

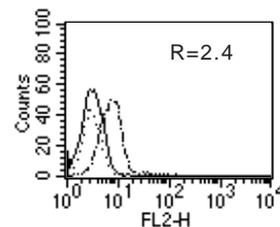
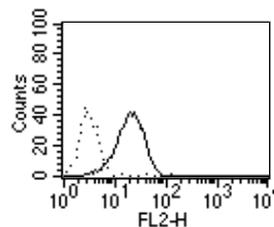
**Association of the tetraspanin CD151 with the laminin-binding integrins  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$  and  $\alpha 7\beta 1$  in cells in culture and in vivo**

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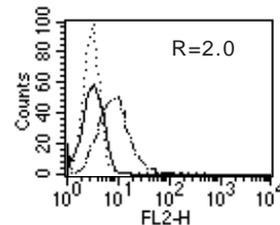
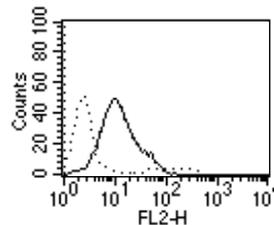
In the printed version of this article, Fig. 10 was incorrect. The correct figure is shown below.

**Fig. 10.** Expression of laminin-binding integrins on transfected K562 cells increases CD151 cell surface expression. Flow cytometric analysis of wild-type K562 cells endogenously expressing  $\alpha 5\beta 1$  and transfected K562 also expressing  $\alpha v\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha 7X1\beta 1$  or  $\alpha 7X2\beta 1$ . Solid lines in the left panels indicate staining with anti-integrin antibodies: anti- $\alpha 5$  mAb Sam-1 (K562 cells), anti- $\alpha v$  mAb 13C2 (K562- $\alpha v$ ), anti- $\alpha 3$  mAb J143 (K562- $\alpha 3$ ), anti- $\alpha 6$  mAb GoH3 (K562- $\alpha 6$ ), anti- $\beta 4$  mAb 439-9B (K562- $\alpha 6\beta 4$ ) and anti- $\alpha 7$  mAb CA25 (K562- $\alpha 7BX1$  and K562- $\alpha 7BX2$ ). Staining of cells with anti-CD151 antibodies is shown in the right panels. Solid lines represent staining of cells with mAb TS151R and broken lines with mAb P48. A negative control (dotted line) with secondary goat anti-mouse or rat IgG alone is shown in each panel. R represents the ratio of the mean fluorescence intensity of cells stained with mAb P48 and with secondary antibody alone. A three- to sixfold increase of CD151 expression is observed in transfected cells that ectopically express lamininbinding integrins. The TS151R epitope is masked in all cells expressing one of the laminin-binding integrins.

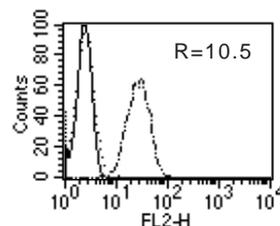
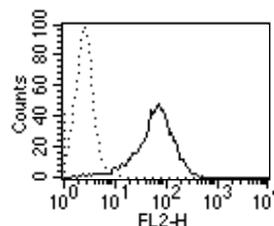
K562- $\alpha 5$



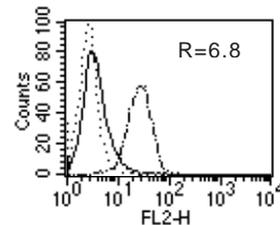
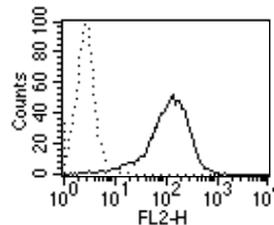
K562- $\alpha v$



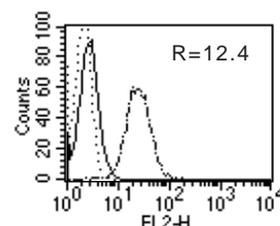
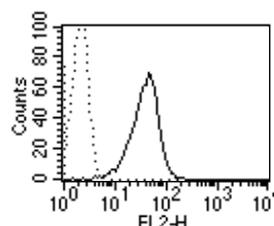
K562- $\alpha 3$



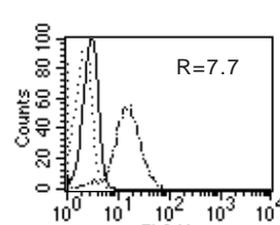
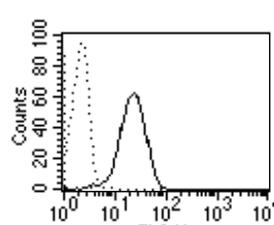
K562- $\alpha 6$



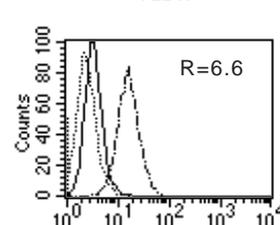
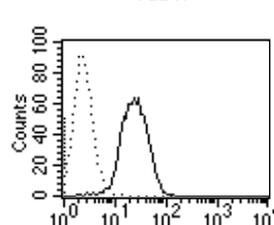
K562- $\alpha 6\beta 4$



K562- $\alpha 7BX1$



K562- $\alpha 7BX2$



anti-integrin

anti-CD151

# Association of the tetraspanin CD151 with the laminin-binding integrins $\alpha 3\beta 1$ , $\alpha 6\beta 1$ , $\alpha 6\beta 4$ and $\alpha 7\beta 1$ in cells in culture and in vivo

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## Summary

CD151 is a cell surface protein that belongs to the tetraspanin superfamily. It forms complexes with the laminin-binding integrins  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  and is co-distributed with these integrins in many tissues at sites of cell-matrix interactions. In this study we show that CD151 can also form stable complexes with the laminin-binding integrin  $\alpha 7\beta 1$ . The strength of this interaction is comparable to that between CD151 and  $\alpha 3\beta 1$ . Complexes of  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 7\beta 1$  with CD151 are equally well formed with all splice variants of the  $\alpha 3$ ,  $\alpha 6$  and  $\alpha 7$  subunits, and complex formation is not affected by mutations that prevent the cleavage of the integrin  $\alpha 6$  subunit. Like the expression of  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$ , expression of  $\alpha 7\beta 1$  in K562 cells results in increased levels of CD151 at its surface. Two non-integrin laminin receptors, dystroglycan and the polypeptide on which the Lutheran blood group antigens are expressed, are also often colocalized with CD151, but no association with

CD151- $\alpha 3\beta 1$  complexes was found with biochemical analysis.

The anti-CD151 antibody TS151R detects an epitope at a site at which CD151 interacts with integrins, and therefore it cannot react with CD151 when it is bound to an integrin. Comparison of the staining patterns produced by TS151R with that by of an anti-CD151 antibody recognizing an epitope outside the binding site (P48) revealed that most tissues expressing one or more laminin-binding integrins reacted with P48 but not with TS151R. However, smooth muscle cells that express  $\alpha 7\beta 1$  and renal tubular epithelial cells that express  $\alpha 6\beta 1$  were stained equally well by TS151R and P48. These results suggest that the interactions between CD151 and laminin-binding integrins are subject to cell-type-specific regulation.

Key words: Tetraspanin, Laminin receptor, Integrin, Dystroglycan, Lutheran blood group antigens

## Introduction

CD151 is a transmembrane molecule that has been recently characterized as a member of the evolutionary conserved tetraspanin superfamily (Wright and Tomlinson, 1994; Maecker et al., 1997). Tetraspanin molecules, so called because they cross the plasma membrane four times, have two extracellular loops and three cytoplasmic domains including the C- and N-terminus. Many interactions among tetraspanins and between tetraspanins, integrins and other molecules have been described (Berditchevski et al., 1996; Mannion et al., 1996; Hasegawa et al., 1998; Fitter et al., 1999; Sincock et al., 1999). Hence it is thought that one function of tetraspanins is to facilitate the coupling of signaling pathways by assembling and connecting specific cell surface proteins into large molecular complexes.

On the basis of the stability of the formed complexes, interactions of tetraspanins can be classified into three categories (Claas et al., 2001). CD151- $\alpha 3\beta 1$  complexes represent the exceptionally strong, highly stable and selective level 1 association. Most interactions between tetraspanins and integrins, for example, the association of CD151 with  $\alpha 6\beta 4$ , are less strong and considered to be level 2; they are numerous,

fairly stable and selective. CD151- $\alpha 6\beta 1$  interactions are either level 1 or level 2, probably depending on the cell type in which these molecules are expressed. Level 3 interactions, which occur mostly between tetraspanins and molecules other than tetraspanins or integrins, are indirect, numerous and are the least stable of the interactions. The level 1 interaction of CD151 with  $\alpha 3$  occurs between the large extracellular loop of CD151 and the extracellular membrane proximal region of the  $\alpha 3$  subunit (Yauch et al., 2000). A CD151 epitope recognized by the mAb TS151R is located at this site of interaction (Serru et al., 1999; Yauch et al., 2000). Recently, it was shown that the binding site on CD151 for tetraspanins is different from the one involved in the binding to integrins (Berditchevski et al., 2001).

Integrins are heterodimeric cell surface receptors consisting of a non-covalently associated  $\alpha$  and  $\beta$  subunit, which link the extracellular matrix to the cytoskeleton (Hynes, 1992; Schwartz et al., 1995; van der Flier and Sonnenberg, 2001). The integrins  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 7\beta 1$  bind preferentially to laminin and contain sequential and structural homologies. During maturation, the  $\alpha 3$ ,  $\alpha 6$  and  $\alpha 7$  integrin subunits are post-translationally cleaved into a heavy and a light chain.

Furthermore, the mRNAs for these integrin subunits can be alternatively spliced, leading to extracellular and cytoplasmic variants (Collo and Quaranta, 1993; Hogervorst et al., 1993; de Melker et al., 1997). The ligand binding and signaling function of these integrin variants is potentially different, which during development may lead to changes in function when variants are switched (Ziober et al., 1993; Thorsteindóttir et al., 1995; Schober et al., 2000).

While much is known about the function and biochemical properties of integrins, the exact function of the associated tetraspanins is still elusive but is gradually becoming unraveled. Previously, we have shown that CD151 is a component of hemidesmosomes, specialized structures in the basal epidermal cell layer that link intermediate filaments to the extracellular matrix (Sterk et al., 2000). Others have shown the involvement of CD151 in cell adhesion (Hasegawa et al., 1998; Fitter et al., 1999), (tumor) cell migration and metastasizing (Yáñez-Mó et al., 1998; Yauch et al., 1998; Sincostack et al., 1999; Sugiura and Berditchevski, 1999; Berditchevski and Odintsova, 1999; Testa et al., 1999), vesicular transport of integrins (Sincostack et al., 1999), signaling (Berditchevski et al., 1997; Yauch et al., 1998; Sugiura and Berditchevski, 1999; Berditchevski and Odintsova, 1999; Zhang et al., 2001), cell polarization (Yáñez-Mó et al., 2001), wound healing (Peñas et al., 2000) and neurite outgrowth (Stipp and Hemler, 2000).

In vivo, CD151 is expressed by a large variety of cells of epithelial and mesenchymal origin. The tissue distribution of CD151 shows a striking overlap with that of the combined patterns of expression of  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$  and  $\alpha 7\beta 1$  - all laminin-binding integrins (Sincostack et al., 1997). This suggests that CD151 preferentially interacts with the laminin-binding integrins.

Laminin is a basement membrane component that, by interaction with its cell surface receptors, influences cell shape, movement and differentiation (Colognato and Yurchenko, 2000). Besides representing a ligand for integrins, laminin also interacts with the recently discovered Lutheran (LU) molecule expressed on erythrocytes (El Nemer et al., 1998; Parsons et al., 2001; Udani et al., 1998) and with dystroglycan (DG), an integral component of the dystrophin glycoprotein complex (DGC) originally detected in skeletal muscle and subsequently in epithelia (Ibraghimov-Breskovnaya et al., 1992; Durbeej and Campbell, 1999).

In the present study we investigated the expression of various tetraspanins and laminin receptors in tissues that strongly express one or more of the laminin-binding integrins, such as kidney ( $\alpha 3\beta 1$  in glomerular cells;  $\alpha 6\beta 1$  in tubular cells), skin ( $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ ) and muscle ( $\alpha 7\beta 1$ ). At sites of contacts between cells and the matrix, CD151 is the only one of the tetraspanins tested that is always colocalized with the laminin-binding integrins. The intimate relation between CD151 and the laminin-binding integrins is also stressed by their high stoichiometric interaction and the increase of CD151 cell surface expression in K562 cells transfected with one of these integrins. By immunoprecipitation it was demonstrated for the first time that CD151 is associated with  $\alpha 7\beta 1$ , and that their firm association is comparable to that of the association between  $\alpha 3\beta 1$  and CD151. Cytoplasmic or extracellular splice variants or mutations preventing cleavage of the integrin  $\alpha$  subunit were found to have no influence on the formation of

integrin-CD151 complexes. Although there is a considerable overlap in expression between the laminin receptors from the three different receptor families and CD151, no interaction between CD151 and either LU or DG could be identified *in vitro*. Finally, we provide evidence that the same site on CD151 interacts with all the different laminin-binding integrins. Furthermore, the exposure of a CD151 epitope involved in this binding is cell type specific and may vary depending on the (patho)physiological status of the tissues involved.

## Materials and Methods

### Antibodies

Mouse mAbs J8H against the extracellular domain of the human  $\alpha 6$  integrin subunit and 29A3 against the cytoplasmic domain of the human  $\alpha 3A$  subunit have been described previously (Hogervorst et al., 1993; de Melker et al., 1997). The hybridomas producing the mouse mAbs J143, recognizing an extracellular epitope on the human  $\alpha 3$  integrin subunit (Kantor et al., 1987) and TS2/16 against  $\beta 1$  (Hemler et al., 1984), were obtained from the American Type Culture Collection (Rockville, MD). Sam-1 is a mouse mAb against  $\alpha 5$  (Keizer et al., 1987), and GoH3 is a rat mAb against  $\alpha 6$  (Sonnenberg et al., 1987). The mouse mAbs CA25 and 13C2 against  $\alpha 7$  and  $\alpha v$  were generously provided by R. H. Kramer (University of California San Francisco, San Francisco, CA) and M. Horton (Imperial Cancer Research Fund, London, UK), respectively. Rat mAb 439-9B against  $\beta 4$  (Falcioni et al., 1988) was purchased from Pharmingen (San Diego, CA). The rabbit polyclonal antibodies against the cytoplasmic domains of  $\alpha 3A$  (DiPersio et al., 1995),  $\alpha v$  (Defilippi et al., 1991) and  $\alpha 7B$  were kind gifts from M. DiPersio (Albany Medical College, Albany, NY), G. Tarone (Università di Torino, Torino, Italy) and U. Mayer (University of Manchester, Manchester, UK), respectively. Mouse mAbs against  $\alpha$ -dystroglycan were obtained from Novocastra (Newcastle, UK). Polyclonal antibody AP83 against  $\beta$ -dystroglycan was kindly provided by K. P. Campbell and M. Durbeej (Howard Hughes Medical Institute, Iowa, USA). Mouse mAb LU4F2 (workshop 70597) against Lutheran blood group antigens was a kind gift from E. van der Schoot (Central Laboratory for the Blood Transfusion Service, Amsterdam, The Netherlands). The mouse mAbs P48 and P49, also known as 11B1.G4 and 14A2.H1, respectively, were clustered as CD151 in the VI International Leukocyte Typing Workshop (Ashman et al., 1997; Sincostack et al., 1997).

The other mouse mAbs against CD151 were all gifts: 5C11 from M. E. Hemler (Dana-Farber Cancer Institute, Boston, MA), 8C3 from K. Sekiguchi (Osaka University, Osaka, Japan), Sfa-1 from H. Hasegawa (Hasegawa et al., 1996) (Ehime University, Ehime, Japan), LIA1/1 from F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain), IIG5 from M. Humphries (The University of Manchester, Manchester, UK) and TS151 and TS151R (Yauch et al., 2000; Serru et al., 1999) from E. Rubenstein (Hôpital Paul Brousse, Villejuif Cedex, France). The mouse mAbs MEM62 (CD9; (Hořejší and Vlček, 1991)), MEM53 (CD53 (Angelisová et al., 1994)), M38 (CD81 (Imai and Yoshie, 1993)) and C33 (CD82 (Imai and Yoshie, 1993)), were generously provided by V. Hořejší (Institute of Molecular Genetics, Prague, Czech Republic). F. Berditchevsky (The University of Birmingham, Birmingham, UK) kindly supplied the mouse mAb 6H1 (CD63 (Berditchevsky et al., 1995)). The sheep anti-mouse and donkey anti-rabbit horseradish-peroxidase-coupled secondary antibodies were purchased from Amersham (Harlington Heights, IL). FITC- or Texas Red-conjugated goat anti-mouse, goat anti-rat or goat anti-rabbit secondary antibodies were obtained from Rockland (Gilbertsville, PA). The FITC- and TRITC-conjugated goat anti-mouse isotype specific antibodies were obtained from Nordic Immunochemical Laboratory (Tilburg, The Netherlands). Phycoerythrin-conjugated goat anti-mouse or anti-rat antibodies were obtained from Jackson laboratories (Westgrove, PA).

### Generation of retroviral expression constructs and stable cellular transduction

The full-length c-DNA encoding the X1 and X2 splice variants of the extracellular domains of human  $\alpha 7B$  were a kind gift from D. Mielenz and K. von dem Mark (University of Erlangen, Erlangen, Germany).  $\alpha 7BX1$  and  $-X2$  c-DNA was released from pUC-18 or pcDNA3 respectively, by digesting with *EcoRI*. The resulting fragments were ligated into the retroviral LZRS-IRES-zeo expression vector, a modified LZRS retroviral vector conferring resistance to zeocin (Kinsella and Nolan, 1996; van Leeuwen et al., 1997), to give an LZRS-IRES-zeo- $\alpha 7BX1$  or  $-X2$  vector. These constructs were then introduced into the Phoenix packaging cells (Kinsella and Nolan, 1996) by the calcium phosphate precipitation method and virus containing supernatant was collected. K562 cells were infected with the recombinant virus by the DOTAP method (Boehringer Mannheim Corp.). After incubation for 8 hours at 37°C, infected cells were grown in medium as described below for the wild-type K562 cells. Cells expressing  $\alpha 7$  at their surface were analyzed and sorted using FACSscan® flow cytometer (Becton Dickinson, Mountain View, CA).

### Cell lines

The human erythroleukemic cell line K562 was maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Paisly, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Life Technologies). K562 cells stably expressing  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  were established as described previously (Delwel et al., 1993; Niessen et al., 1994). An established cell line of human glomerular visceral epithelial cells (Krishnamurti et al., 1996) was cultured in medium made up of 1 volume of each Dulbecco's modified Eagle's medium (DMEM) (ICN, Costa Mesa, CA) and Hams F-10 (Life Technologies), supplemented with 5% Nu serum (Becton Dickinson, Bedford, MA), 25 ng/ml prostaglandin E<sub>1</sub>, 0.5 nM tri-iodothyronine, 10 nM sodium selenite, 5 µg/ml transferrin, 50 nM hydrocortisone, 5 µg/ml insulin (all hormones from Sigma, St. Louis, MO), 100 U/ml penicillin and 100 µg/ml streptomycin. Human proximal tubular epithelial cells (PTEC) also known as HK-2 cells are HPV 16-immortalized cells purchased from the American Type Culture Collection. Cells were grown in conditioned medium consisting of 1 volume of DMEM and 1 volume Ham F-12, supplemented with 5% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 20 ng/ml tri-iodothyronine, 5 ng/ml hydrocortisone, 5 ng/ml prostaglandin E<sub>1</sub> and 5 ng/ml epidermal growth factor (all from Sigma, St. Louis, MO). Cells were grown at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere.

### Immunoprecipitation of <sup>125</sup>I-labeled cells

Transfected K562 cells, stably expressing  $\alpha 3A$ ,  $\alpha 3B$ ,  $\alpha 6A$ ,  $\alpha 6B$  or  $\alpha 6A^{RGGD}$  mutant, were surface labeled with <sup>125</sup>I by the lactoperoxidase method as previously described (Sonnenberg et al., 1987). Cells were solubilized in lysis buffer containing 1% (w/v) CHAPS (Sigma), 5 mM MgCl<sub>2</sub>, 25 mM Hepes, pH 7.5, 150 mM NaCl and proteinase inhibitors (1 mM phenylmethanesulphonyl fluoride, 10 µg/ml soybean trypsin inhibitor and 10 µg/ml leupeptin). Lysates were clarified at 20,000 g and precipitated with protein A-sepharose beads CL-4B (Amersham Pharmacia Biotech Inc., Uppsala, Sweden), which had previously been sequentially incubated with rabbit anti-mouse IgG and the precipitating antibody. After incubation for 1.5 hour at 4°C, the beads carrying the immune complexes were washed and treated with sodium dodecyl sulfate (SDS) sample buffer. Precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

### Immunoblotting

Wild-type and transfected K562 cells stably expressing  $\alpha v\beta 1$ ,  $\alpha 3\beta 1$ ,

$\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha 7X1\beta 1$  or  $\alpha 7X2\beta 1$ , and podocytes and PTEC were lysed in 1% (w/v) CHAPS buffer with proteinase inhibitors. Alternatively, cells were lysed in 1% (v/v) Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1 mM EDTA. Lysates were incubated for 1 hour at 4°C with Gamma Bind-G Sepharose beads or protein A-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden), previously incubated overnight at 4°C with the precipitating antibodies. The beads carrying the immune complexes were washed three times with lysis buffer and two times with PBS at 4°C. The immune complexes were eluted by the addition of sample buffer, heated at 95°C or 65°C and separated by SDS-PAGE under reducing (2%  $\beta$ -mercaptoethanol) or non-reducing conditions. After electrophoresis, gels were electrophoretically transferred to a PVDF membrane (Immobilon-P, Bedford). Blots were stained with Coomassie blue to indicate the markers, destained in 45% methanol, 5% acetic acid in demineralized water and blocked for 30 minutes with 5% dry milk in TBST-buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.3% Tween-20). Subsequently blots were incubated for 1 hour with primary antibodies in 0.5% dry milk in TBST. Primary antibodies were mAbs 29A3 against  $\alpha 3$  (neat supernatant) or 8C3 against CD151 (1:1,000), or polyclonal antibodies against  $\alpha 3$  (1:100),  $\alpha 7B$  (1:100),  $\alpha v$  (1:100) or  $\beta$ -dystroglycan (1:100). After washing three times with TBST/0.5% dry milk, blots were incubated for an additional hour with secondary sheep anti-mouse, donkey anti-rabbit or goat anti-rat Ig-horseradish-peroxidase-coupled, diluted 1:5,000 or 1:1,000 respectively, in 0.5% dry milk in TBST. After washing three times with TBST, proteins were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech.), as described by the manufacturer.

### Analyses of integrin and CD151 surface expression

The surface expression of integrin subunits and CD151 on wild-type and transfected K562 cells was assessed by flow cytometry.  $5 \times 10^5$  cells were washed three times with PBS containing 2% FCS (PBS/FCS), followed by incubation with the primary antibody or antibodies for 1 hour. The cells were washed three times with PBS/FCS and then incubated with phycoerythrin-conjugated goat anti-mouse or anti-rat antibodies for another hour. After another three washes with PBS/FCS, cells were resuspended in PBSA and analyzed with a FACSscan® flow cytometer.

### Immunohistochemistry and immunofluorescence of tissue sections

Cryostat sections (3 µm thick) from human kidney, skin, smooth, striated or heart muscle were fixed in acetone for 10 minutes at 4°C. For immunoperoxidase staining, endogenous peroxidase was blocked by 0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and 0.3% H<sub>2</sub>O<sub>2</sub> in PBS for 10 minutes. For blockage, PBS containing 2% BSA (PBSA) was used for 10 minutes, followed by overnight incubation with the primary antibody at 4°C. Anti-rabbit or anti-mouse horseradish-peroxidase-conjugated secondary antibodies (PowerVision, Technologies Co., Daly City, CA) were added for 30 minutes and visualized by hydrogen peroxide and 3',3'-amino-9-ethyl carbazole (Sigma Chemical Co.). Sections were counterstained with hematoxylin and mounted in Depex (British Drug House Chemicals, Poole, UK). In between steps, sections were washed three times with PBS. For double-labeled immunofluorescence analyses, sections were blocked in PBSA for 30 minutes and incubated with the primary antibody diluted in PBSA overnight at 4°C. The sections were washed three times with PBSA and then, depending on the primary antibodies used, incubated with different combinations of goat anti-mouse, goat anti-rat or goat anti-rabbit FITC- or Texas-Red-conjugated antibodies for 45 minutes. After washing twice with PBS, sections were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) and viewed under a Leica TCS NT confocal laser-scanning microscope equipped with an Ar/Kr

laser (Leica Microsystems, Heidelberg, Germany). Unless otherwise mentioned, both procedures were performed at room temperature.

## Results

### Co-distribution of CD151 and the various laminin receptors in human kidney, skin and different kinds of muscle

Interactions between tetraspanins and integrins have been defined in biochemical assays, but there is limited information on the associations of these proteins in tissues. The tetraspanin CD151 has been shown to form tight complexes with  $\alpha 3\beta 1$  (Yauch et al., 1998), but in addition it may also associate with other laminin-binding integrins (Fitter et al., 1999; Sincock et al., 1999; Sterk et al., 2001). To assess its distribution relative to that of laminin-binding integrins in vivo, we determined the expression in tissues that express different laminin binding integrins; kidney (glomeruli:  $\alpha 3\beta 1$ ; tubuli:  $\alpha 6\beta 1$ ), skin ( $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ ) and muscle ( $\alpha 7\beta 1$ ). In these tissues the distribution of other tetraspanins and of the laminin receptors DG and LU was also assessed.

Analysis of kidney tissues by immunoperoxidase staining, using mAb P48, revealed strong expression of CD151 in glomeruli, at the basal surface of tubular epithelial cells and in vascular smooth muscle cells (Fig. 1A). Double immunofluorescence analysis revealed a striking colocalization with  $\alpha 3\beta 1$  (Fig. 2A) and DG (Fig. 2B) within glomeruli in a predominant, continuous GBM-bound pattern. Along the GBM, DG was only partially co-distributed with  $\alpha 3\beta 1$  (Fig. 2C), whereas LU was absent from the glomerulus but strongly expressed in the subendothelial matrix and along the basal side of the tubules, colocalized with  $\alpha 6\beta 1$  (Fig. 2D). Like CD9 and CD63, the LU molecule is also focally distributed throughout the cytoplasm of tubular epithelial cells (not shown). Transport of LU to the apical or basolateral side of polarized cells has been described before (El Nemer et al., 1999). This, together with the presence of LU at the site of cell-cell contacts between keratinocytes (see below), suggests that LU is not merely a laminin receptor but has other functions as well. The tetraspanins CD9 (not shown), CD63 (Fig. 2E) and CD81 (Fig. 2F) are present in glomerular cells but are not colocalized with

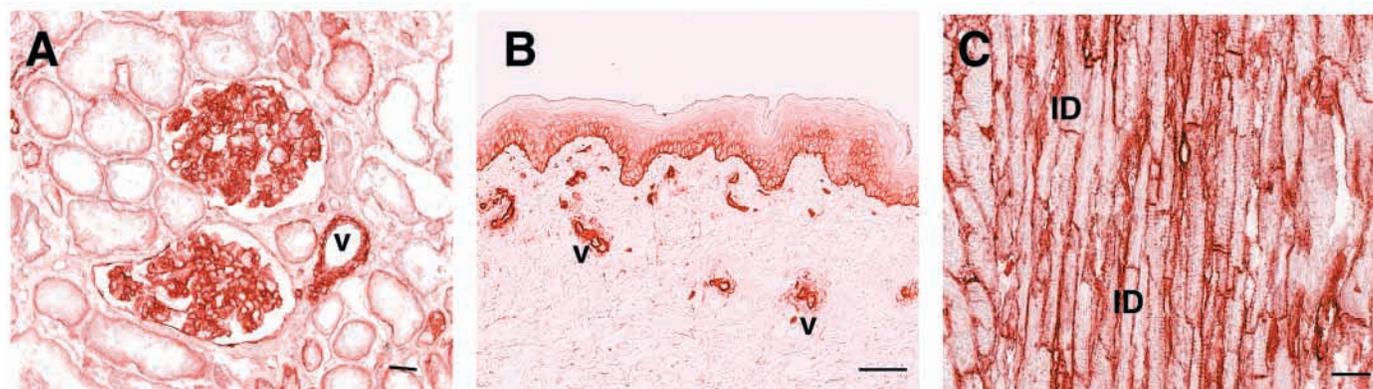
$\alpha 3\beta 1$  along the GBM. Glomerular cells do not express CD53 or CD82, which however are present on a subset of the distal tubules together with CD9 and CD81, the latter being weakly expressed by all tubular cells (not shown).

Immunolocalization of CD151 in skin revealed strong reactivity at the basolateral surface of basal keratinocytes (Fig. 1B), where the integrins  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  are also concentrated (Sterk et al., 2000). Only very small amounts of DG are present at the dermo-epidermal junction, whereas LU is focally expressed on all basal keratinocyte surfaces (not shown). The tetraspanins CD9 and CD81 are not only present in the basal cell layer but also in the more superficial epidermal cell layers. They are absent from the dermo-epidermal junction but are concentrated at inter-keratinocytic contacts (not shown). CD63 is only expressed by melanocytes present between the basal keratinocytes (not shown).

Skeletal, cardiac and smooth muscle cells all express CD151 at their plasma membrane (Fig. 1C, Fig. 9A-C). Additionally, CD151 is present at the costamers in skeletal and cardiac muscle and the intercalated discs in the heart. In these cells, it is colocalized with  $\alpha 7\beta 1$  (Fig. 3A,C) and DG (not shown). Besides CD151, smooth muscle cells express the tetraspanins CD9 and CD81 and some CD53 and CD82 (not shown). Small amounts of CD63 and CD81 are present at the sarcolemma of skeletal and heart muscle and at the intercalated discs. LU is not expressed in myocytes (not shown). In peripheral nerves, also present in the smooth muscle sections,  $\alpha 6\beta 4$ ,  $\alpha 7\beta 1$ , LU, CD9, CD63, CD81 and CD151 are all localized at the endo- and perineurium (not shown). In summary, CD151 is extensively colocalized with various laminin receptors, both integrins and non-integrins, at sites of cell-matrix interactions.

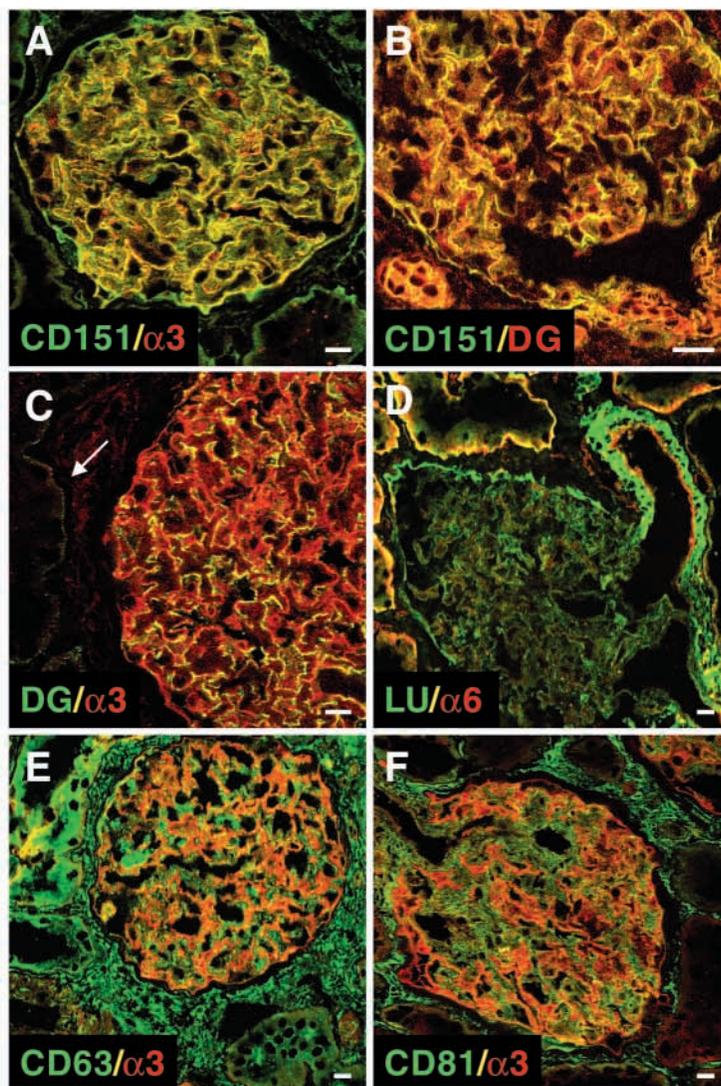
### $\alpha 3$ and $\alpha 6$ are coprecipitated with CD151

To determine whether CD151 is also able to associate with the integrin  $\alpha 3$  and  $\alpha 6$  subunits, K562 cells stably transfected with  $\alpha 3$  or  $\alpha 6$  were  $^{125}\text{I}$ -surface-labeled and lysed in 1% CHAPS. As shown in Fig. 4, antibodies against  $\alpha 3$  precipitated  $\alpha 3\beta 1$  and antibodies against  $\alpha 6$ ,  $\alpha 6\beta 1$  from lysates of  $\alpha 3$ - and  $\alpha 6$  transfected K562 cells, respectively. The transfected integrins were also present in precipitates prepared with antibodies



**Fig. 1.** Immunohistochemical distribution of CD151 in kidney, skin and heart. Cryostat sections of human kidney (A), skin (B) and heart tissue (C) were stained by immunoperoxidase reaction with anti-CD151 (P48). Intense staining is seen in the glomeruli and the epithelia of kidney tubules (A), keratinocytes of the skin (B) and in vascular smooth (A,B) and cardiac muscle cells (C). Note that CD151 is localized on the basolateral surface of epithelia and at the sarcolemma of cardiac myocytes. It is also detected at costameres and intercalated discs (ID). v, vessel. Bars, 50 (A), 100 (B) and 10  $\mu\text{m}$  (C).

**Fig. 2.** Colocalization of CD151 with laminin receptors  $\alpha 3\beta 1$  and dystroglycan in the glomerulus and with  $\alpha 6\beta 1$ , dystroglycan and the Lutheran molecule in tubular epithelial cells. Cryostat sections of human kidney were processed for double immunofluorescence confocal microscopy using P48 against CD151 (A,B), polyclonal antibodies against  $\alpha 3$  (A,C,E,F), polyclonal (AP83) and monoclonal antibodies against dystroglycan (DG) (B,C, respectively), LU4F2 against the Lutheran blood group molecule (LU) (D), GoH3 against  $\alpha 6$  (D), 6H1 against CD63 (E) and M38 against CD81 (F). Composite images were generated by superimposition of the green and red signals, with areas of overlap appearing as yellow. In the glomerulus, CD151 is co-distributed with laminin-receptors  $\alpha 3\beta 1$  and DG but not with LU. The distribution pattern of  $\alpha 3\beta 1$  is mainly restricted to the GBM side, where it completely overlaps with that of CD151 and partly with that of DG. CD151 is also present in the cytoplasm of cells and at cell surfaces other than the GBM side. Although prominently present in the cytoplasm of glomerular cells, CD63 and CD81 show hardly any overlap with  $\alpha 3\beta 1$  at the GBM side. In tubular epithelial cells, CD151 is colocalized with  $\alpha 6\beta 1$ , DG (arrow) and LU. CD81 is only present at a low level, and CD63 is mainly present throughout the cytoplasm. Note the prominent staining with anti-LU in the subendothelial matrix and with  $\alpha 6\beta 1$  present on the endothelial lining. Bar, 10  $\mu\text{m}$ .



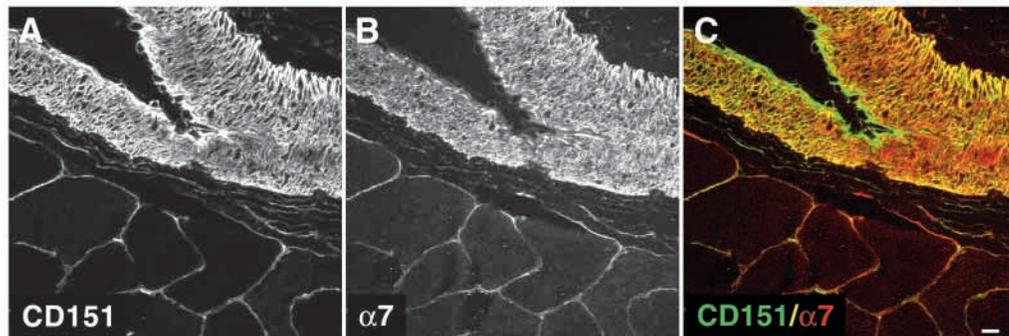
against CD63, CD81 and CD151. However, although large amounts of  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  were coprecipitated with CD151, only small amounts were coprecipitated with CD63 or CD81. This difference is not due to differences in the level of expression of the proteins, as all three tetraspanins proved to be present at substantial levels in K562 cells, as shown by FACS analysis (Yauch et al., 1998) (L.M.T.H. and A.S., unpublished). Rather, it is the result of the high stoichiometric binding of CD151 to the laminin-binding integrins, which indicates the unique and specific role of CD151 in the formation of these stable complexes. Furthermore, immunoprecipitates of CD63 and CD151 from the two transfected K562 cell lines, contained a protein of the size of CD81 (23 kDa). Because it is known that tetraspanins can interact with other tetraspanins, we suspect that CD81 is coprecipitated because it is associated with CD63 and CD151, and not, as in the case of CD151, with integrins. CD63, which migrates as a smear between 45-60 kDa on gels, could not be detected in the various

immunoprecipitations, probably because it is labeled only poorly with  $^{125}\text{I}$ . As anticipated, similar amounts of the  $\alpha 3$  and  $\alpha 6$  subunits were coprecipitated with CD151 from lysates of K562 cells transfected with the cytoplasmic A or B variants of  $\alpha 3$  or  $\alpha 6$  (not shown). Thus, the formation of CD151- $\alpha 3\beta 1$  or

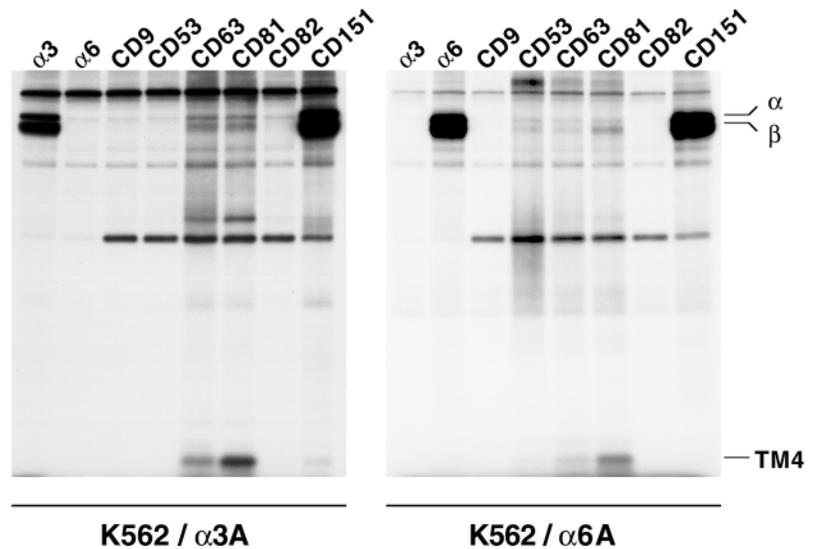
**Fig. 3.** Colocalization of CD151 with the laminin receptor  $\alpha 7\beta 1$  in smooth and skeletal muscle.

Cryostat sections of human smooth and striated muscle were processed for double immunofluorescence confocal microscopy using P48 against CD151 (A) and polyclonal antibodies against  $\alpha 7\beta 1$  (B). The composite image (C) was generated by superimposition of the green and red signals, with areas of overlap appearing as yellow.

The distribution pattern of CD151 and  $\alpha 7\beta 1$  completely overlap both at the sarcolemma of striated skeletal muscle (lower half) and on smooth muscle cells present in vessel walls (upper half). Note the prominent presence of CD151 in endothelial cells and the small amounts of CD151 in the stroma. Bar, 10  $\mu\text{m}$ .



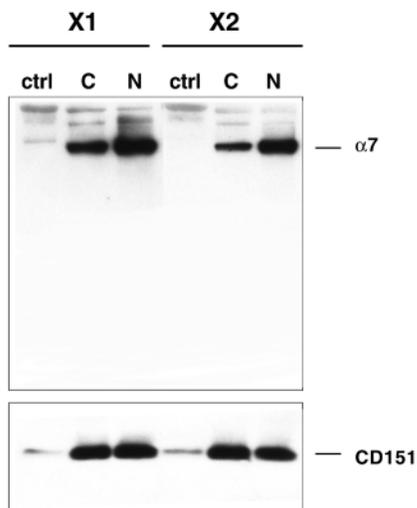
**Fig. 4.** Association of CD151 with  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  in transfected K562 cells. Transfected K562 cells stably expressing  $\alpha 3\beta 1$  or  $\alpha 6\beta 1$  were  $^{125}\text{I}$ -surface-labeled, extracted with 1% CHAPS and lysates were subjected to immunoprecipitation with antibodies. Antibodies used were the anti-integrin antibodies J143 against the  $\alpha 3$ - and J8H against the  $\alpha 6$  subunit and the anti-tetraspanin antibodies MEM62 against CD9, MEM53 against CD53, 6H1 against CD63, M38 against CD81, C33 against CD82 and 5C11 against CD151. Both  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  are present in immunoprecipitates prepared with antibodies against CD63, CD81 and CD151. However, the amount of integrin that is coprecipitated with CD151 is much greater than with the other tetraspanins, reflecting the stability and selectivity of the CD151-integrin binding. The formation of multimolecular complexes is reflected by the additional presence of CD81 (TM4) precipitated by antibodies against CD63 and CD151.



CD151- $\alpha 6\beta 1$  complexes is not influenced by the nature of the splice variants in the integrin molecules.

CD151 stably associates with the two extracellular variants (X1 and X2) of  $\alpha 7\text{B}$

Association of CD151 with either splice variant of the

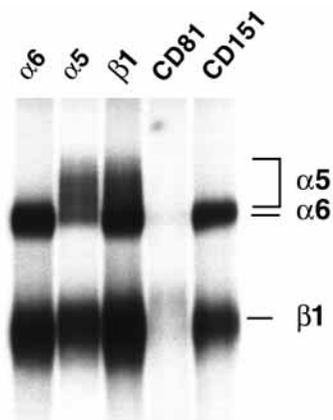


**Fig. 5.** CD151 is strongly associated with both splice variants (X1 and X2) of  $\alpha 7\text{B}\beta 1$  in transfected K562 cells. K562 cells stably expressing the  $\alpha 7\text{B}\beta 1$  extracellular splice variant X1 or X2 were lysed in 1% CHAPS (C) or 1% Nonidet P-40 (N). Lysates were subjected to immunoprecipitation with mAb P48 against CD151 (upper panel) or with polyclonal antibodies against  $\alpha 7\text{B}$  (lower panel). The presence of  $\alpha 7\text{B}$  or CD151 in the immune complexes was detected by immunoblotting with anti- $\alpha 7\text{B}$  or anti-CD151 (mAb 8C3) antibodies. No immunoreactive bands corresponding to  $\alpha 7\text{B}$  were detected in control (ctrl) immunoprecipitates (upper panel). As a further control (ctrl), the presence of CD151 in total lysates is shown (lower panel). Note that  $\alpha 7\text{B}$  is coprecipitated with CD151 and vice versa, and that this is independent of the extracellular splice variant or the lysis buffer used.

extracellular domain X1 or X2 of the laminin receptor  $\alpha 7\beta 1$  was investigated in coimmunoprecipitation experiments; the strength of the interaction was also tested by using different lysis buffers. CHAPS is considered to be a mild detergent in which weak interactions between different molecules are not disrupted, whereas Nonidet P-40 is more stringent leaving only stable complexes intact. As shown in Fig. 5 (upper panel),  $\alpha 7$  is coprecipitated with CD151 from K562 cells stably expressing either the X1 or X2 splice variant, and vice versa CD151 is co-precipitated with  $\alpha 7$  (lower panel). The  $\alpha 7$  subunit and CD151 were precipitated in comparable amounts irrespective of the lysis buffer used. Thus, CD151 not only associates with the  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  integrins but also with  $\alpha 7\beta 1$ , the strength of this interaction being comparable with that with  $\alpha 3\beta 1$ .

No influence of extracellular cleavage from the integrin  $\alpha$  subunit on CD151 binding

The extracellular domain of the integrin  $\alpha 3$ ,  $\alpha 6$  and  $\alpha 7$  subunits is cleaved during biosynthesis into a heavy and a light chain. This has been shown to be required for proper inside-out signaling by  $\alpha 6\beta 1$  in K562 cells but has no influence on ligand binding (Delwel et al., 1996). When the RKKR sequence (amino acids 876-879) is mutated to RGGR, cleavage into a heavy and a light chain cannot occur. For the formation of stable complexes of tetraspanins and integrins, their extracellular domains are essential (Yauch et al., 2000). To determine whether cleavage influences the binding of integrins to CD151, K562 cells were transfected with the  $\alpha 6\text{A}^{\text{RGGR}}$ -mutant (uncleaved). Cells were  $^{125}\text{I}$ -surface-labeled, lysed in 1% CHAPS and the lysates precipitated with antibodies against  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ , CD81 or CD151. As shown in Fig. 6,  $\alpha 6\beta 1$  could be precipitated by antibodies against  $\alpha 6$  or  $\beta 1$  as well as by antibodies against CD81 or CD151. Consistent with previous results (Fig. 4), the amount of  $\alpha 6$  precipitated by anti-CD81 is much smaller than that precipitated by anti-CD151. The  $\alpha 5\beta 1$  integrin that is endogenously present on K562 cells is only precipitated by antibodies against  $\alpha 5$  or  $\beta 1$ , not by antibodies against either of the two tetraspanins, CD81 or CD151.



**Fig. 6.** The absence of cleavage of the integrin  $\alpha 6$  subunit on the association with CD151 in transfected K562 cells has no effect. Transfected K562 cells, stably expressing  $\alpha 6A^{RGGGR}\beta 1$  (uncleaved  $\alpha 6A$ ), were  $^{125}I$ -surface-labeled, lysed in 1% CHAPS and immunoprecipitated with anti-integrin subunit or anti-tetraspanin antibodies. Antibodies used for immunoprecipitation were J8H directed against  $\alpha 6$ , Sam-1 against  $\alpha 5$ , TS2/16 against  $\beta 1$ , M38 against CD81 and 5C11 against CD151. Note that  $\alpha 6A^{RGGGR}\beta 1$  is coprecipitated with CD151 and to a lesser extent with CD81. The integrin  $\alpha 5\beta 1$  is not coprecipitated with tetraspanins; the typical smear around 150 kDa representing  $\alpha 5$  is not detected in the precipitates obtained with antibodies against these molecules.

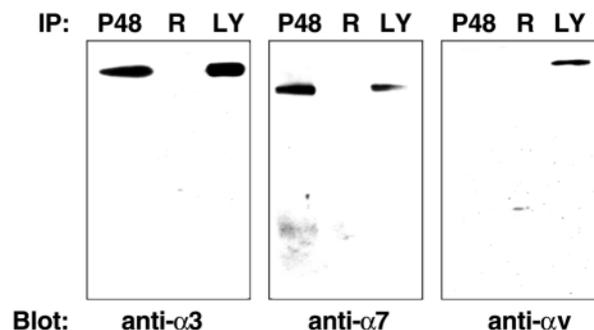
#### Availability of the CD151 epitope recognized by antibody TS151R in vitro and in vivo

TS151R is a monoclonal antibody directed against an epitope on CD151 that is located at the binding site for  $\alpha 3$ , whereas P48 recognizes an epitope outside this binding site (Serru et al., 1999; Yauch et al., 2000). To investigate whether the CD151 epitope recognized by TS151R is also involved in the binding to  $\alpha 7\beta 1$ , lysates of K562 cells stably expressing the integrin  $\alpha 7\beta 1$  were subjected to immunoprecipitation with mAb TS151R, and the presence of  $\alpha 7$  was tested by immunoblotting. Immunoprecipitations prepared from lysates of K562 cells that were transfected with cDNAs encoding the  $\alpha 3$ - or  $\alpha v$ -subunits served as positive and negative controls, respectively. As shown in Fig. 7, the  $\alpha 3$  and  $\alpha 7$  integrins were only precipitated by P48 and not by TS151R. The  $\alpha v\beta 1$  integrin was not precipitated by either of the antibodies. Thus,

**Table 1. Differences in the recognition of CD151 by P48 compared to TS151R**

| Tissue     | Laminin-binding integrin | Reactivity with antibody |        |
|------------|--------------------------|--------------------------|--------|
|            |                          | P48                      | TS151R |
| Kidney     |                          |                          |        |
| Glomerulus | $\alpha 3\beta 1$        | +++                      | ±      |
| Tubules    | $\alpha 6\beta 1$        | ++                       | ++     |
| Skin       | $\alpha 3\beta 1$        | +++                      | -      |
|            | $\alpha 6\beta 4$        | +++                      | ±      |
| Muscle     |                          |                          |        |
| Smooth     | $\alpha 7\beta 1$        | +++                      | +++    |
| Striated   | $\alpha 7\beta 1$        | ++                       | -      |
| Heart      | $\alpha 7\beta 1$        | +++                      | ±      |

Reactivity of various tissues with CD151-specific antibodies; +++, very strong staining; ++, strong staining; ±, weak staining; -, no staining.



**Fig. 7.** Monoclonal antibody TS151R does not recognize CD151 when it is bound to  $\alpha 3\beta 1$  or  $\alpha 7\beta 1$ . Transfected K562 cells stably expressing  $\alpha 3\beta 1$  (left),  $\alpha 7\beta 1$  (middle) or  $\alpha v\beta 1$  (right) were lysed in 1% Nonidet P-40 and subjected to immunoprecipitation with the mAbs P48 or TS151R, followed by immunoblotting with the indicated polyclonal antibodies against  $\alpha 3$ ,  $\alpha 7$  or  $\alpha v$ . Immunoblot analysis of the lysates (LY) show the level of expression of each integrin.

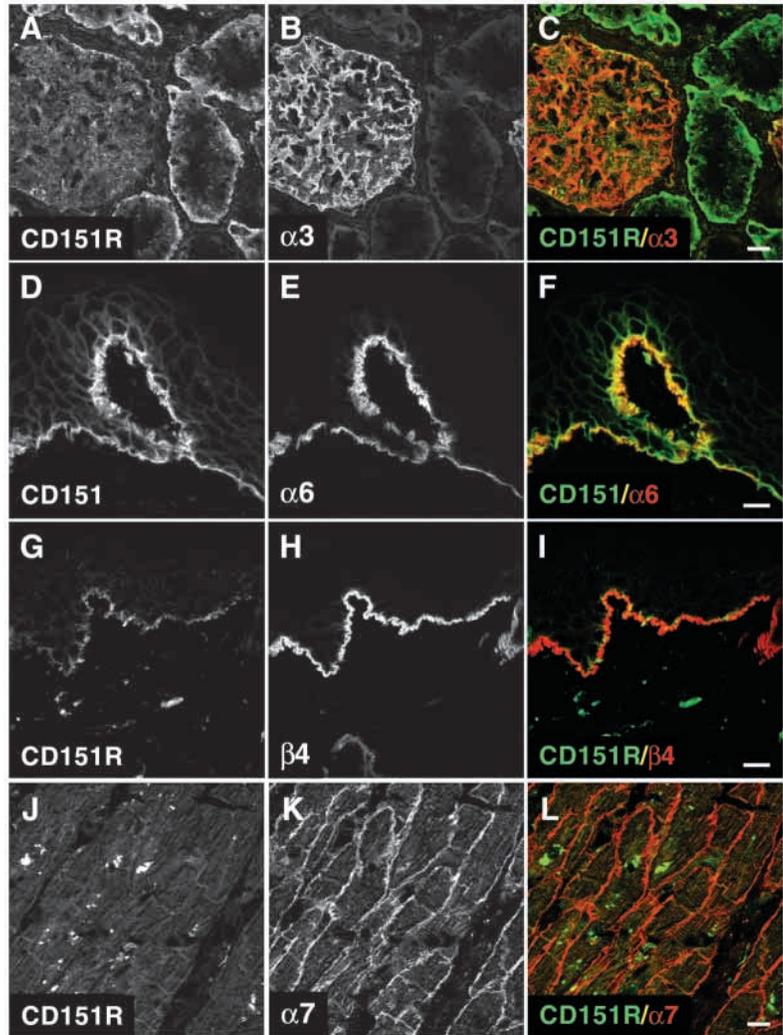
the TS151R epitope is not only masked when CD151 is associated with  $\alpha 3\beta 1$  but also when it is bound to  $\alpha 7\beta 1$ .

Next we compared the distribution of the CD151 epitope recognized by TS151R with that by P48 (Table 1). Kidney sections incubated with TS151R (Fig. 8A,C) show hardly any fluorescent staining in the glomerular cells that express  $\alpha 3\beta 1$  (Fig. 8B,C), whereas in the tubuli expressing  $\alpha 6\beta 1$ , staining is comparable to that of mAb P48. In the skin, where CD151 is not only associated with  $\alpha 3\beta 1$  but also with  $\alpha 6\beta 4$  (Fig. 8E,F,H,I), the staining by TS151R (Fig. 8G,I) is weak compared with that by mAb P48 (Fig. 8D,F). Staining of the different muscle tissues expressing  $\alpha 7\beta 1$  varied: all three muscle tissues were strongly stained by P48 (Fig. 1C, Fig. 9A,B), smooth muscle cells were also strongly stained by TS151R (Fig. 9C), cardiomyocytes only weakly at the intercalated discs (Fig. 8J,L), and striated muscle cells were not stained at all by this antibody (Fig. 9D). Staining of cardiomyocytes for  $\alpha 7$  expression is shown in Fig. 8K,L. For further comparison and the possible recognition of CD151 epitopes involved in the binding of integrins, all the above tissues were incubated with a panel of other antibodies against CD151. This resulted in staining patterns comparable to those produced by P48 (not shown). Thus, binding of CD151 to laminin-binding integrins leads to masking of the TS151R epitope in many, but not all, types of cells in vivo.

#### Level of CD151 cell surface expression is integrin dependent

Previously it has been shown that expression of  $\alpha 3\beta 1$  in K562 cells results in an increase in the surface expression of CD151 (Yauch et al., 1998). To determine whether this ability of  $\alpha 3\beta 1$  to induce CD151 expression is a general property of laminin-binding integrins, the surface expression of CD151 was assessed by flow cytometry (FACSscan) of transfected K562 cells stained with mAb P48. As shown in Fig. 10, the expression of transfected  $\alpha v$  had little or no effect on the expression of CD151. However, expression of  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha 7X1\beta 1$  or  $\alpha 7X2\beta 1$  in K562 cells increased the expression of CD151 three- to sixfold. Staining of the cells with TS151R revealed that the CD151 epitope, which is

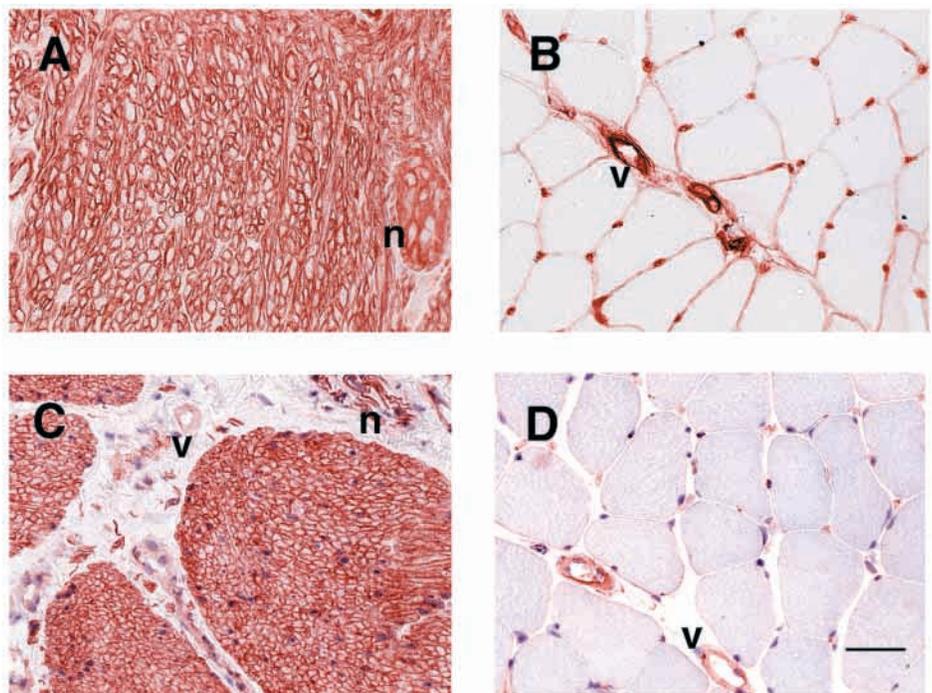
**Fig. 8.** Reactivity of CD151 with antibody TS151R is cell type specific. Cryostat sections of human kidney, skin and heart muscle were processed for double immunofluorescence confocal microscopy using TS151R (A,G,J) and P48 (D) against CD151, GoH3 against  $\alpha 6$  (E), 439-9B against  $\beta 4$  (H) and polyclonal antibodies against  $\alpha 3$  (B) or  $\alpha 7$  (K). The composite images (C,F,I, L) were generated by superimposition of the green and red signals, with areas of overlap appearing as yellow. TS151R against CD151 reacts with CD151 in tubular epithelial cells but hardly reacts in glomerular cells (A-C), keratinocytes (G-I) and cardiomyocytes (J-L). P48 against CD151 is used in the skin for comparison and shows a prominent staining especially at the dermo-epidermal junction (D-F). Bar, 10  $\mu$ m.



recognized by this antibody, is masked, and it is not dependent on the type of laminin-binding integrin that the K562 cells were transfected with. These results are consistent with the results of the above biochemical study showing that the TS151R epitope is not expressed on K562 cells expressing different laminin-binding integrins. However, also no CD151 was detected with TS151R on untransfected K562 cells. We therefore cannot exclude non-reactivity of this antibody in fluorescence studies, because of low affinity when the number of available epitopes is restricted. Furthermore, the results indicate that the surface levels of CD151 on cells are strongly influenced by the expression of the laminin-binding integrins.

#### Lack of complex formation between CD151 and the Lutheran molecule or dystroglycan

The colocalization of CD151 with the different kinds of laminin receptors, DG, LU and integrins, may suggest a role for CD151 in linking these three kinds of laminin receptors to each other. To investigate whether CD151, DG and LU are not only colocalized but also complexed, cultured human podocytes and proximal tubular epithelial cells (PTEC) were used for immunoprecipitation studies (Fig. 11). FACS analysis showed that both cell lines express  $\alpha 3\beta 1$ , CD151, DG



**Fig. 9.** Immunohistochemical staining of smooth and striated muscle tissue with P48 and TS151R against CD151. Cryostat sections of human smooth (A,C) and striated muscle (B,D) were stained by immunoperoxidase reaction with P48 (A,C) or TS151R (B,D). Both types of muscle cells were strongly stained by P48. Intense staining of CD151 by TS151R was only seen in smooth muscle cells, no staining is found in skeletal muscle. n, nerve; v, vessel. Bar, 10  $\mu$ m.

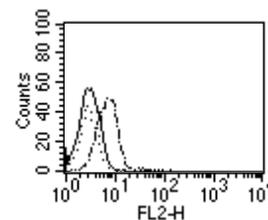
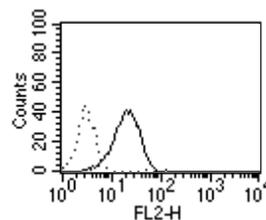
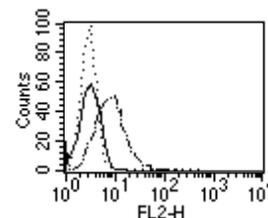
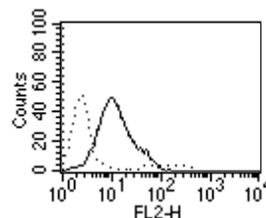
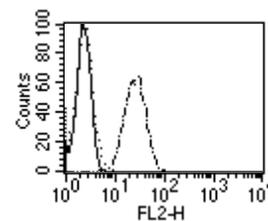
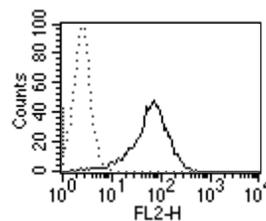
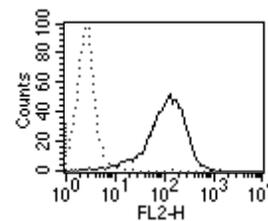
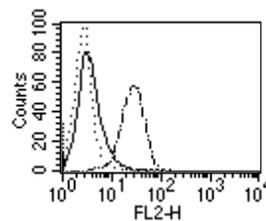
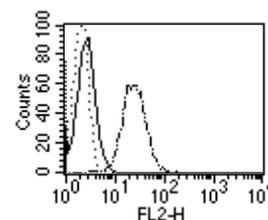
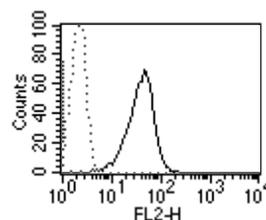
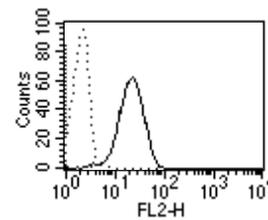
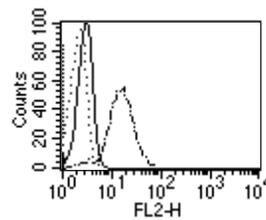
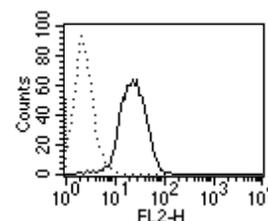
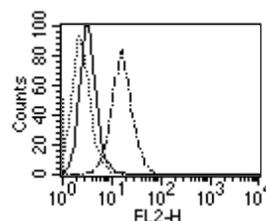
and LU, although LU is only weakly expressed by podocytes. These expression profiles correspond with those of podocytes and PTECs in vivo. Lysates from cells prepared with 1% CHAPS were incubated with antibodies against  $\alpha 3$ , CD151, LU or  $\beta$ -DG. Subsequently the presence of  $\alpha 3$  (light chain, 30 kDa) or  $\beta$ -DG (43 kDa) in the precipitates was assessed by immunoblotting using specific antibodies. Protein bands corresponding to  $\alpha 3$  could be detected on the immunoblots of the immunoprecipitates containing  $\alpha 3\beta 1$  or CD151 from both cell lysates (left panels). LU or DG could not be coprecipitated with  $\alpha 3\beta 1$  with any of the cell lysates. P48 against CD151 and antibodies against  $\alpha 3$  or LU did not coprecipitate DG (right panels) either. This demonstrates that in the tested cell lines, CD151 bound to the laminin-binding integrins does not associate with the other laminin receptors LU or DG and therefore cannot serve as a link between them.

### Discussion

In a former study we have shown that CD151 is associated with  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  in the basal keratinocyte layer of the skin and in hemidesmosomes (Sterk et al., 2000). Now we have investigated the association of CD151 with various laminin receptors in kidney, skin and muscle tissues selected for the laminin-binding integrins they express.

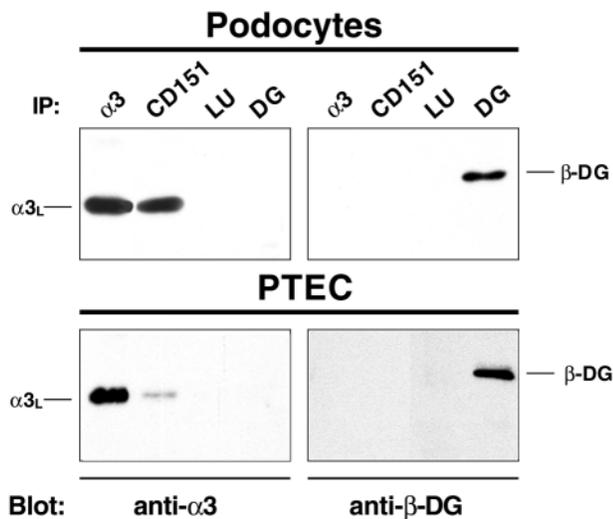
As indicated by this study, there are different distribution patterns of CD151. In the first pattern most of the CD151 is colocalized with the laminin-binding integrins present and thus is mainly expressed at the site of cell-matrix interactions where there is a basement membrane

**Fig. 10.** Expression of laminin-binding integrins on transfected K562 cells increases CD151 cell surface expression. Flow cytometric analysis of wild-type K562 cells endogenously expressing  $\alpha 5\beta 1$  and transfected K562 also expressing  $\alpha v\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 6\beta 4$ ,  $\alpha 7X1\beta 1$  or  $\alpha 7X2\beta 1$ . Solid lines in the left panels indicate staining with anti-integrin antibodies: anti- $\alpha 5$  mAb Sam-1 (K562 cells), anti- $\alpha v$  mAb 13C2 (K562- $\alpha v$ ), anti- $\alpha 3$  mAb J143 (K562- $\alpha 3$ ), anti- $\alpha 6$  mAb GoH3 (K562- $\alpha 6$ ), anti- $\beta 4$  mAb 439-9B (K562- $\alpha 6\beta 4$ ) and anti- $\alpha 7$  mAb CA25 (K562- $\alpha 7BX1$  and K562- $\alpha 7BX2$ ). Staining of cells with anti-CD151 antibodies is shown in the right panels. Solid lines represent staining of cells with mAb TS151R and broken lines with mAb P48. A negative control (dotted line) with secondary goat anti-mouse or rat IgG alone is shown in each panel. R represents the ratio of the mean fluorescence intensity of cells stained with mAb P48 and with secondary antibody alone. A three- to sixfold increase of CD151 expression is observed in transfected cells that ectopically express laminin-binding integrins. The TS151R epitope is masked in all cells expressing one of the laminin-binding integrins.

K562- $\alpha 5$ K562- $\alpha v$ K562- $\alpha 3$ K562- $\alpha 6$ K562- $\alpha 6\beta 4$ K562- $\alpha 7BX1$ K562- $\alpha 7BX2$ 

anti-integrin

anti-CD151



**Fig. 11.** No complexes are formed between dystroglycan or the Lutheran blood group molecule and CD151- $\alpha 3\beta 1$  complexes in human podocytes and proximal tubular epithelial cells in culture. Human podocytes (upper panels) and proximal tubular epithelial cells (PTEC) (lower panels) expressing the laminin receptors  $\alpha 3\beta 1$ , dystroglycan (DG) and Lutheran blood group antigens (LU) were lysed in 1% CHAPS and precipitated with polyclonal antibodies against  $\alpha 3$  or DG (AP83) and mAbs against CD151 (P48) or LU (LU4F2). The immunoprecipitates were analyzed by immunoblotting for the presence of  $\alpha 3$  and DG using specific antibodies. Note that DG is not coprecipitated with  $\alpha 3\beta 1$  or CD151 and that  $\alpha 3\beta 1$  is not seen in the precipitates obtained by antibodies against LU or DG.  $\alpha 3_L$ ,  $\alpha 3$  light chain;  $\beta$ -DG,  $\beta$ -dystroglycan.

containing at least one laminin isoform (Miner et al., 1997; Aumailley and Rouselle, 1999). Thus, in glomerular cells, CD151 and  $\alpha 3\beta 1$  are colocalized at the GBM side of cells. In renal tubular epithelial cells, CD151 and  $\alpha 6\beta 1$  are concentrated at the tubular basement membrane, and in skin CD151 and  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  are present at the dermo-epidermal junction. Finally in myocytes, CD151 together with  $\alpha 7\beta 1$  is expressed at the sarcolemma. The second pattern is seen in cells such as endothelial and most hematopoietic cells in which the amount of laminin-binding integrins is limited. As shown by others, these cells contain, in addition to a considerable pool of unbound CD151, CD151 that is weakly associated with  $\alpha 5\beta 1$  and other surface molecules (Hasegawa et al., 1998; Fitter et al., 1999; Sincock et al., 1999). The third distribution pattern is seen in fibroblasts (stromal cells) and K562 cells, which contain CD151 but no laminin-binding integrins.

In addition to the previously reported association between CD151 and the laminin-binding integrins  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$ , we demonstrate here that CD151 also interacts with  $\alpha 7\beta 1$ . Like CD151- $\alpha 3\beta 1$  complexes, CD151- $\alpha 7\beta 1$  complexes remained intact after Nonidet P-40 lysis of transfected K562 cells, which classifies their interaction as level 1. Also using transfected K562 cells, it was shown that CD151- $\alpha 6\beta 1$  and CD151- $\alpha 6\beta 4$  (Sterk et al., 2000) interact at level 2, the interaction not being resistant to Nonidet P-40 treatment. A correlation between the presence of the laminin-binding integrins and CD151 expression was established by FACS analysis, which showed an increase of CD151 expressed at the

cell surface of K562 cells after transfection of either of the laminin-binding integrins.

CD151 does not link the different laminin receptors with each other

Two non-integrin receptors for laminin are DG and LU. DG was originally isolated from skeletal muscle as a component of the large oligomeric DGC, which amongst other proteins contains sarcospan and dystrophin (Durbeej and Campbell, 1999). The third type of laminin receptor is the molecule expressing the LU antigens, which is able to bind to the laminin  $\alpha 5$  chain present in the subendothelial matrix (Parsons et al., 2001).

Our *in vivo* results show a considerable overlap in the distribution of these different types of laminin receptors. Hence, a tight regulation and precise coordination of the signaling mechanisms by the different receptors seems essential. An example of an interaction between the different laminin receptors is shown in glomerulogenesis, during which a switch occurs in expression of the laminin-binding integrins from  $\alpha 6\beta 1$  to  $\alpha 3\beta 1$  simultaneously with a switch in the deposition of laminin isoforms, that is, from laminin-1 to laminin-11 (Sterk et al., 1998). Others have shown that the symptoms induced by the absence of dystrophin, which disturb the function of the laminin receptor DG, can be partly compensated by increasing the expression of  $\alpha 7\beta 1$  (Burkin et al., 2001). Moreover, myocytes of patients with Duchenne's disease lacking dystrophin and of dystrophin-deficient mice express an increased amount of  $\alpha 7\beta 1$  (Hodges et al., 1997). This indicates that when one laminin receptor is lacking, another laminin receptor can (partially) compensate for it. A protein present in the DGC that is homologous to tetraspanins is sarcospan (Crosbie et al., 1999). Although sarcospan is a component of the DGC and has a function in maintaining the integrity of muscle tissue, sarcospan-deficient mice have only mild symptoms of muscular atrophy (Lebakken et al., 2000). Moreover, epithelial cells expressing DG do not contain sarcospan (Durbeej and Campbell, 1999). These data suggest that in sarcospan-deficient mice and epithelia expressing DG, other tetraspanins may compensate for the absence of sarcospan. We demonstrate that, except in the skin, in all tissues tested, DG, the laminin-binding integrins and CD151 are co-distributed. Furthermore, this study shows that CD151 is the only known tetraspanin in the glomerulus that is present at the side of the basement membrane, together with DG. Therefore and analogous to the sarcolemma, the sarcospan function in the glomerulus could be taken over by CD151, thereby connecting the integrin family with the DGC. However, despite their co-distribution, coimmunoprecipitation studies could not demonstrate a physical association between  $\alpha 3\beta 1$ -CD151 complexes and DG or LU in two different cell lines obtained from the kidney, that is, human podocytes and proximal tubular epithelial cells.

Neither the variants of the extracellular or cytoplasmic domains of integrin  $\alpha$  subunits nor the cleavage of  $\alpha 6$  affect binding of CD151 to integrins

The binding between tetraspanins and integrins is established via the extracellular domains of the tetraspanin and the integrin  $\alpha$  subunit (Yauch et al., 2000). Theoretically, splicing of the mRNA for the extracellular or cytoplasmic domains of integrin

subunits or maturation-induced cleavage of the  $\alpha$  chain could influence the association of tetraspanins with integrins and thereby alter integrin signaling. However, biochemically no such differences could be demonstrated for the cytoplasmic A and B variants of the integrin  $\alpha 3$  and  $\alpha 6$  subunits or for the extracellular variants X1 and X2 of  $\alpha 7$ . Furthermore, mutational changes in the extracellular domain of  $\alpha 6$  preventing cleavage seemed to have no influence on the formation of a complex with CD151. This implies that switches of splice variants as described during development (Collo et al., 1993; Thorsteindóttir et al., 1995; Sterk et al., 1998) or cleavage of the  $\alpha$  subunit during maturation have no effect on CD151-integrin binding.

#### All laminin-binding integrins may bind to a single site on CD151

The reactivity of CD151 with TS151R and P48 in immunohistochemistry indicates that masking of the TS151R epitope detected in the biochemical experiments and FACS analysis also occurs in vivo. Glomerular cells expressing  $\alpha 3\beta 1$ , basal keratinocytes expressing  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  and cardiac and skeletal muscle cells expressing  $\alpha 7\beta 1$  either did not or only weakly react with TS151R, although these cell types were strongly stained by mAb P48. A likely interpretation of this finding is that in these tissues most if not all of the CD151 is bound to a laminin-binding integrin, leading to masking of the TS151R epitope. However, renal tubular and smooth muscle cells, which express  $\alpha 6\beta 1$  and  $\alpha 7\beta 1$ , respectively, were stained equally well by the two antibodies. The reason for this observation is not clear, but there are several explanations. First, the strength of the interaction between CD151 and integrins might be regulated from within the cell and/or by the microenvironment, by which the tightness of the binding may be influenced and thus the exposure of the TS151R epitope. Second, CD151 may not directly interact with one of the laminin-binding integrins but indirectly by forming a tetraspanin web with other molecules. In fact, different binding sites have recently been described on CD151 for tetraspanins and integrins (Berditchevski et al., 2001). Third, CD151, in addition to being complexed with a laminin-binding integrin, could also be bound to other cell surface molecules, as has been described for different tetraspanins and MHCII, DRAP27 and HB-EGF (Angelisova et al., 1994; Nakamura et al., 1995). This binding should, in that case, involve another part of the binding site than that which interacts with integrins. Fourth, in kidney sections from HIV patients, we found that the amount of CD151 reactive with TS151R is greatly increased in the glomeruli and comparable with that reactive with P48 (L.M.T.S., unpublished). This indicates that under certain pathophysiological conditions the TS151R epitope on CD151, which is normally complexed with  $\alpha 3\beta 1$ , becomes exposed. Finally, a difference in affinities of the two antibodies for CD151, as suggested by our FACS data, may have influenced their reactivity.

In summary, we demonstrate extensive co-distribution of various kinds of laminin receptors in different tissues. We confirm previous findings that CD151 binds to the laminin-binding integrins  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  and show for the first time that it also interacts with  $\alpha 7\beta 1$ . CD151 does not bind to the laminin receptors DG or LU. Moreover, we show that variants

of the cytoplasmic or extracellular domains bind equally well to CD151 and that cleavage of the integrin  $\alpha$  subunits does not alter their binding. Studies with the mAb TS151R, which has previously been shown to recognize an epitope at the binding site of CD151 with  $\alpha 3\beta 1$ , indicate that the same site is involved in the binding to  $\alpha 7\beta 1$  and possibly also to  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$ . The finding that in immunohistochemical studies few cells and tissues, irrespective of the laminin-binding integrins they express, reacted with TS151R, suggests that the strength of interaction of CD151 is subject to regulation either from within the cell and/or by the cell's environment.

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