoclonal antibodies against rat α3β1 in vivo. This study was done in anticipation of the development of the conditional mutant mice for the α3 subunit. The results with the conventional mutant mice already indicated that α3β1 is involved in glomerular basement membrane assembly, determining the shape of the podocyte by arrangement of the cytoskeleton and the selectivity of glomerular permeability (Kreidberg et al., 1996). As the antibodies directed against α3β1 were thought to interfere with the function of α3β1 at the protein level, they were expected to have a pathophysiological effect. Two different kinds of antibodies were used: The Ralph 3.2 antibodies that were known to block cell-matrix adhesion and the Ralph 3.3 antibodies that were not. In order to study a possible additional effect on the developing kidney, the antibodies were also administered to adult rats of which a subgroup was pregnant. The antibodies were not only administered as a single bolus, but also via a diffusion pump, which guaranteed a continuous delivery of antibodies during at least one week. In this way, breakdown and washout of antibodies were at least partially circumvented. Additionally, the continuous administration of antibodies would increase the availability of the α3 epitopes for the blocking antibodies that otherwise would be occupied by adhesion of the podocyte to the GBM. After all, these α3 epitopes, involved in cell adhesion, would only become exposed just after cell surface expression and before they actually would bind to the GBM.

Unfortunately the possibility to investigate the many aspects in which α3β1 could be involved was at some point seriously restricted as the availability of antibodies selectively reacting with rat proteins such as nephrin, podocin, ZO-1 and cadherin, was limited. Furthermore, the lack of polyclonal antibodies necessary for double-label CLSM analysis was experienced as a drawback.
Nevertheless, we showed in agreement with its expression at the lateral side of the podocyte footprocesses, that α3β1 is colocalized with ZO-1, P-cadherin, podocin and synaptopodin, molecules of which the majority were proven to be present at the interpodocytic slit diaphragms. Except for the presence of α3β1 at interpodocytic slit junctions, the presence of α3β1 at interendothelial adherens junctions was shown by its colocalization with VE-cadherin. Colocalization of α3β1 with adherens junction proteins was not only shown by indirect immunofluorescence combined with CLSM analysis in kidneys of untreated rats, but also by in situ detection of mouse antibodies in adult and newborn or fetal rats that had been treated with the non-blocking antibodies. The binding of the non-blocking antibodies in vivo had not induced obvious differences in podocyte morphology nor was there any change in the expression of any of the adherens junction proteins tested, the other laminin receptor present, i.e. dystroglycan, and the phosphorylated signaling molecules p-FAK and p-ERK. Furthermore, there was no proteinuria, which made disturbances in the selective permeability unlikely. Similar to the adult situation, the non-blocking antibodies present in developing kidney of newborn rats had not altered the expression of any of the proteins studied, nor was there any obvious difference in glomerulogenesis.

In contrast with the non-blocking antibodies, only limited amounts of the blocking antibodies were recovered in adult animals in situ. Another difference is that most of the blocking antibodies were mainly present in the endothelium in stead of at the podocytic side of the GBM where most of the non-blocking antibodies were recovered. In endothelial cells the blocking antibodies were not only present at the cell surface, but also in their cytoplasm, obviously as a result of internalization. Despite additional morphological alterations shown by electron microscopical examination, this had not disturbed the integrity of the endothelial lining, while no proteinuria was present. The reason for the almost complete absence of blocking antibodies at the podocytic side of the GBM is not clear, but is likely due to instability of the monoclonal antibodies, combined with endocytosis and degradation by endothelial cells to which they had bound.

Shown by the presence of non-blocking antibodies in newborn rat, antibodies applied to the mother were able to cross the placental barrier. Nevertheless, no blocking antibodies were recovered in the glomeruli of newborn rats. Again this was probably due to a combination of antibody breakdown and internalization by endothelial cells in the mother.

During glomerulogenesis, the temporal and spatial expression of the adherens junction proteins tested, differed. General cell-cell receptors such as ZO-1, P-cadherin and α3β1 and cell-matrix receptors such as α3β1 and dystroglycan are expressed from the early stages onward. In contrast, the specific podocyte markers such as podocin and synaptopodin appear relatively late, i.e. from the capillary stage onward, when actual podocyte differentiation with the formation of footprocesses takes place.
Future perspectives

Based on their state of adhesion, mechanical forces and the concentration of soluble growth factors, integrins may enable cells to modulate their functional behavior by integrating cell adhesion with cytoskeletal arrangement and signaling cascades. As a result, an integrin can be considered a spider in a web constructed of a still increasing amount of structural proteins and signaling molecules to which it is connected. So far, most of the information on integrins is based on biochemical data and the use of transgenic mice. Due to the development of recent techniques, integrins can now be analyzed in living cells too. For example FRET (fluorescence resonance energy transfer) can be used to monitor real-time integrin conformational changes or to study the interaction of the integrin with other proteins; fluorescence recovery after photobleaching (FRAP) can be used to study its energy dependent movement and AFM (Atomic Force Microscopy) allows adhesion forces between integrins and its ligands to be measured and mapped. Also crystallography studies on the different types of integrins with or without binding to a ligand will clarify certain obscurities on the function of an integrin that can be deduced from its conformation. The discovery of tetraspanins that are able to bind to many structural and signaling proteins and to which integrins are often bound may in potency even further enhance the functional diversification of the integrins. As far as laminin-binding integrins are concerned, tetraspanin CD151 is of special interest because of their exclusive direct interaction and their symbiosis in signaling. The exceptional stoichiometric binding of CD151 with $\alpha_3\beta_1$ may additionally explain some of the putative functions of $\alpha_3\beta_1$ other than cell-matrix adhesion. Because most cells express CD151, its function is not easy to study. Therefore researchers in the field of tetraspanins and integrins are eagerly awaiting the results obtained with CD151 (conditional) mutant mice or the results from the CD151 inhibition at the RNA level by using small interference RNAs. Clearly much in vitro work is needed on CD151/$\alpha_3\beta_1$ signaling and its role in hemidesmosome assembly by means of e.g. tagged proteins or transfection studies with mutated CD151. The role of $\alpha_3\beta_1$/CD151 complexes in the podocyte can best be studied in vivo, because cells in culture have the disadvantage that they do not properly form foot processes and slits, which are so essential in the function of podocytes in vivo. Although $\alpha_3\beta_1$, by its cell-matrix and cell-cell interactions, is playing a pivotal role in the glomerulus, the precise interactions with other proteins present and its possible influence on signaling events is still unknown. The conditional $\alpha_3$ mutant mice made in our laboratory, will clarify to some extent not only the function of the $\alpha_3\beta_1$ integrin, but also that of the associated tetraspanin CD151. A combination with micro-array analysis and proteomics of e.g. glomerular cells will give a plethora of results, which can serve as a source for new ideas and additional experiments for years to come.