Interactions of primary fibroblasts and keratinocytes with extracellular matrix proteins: contribution of $\alpha_2\beta_1$ integrin

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Accepted 25 January 2006
Journal of Cell Science 119, 1886-1895 Published by The Company of Biologists 2006
doi:10.1242/jcs.02921

Summary

The $\alpha_2\beta_1$ integrin is a collagen-binding protein with very high affinity for collagen I. It also binds several other collagens and laminins and it is expressed by many cells, including keratinocytes and fibroblasts in the skin. In the past, $\alpha_2\beta_1$ integrin was suggested to be responsible for cell attachment, spreading and migration on monomeric collagen I and contraction of three-dimensional collagen lattices. In view of these functions, normal development and fertility in integrin α_2-deficient mice, which we generated by targeting the integrin α_2 gene, came as a surprise. This suggested the existence of compensatory mechanisms that we investigate here using primary fibroblasts and keratinocytes isolated from wild-type and α_2-deficient mice, antibodies blocking integrin function and downregulation of integrin α_2 expression. The results show that the $\alpha_2\beta_1$ integrin is absolutely required for keratinocyte adhesion to collagens whereas for fibroblasts other collagen-binding integrins partially back-up the lack of $\alpha_2\beta_1$ in simple adhesion to collagen monomers. A prominent requirement for $\alpha_2\beta_1$ integrins became apparent when fibroblasts executed mechanical tasks of high complexity in three-dimensional surroundings, such as contracting free-floating collagen gels and developing isometric forces in tethered lattices. The deficits observed for α_2-deficient fibroblasts appeared to be linked to alterations in the distribution of force-bearing focal adhesions and deregulation of Rho-GTPase activation.

Key words: Fibroblast, Keratinocyte, Adhesion, Migration, Force, RhoGTPase

Introduction

The collagen-rich matrix of the skin provides a mechanical scaffold for cell adhesion and a source of biological cues controlling cell behavior and signaling. Earlier studies with tissue-extracted collagens found in the skin have demonstrated that collagen I (Grinnell and Bennett, 1981; Mauch et al., 1986; Santoro, 1986), collagen IV (Aumailley and Timpl, 1986), collagen V (Ruggiero et al., 1994) and collagen VI (Aumailley et al., 1989; Pfaff et al., 1993) are excellent cell-adhesion substrates. Cellular interactions with these collagens under native conformation are mediated by integrins of the β_1 family, including the rather ubiquitous $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (Wayner and Carter, 1987; Takada et al., 1988; Kramer and Marks, 1989) and the more recently discovered $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$ with more restricted distribution (Camper et al., 1998; Velling et al., 1999). These integrins belong to a subclass of the β_1 family characterized by an inserted domain (I domain) with homology to von Willebrand factor A domain and interacting with the ligand (Emsley et al., 2000; White et al., 2004). In vitro studies with purified integrins or their recombinant I domain and collagen monomers showed that $\alpha_1\beta_1$ integrin has higher affinity for collagen IV than collagen I and, conversely, $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins bind collagen I more efficiently than collagen IV (Kern et al., 1993; Tiger et al., 2001; Tulla et al., 2001). Collagens are, however, not the only extracellular matrix (ECM) ligands for $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins and both bind laminin 1 (Elices and Hemler, 1989; Goodman et al., 1991; Pfaff et al., 1994; Ettner et al., 1998; Colognato et al., 1997). Additional interactions between $\alpha_1\beta_1$ integrin and matrilin-1 (Makihira et al., 1999) and between $\alpha_2\beta_1$ integrin and decorin (Guidetti et al., 2002), chondroadherin (Camper et al., 1997) and laminin 5 (Orian-Rousseau et al., 1998; Decline and Rousselle, 2001) have been reported.

Integrins are heterodimeric cell-surface receptors transducing outside-in and inside-out biological and mechanical cues between the ECM and the cell machinery, thereby regulating diverse cellular activities such as adhesion, migration, differentiation, apoptosis and expression of specific genes. Integrins execute these functions by establishing a physical link between the ECM and the actin cytoskeleton at specific membrane locations, the focal adhesions (Ingber, 1997; Grinnell, 2003; Danen and Sonnenberg, 2003; Humphries et al., 2004). The dynamics of both the actin cytoskeleton and focal adhesions is under the control of binary molecular switches of the Rho subfamily of small GTPases, in

particular the trio RhoA, Rac1 and Cdc42 (Hall, 1992). By switching between soluble, inactive, GDP-bound and membrane-anchored, active conformations, RhoA, Rac1 and Cdc42 control actin polymerization and hence the cytoskeleton stiffness, allowing it to modulate and counterbalance forces applied to, or generated by cells during movement or matrix remodeling (Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1996; Nobes and Hall, 1999; Wiesner et al., 2005).

Based on cell culture experiments, it has been suggested that optimal fibroblast proliferation requires $\alpha_1\beta_1$ integrins (Pozzi et al., 1998) whereas $\alpha_2\beta_1$ integrins are involved in several aspects of matrix remodeling, including formation of collagen fibrils (Klein et al., 1991; Li et al., 2003; Jokinen et al., 2004), contraction of collagen matrices (Schiro et al., 1991; Langholz et al., 1995; Jenkins et al., 1999) and induction of collagenase activity (Schiro et al., 1991; Langholz et al., 1995), which agrees with upregulation of the latter during scar contraction (Wu et al., 2003). In addition, both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins regulate collagen synthesis, but in opposite ways (Langholz et al., 1995; Riikonen et al., 1995). Less is known about $\alpha_{11}\beta_1$ integrin function. It may also regulate collagen fibril organization as well as fibroblast migration (Tiger et al., 2001; Popova et al., 2004). Thus $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins may partially compensate each other, as underscored by the subtle phenotypes of mice with targeted deletion of either α_1 or α_2 subunits (Gardner et al., 1996; Holtkötter et al., 2002; Chen et al., 2002). Indeed, skin fibroblasts co-express $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins (Voigt et al., 1995; Wu and Santoro, 1996; Tiger et al., 2001) and the contribution of each integrin to interactions with different ECM ligands may overlap. By contrast, the repertoire of collagen-binding integrins expressed by keratinocytes is restricted to $\alpha_2\beta_1$ (Watt, 2002). Using primary fibroblasts and keratinocytes isolated from wild-type and α_2-deficient mice, integrin function-blocking antibodies and downregulation of integrin α_2 expression by small-interfering RNAs (siRNAs), we have evaluated the contribution of $\alpha_2\beta_1$ integrin when cells are performing tasks of different complexity, ranging from simple adhesion to a planar surface of ECM proteins to generation of forces in three-dimensional (3D) surroundings.

Results

The $\alpha_2\beta_1$ integrin is dispensable for fibroblast adhesion to collagen I

Dermal fibroblasts isolated from wild-type and α_2-deficient mice and grown as cell monolayers on rigid tissue culture support did not reveal any obvious differences under phase-contrast microscopy (not shown). The two cell strains were compared for adhesion to different ECM proteins. In 30-minute-long assays, both type of fibroblasts adhere equally well to laminin 1, laminin 5 and collagen IV, whereas adhesion of α_2-deficient fibroblasts to monomeric collagen I was about 35% lower than that of wild-type counterparts (Fig. 1A). Examination of cell spreading on different ECM substrates by phase contrast microscopy showed no obvious difference between α_2-deficient and wild type fibroblasts (shown for collagen I, Fig. 1B,C). To get independent evidence that $\alpha_2\beta_1$ integrins are dispensable for fibroblast adhesion to collagen I, we used RNA interference to downregulate integrin α_2 subunit in wild-type mouse fibroblasts. Immunoblotting detection of integrin α_2 subunit in cell lysates showed that two (siRNA2 and siRNA3) out of three tested siRNAs induced a gradual decrease of integrin α_2 subunit expression over time to reach a minimum (70% of the control) 76 hours post-transfection (not shown). More pronounced downregulation of integrin α2 subunit was obtained by transfecting the fibroblasts concomitantly with siRNA2 and siRNA3 once (Fig. 2A, downregulation to 26% of control level) or twice successively (Fig. 2B, downregulation to 19% of control level). Under these conditions, adhesion of the siRNA-transfected fibroblasts to collagen I was decreased proportionally to the downregulation of the integrin subunit (Fig. 2, compare adhesion curves A and B), with a maximal decrease of 30% compared with controls following two successive rounds of transfection (Fig. 2B). Thus downregulation of integrin α_2 subunit by at least 90% impaired adhesion of fibroblasts to collagen I monomers by only 30%, consistent with the adhesion results obtained with fibroblasts lacking the integrin α_2 subunit.

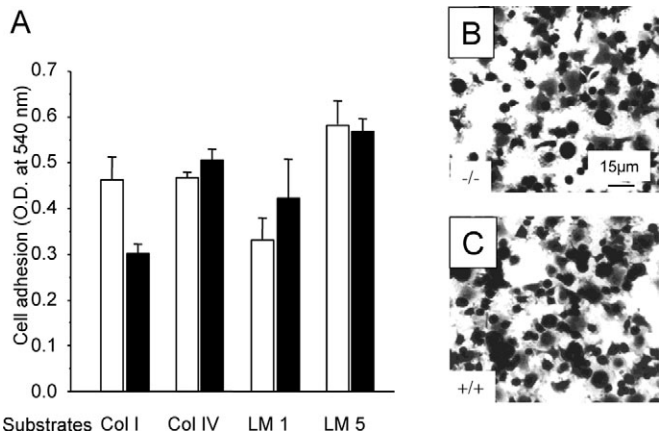

Fig. 1. Adhesion of wild-type and α_2-deficient fibroblasts to extracellular matrix proteins. (A) Equal numbers of wild-type (white columns) and α_2-deficient (black columns) fibroblasts were seeded on triplicate wells coated with optimal concentrations of collagen I (Col I, 40 μg/ml), collagen IV (Col IV, 10 μg/ml), laminin 1 (LM 1, 20 μg/ml) and laminin 5 (LM 5, 5 μg/ml) as indicated. After 30 minutes, adhesion of α_2-deficient fibroblasts to collagen I is slightly lower than that of wild-type cells. The mean average of triplicate wells and s.d. are shown. (B,C) At the end of the adhesion assay, wild type (+/+) and integrin α_2-deficient (–/–) fibroblasts adhering to collagen I were photographed under phase-contrast microscopy.

As collagen-binding integrins co-expressed by fibroblasts may not come into play simultaneously, adhesion of fibroblasts from α_2-knockout mice, characterized by complete absence of the α_2 subunit, were investigated in more detail by varying the length of the assay and the concentration of coated collagen I (Fig. 3). In short-term assays (15 minutes), adhesion of α_2-deficient fibroblasts was markedly reduced at each concentration of collagen compared with wild-type cells (Fig. 3, 15 minutes). In longer assays (30 and 60 minutes), adhesion of mutant cells to collagen I was decreased by about 30% compared with wild-type fibroblasts (Fig. 3). Together these results indicate that $\alpha_2\beta_1$ integrins are important in the very early steps of fibroblast adhesion to collagen I and that later on other receptors are involved and partially compensate for the lack of $\alpha_2\beta_1$ integrins.

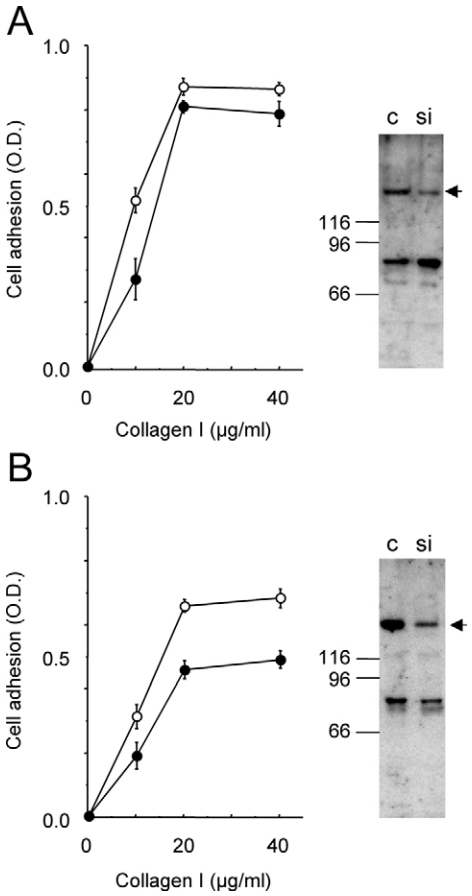

Fig. 2. Adhesion of mouse fibroblasts to collagen I after siRNA downregulation of integrin α_2 expression. Subconfluent wild-type mouse fibroblasts were transfected once (A) or twice (B) with 100 nM each siRNA2 and siRNA3 targeting integrin α_2 subunit or an unrelated siRNA as described in the Materials and Methods. Graphs show adhesion assays (30 minutes) to increasing concentrations of collagen I performed 76 hours after a single (A) or two successive (B) transfections. Equal numbers of control (unrelated siRNA; open symbols) and specific siRNA-transfected (α_2-specific; closed symbols) fibroblasts were seeded on the collagen coats. The mean average of adherent cells in triplicate wells and s.d. are shown. Equal aliquots of fibroblasts transfected with control (c) and specific siRNAs (si) were lysed for immunoblotting analysis of integrin α_2 subunit expression. The blots shown to the right of the graphs correspond to the fibroblasts used for the adhesion assays (arrow indicates the position of integrin α_2 subunit). Densitometry measurement of band intensity in the blots shown in A and B indicate that expression of the α_2 subunit is downregulated to 26% and 19%, respectively, of the corresponding controls. Molecular size markers are indicated in kDa on the left of the blots.

The $\alpha_1\beta_1$ integrin participates in fibroblast adhesion to collagen I, collagen IV and laminin 1

The contribution of $\alpha_1\beta_1$ integrins to fibroblast adhesion to different ECM proteins was examined by using specific integrin function-blocking antibodies. Adhesion of wild-type and α_2-deficient fibroblasts to collagen I, collagen IV, laminin 1 and laminin 5 was inhibited by antibodies against integrin β_1 subunit (Fig. 4), indicating that only integrins of the β_1 family mediate adhesion of primary fibroblasts to the ECM proteins

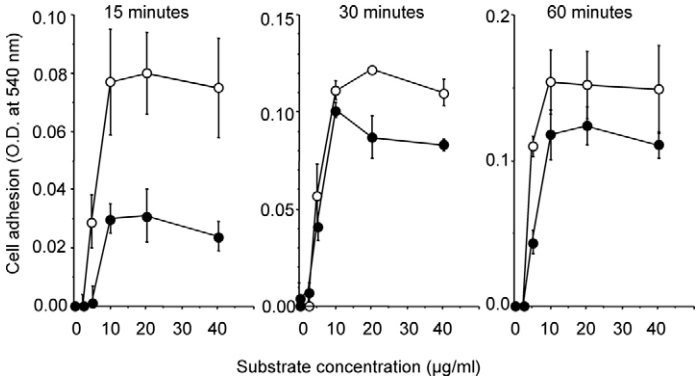

Fig. 3. The $\alpha_2\beta_1$ integrin is important for initial fibroblast adhesion to collagen I. Equal numbers of wild-type (open circles) and α_2-deficient (closed circles) fibroblasts were seeded on triplicate wells coated with increasing concentrations of collagen I and for different periods of time as indicated. After 15 minutes, adhesion of α_2-deficient fibroblasts to collagen I was markedly decreased compared with normal cells and partially rescued at longer incubation times. The means ± s.d. of triplicate wells are shown.

tested. Antibodies against the integrin α_2 subunit had no or marginal effects on wild-type or α_2-deficient fibroblast adhesion to all four substrates (Fig. 4). By contrast, function-blocking antibodies against integrin α1 subunit completely inhibited adhesion of wild-type and α_2-deficient fibroblasts to collagen IV and laminin 1 (Fig. 4), indicating that $\alpha_1\beta_1$ integrin is the major receptor mediating fibroblast adhesion to collagen IV and laminin 1. Function-blocking antibodies against integrin α_1 subunit did not prevent adhesion of wild-type or α_2-deficient fibroblasts to laminin 5 (Fig. 4), which agrees with previous studies showing that cellular interactions with laminin 5 are mediated by $\alpha_3\beta_1$ integrins (Carter et al., 1991; Rousselle and Aumailley, 1994). There was also no effect of the α_1 integrin function-blocking antibody on wild-type fibroblast adhesion to collagen I (Fig. 4). However, the antibody induced a slight and reproducible inhibition of α_2-deficient fibroblast adhesion to collagen I (Fig. 4), indicating the need of both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins for optimal fibroblast adhesion to collagen I.

The $\alpha_2\beta_1$ integrin is required for keratinocyte adhesion to collagen I and IV

The integrin repertoire varies according to cell type. For example, keratinocytes are known to express $\alpha_2\beta_1$, but not $\alpha_1\beta_1$ integrins (Watt, 2002). We therefore isolated keratinocytes from the epidermis of wild-type and α_2-deficient mice. Phase-contrast microscopy revealed that the overall morphology of wild-type and α_2-deficient primary keratinocytes was similar (not shown). We then compared keratinocyte expression of collagen-binding integrins to that of fibroblasts by immunoblotting cell lysates with antibodies against the integrin α_1, α_2, and α_{11} subunits (Fig. 5). As expected, the α_2 subunit was expressed by wild-type keratinocytes and fibroblasts and was absent in cells from α_2-deficient mice (Fig. 5B). The integrin α_1 and α_{11} subunits were expressed in fibroblasts but not in keratinocytes (Fig. 5A,C). Thus keratinocytes represent the model of choice to investigate whether the $\alpha_2\beta_1$ integrin mediates adhesion to collagens when

it is the only collagen-binding integrin present in a cell. Wild-type and α_2-deficient keratinocytes adhered equally well to laminin 1 and laminin 5 (Fig. 5D). By contrast, whereas wild-type cells were able to adhere and spread on collagen I and collagen IV, there was almost no adhesion of α_2-deficient keratinocytes to either collagen (Fig. 5D). Moreover, adhesion of wild-type mouse keratinocytes to collagen I and IV was completely inhibited by a function-blocking antibody against the α_2 subunit (Fig. 5E). The antibody, however, did not inhibit keratinocyte adhesion to laminin 1 (Fig. 5E), which is a strong ligand for α_6-subunit-containing integrins expressed by keratinocytes (Sonnenberg et al., 1990; Rousselle and Aumailley, 1994) and used here as a control.

The $\alpha_2\beta_1$ integrin is important for transmission of forces in fibroblasts

In addition to mediating cell adhesion to the ECM, integrins are important for outside-in and inside-out transmission of mechanical forces, such as during cell migration and matrix remodeling (Ingber, 1997; Palecek et al., 1997; Eastwood et al., 1998; Grinnell, 2003). The mechanical consequences resulting from the absence of $\alpha_2\beta_1$ integrins were first analysed by time-lapse videomicroscopy recording of cell movement on collagen I monomers. Wild-type and α_2-deficient fibroblasts displayed roughly similar speed (0.32±0.13 μm/minute versus 0.29±0.10 μm/minute) and processive indexes (0.53±0.20 versus 0.42±0.22), indicating that the absence of $\alpha_2\beta_1$ integrin does not grossly impair fibroblast locomotion on collagen I. We next examined the properties of the α_2-deficient fibroblasts in assays more specific of fibroblast function in 3D surroundings, i.e. in collagen gels lacking mechanical load and in tethered collagen lattices. In a first set of experiments, wild-type and α_2-deficient mouse fibroblasts were seeded in free-floating collagen gels to test their capacity to exert tractional forces as reflected by gel contraction. Contraction of the free-floating gels by α_2-deficient fibroblasts was delayed compared with wild-type cells and it did not reach the plateau values observed for the controls (Fig. 6A,B). In a second set of experiments, the fibroblasts were cast into tethered collagen lattices and their ability to generate isometric forces against collagen fibrils was monitored over time. The characteristic bi-phasic curves representing force kinetics displayed by the cells within the tethered collagen lattices revealed that α_2-deficient fibroblasts developed forces of significantly lower magnitude than the wild-type cells (Fig. 6C).

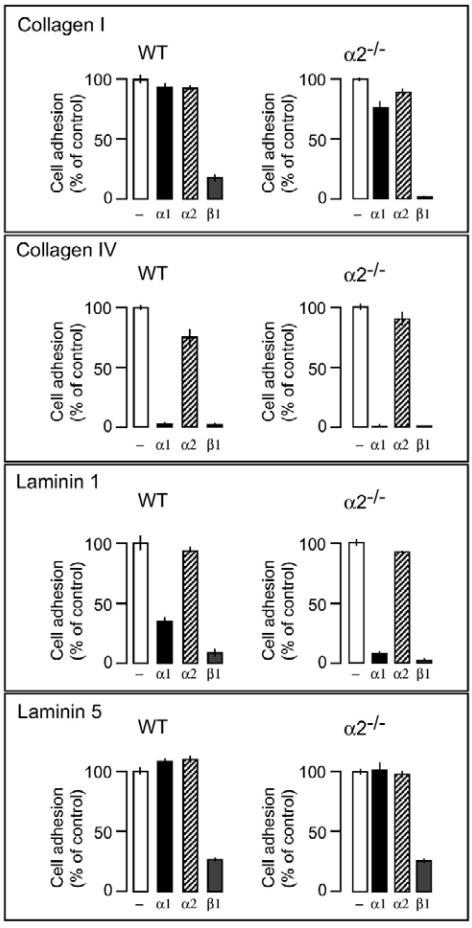

Fig. 4. The $\alpha_1\beta_1$ integrin has a major role for fibroblast adhesion to collagen IV and laminin 1, and to a lesser extent to collagen I. Wild-type and α_2-deficient mouse fibroblasts were seeded on wells coated with collagen I, collagen IV, laminin 1 and laminin 5, as indicated, in the absence (–) or the presence of function-blocking antibodies (20 μg/ml) against integrin α_1, α_2 and β_1 subunits. After 30 minutes of incubation, the extent of cell adhesion was determined as in Fig. 2. The results were expressed as a percentage of the control without antibodies set as 100%.

Alteration of focal adhesions and RhoA/Cdc42 activation in α_2-deficient fibroblasts

Appropriate inside-out and outside-in force transmission by cells requires integrity of both focal adhesions and actin cytoskeleton and finely tuned modulation by Rho GTPases (Hall, 1992; Wiesner et al., 2005). To examine whether absence of the integrin α2 subunit had any effect on the actin cytoskeleton and focal adhesions, we stained fibrillar actin and vinculin, a marker of focal adhesions. Wild-type and α_2-deficient fibroblasts adhering to collagen I displayed a well-developed actin cytoskeleton with numerous stress fibers running across the cell body (Fig. 7). Vinculin-rich focal adhesions were also present in both cell strains, however, with different distributions. In wild-type fibroblasts vinculin patches were observed mostly at the cell periphery at the end of actin stress fibers (Fig. 7). By contrast, vinculin-containing focal adhesions were mainly concentrated at the ventral surface of α_2-deficient fibroblasts (Fig. 7).

Because assembly and turnover of focal adhesions are controlled by the Rho subfamily of GTPases, we analysed activation of these proteins upon fibroblast adhesion to collagen I in the absence of $\alpha_2\beta_1$ integrins. Serum-starved fibroblasts were seeded on collagen I for various periods of time ranging from 60 to 120 minutes and GTP-bound RhoA, Cdc42 and Rac1 were analysed by pull-down assays. At 60 and 90 minutes, the levels of active, GTP-bound RhoA and Cdc42 were higher in α_2-deficient fibroblasts than in wild-type fibroblasts (Fig. 8). By contrast, active, GTP-bound Rac1 levels were similar in wild-type and α_2-deficient fibroblasts (Fig. 8). Thus in the absence of $\alpha_2\beta_1$ integrins, there is a deregulation of Rho GTPase activation in fibroblasts adhering to collagen I associated with diminished generation of tractional and isometric forces.

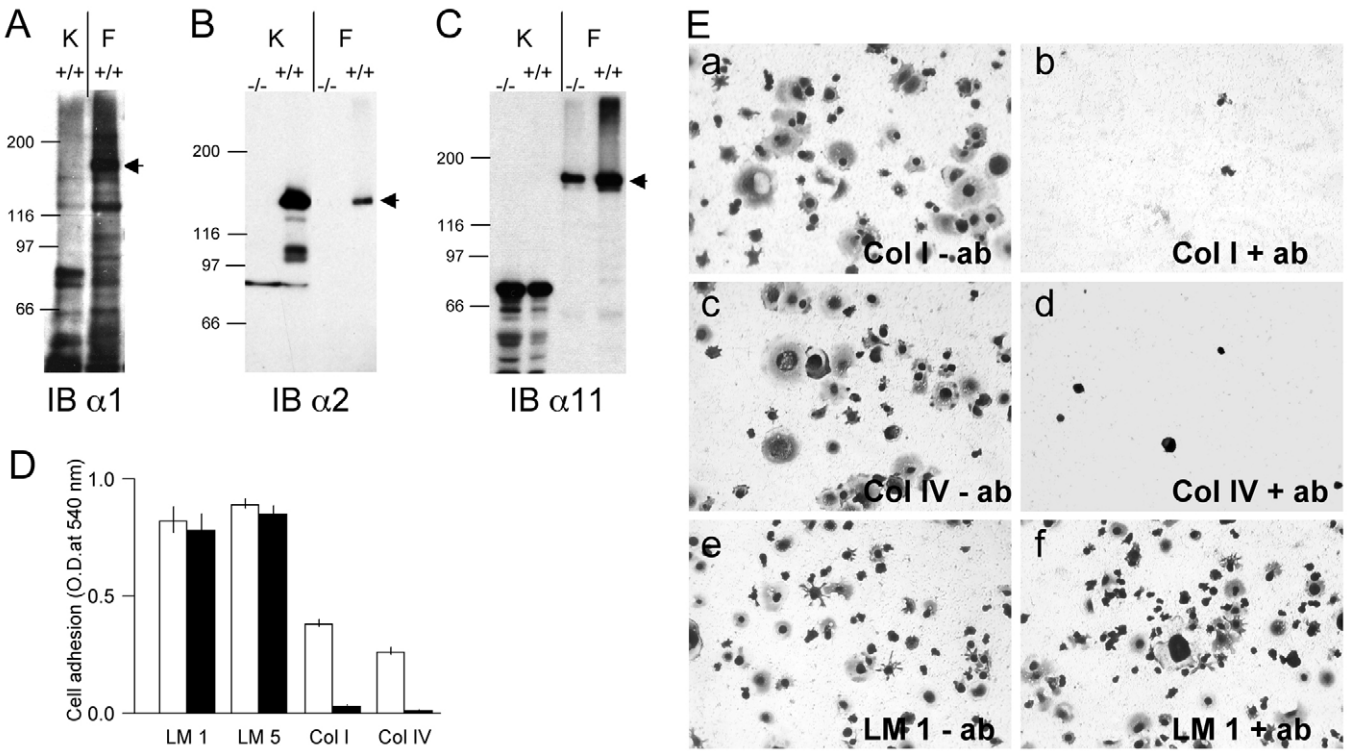

Fig. 5. The $\alpha_2\beta_1$ integrin is indispensable for keratinocyte adhesion to collagens. (A-C) Lysates of primary keratinocytes (K) and fibroblasts (F) from wild type (+/+) and α_2-deficient (–/–) mice were immunoblotted with primary antibodies against the integrin α_1 (A), α_2 (B) and α_{11} (C) subunit as indicated. (D) Equal numbers of wild-type (white columns) and α_2-deficient (black columns) keratinocytes were seeded on triplicate wells coated with collagen I (Col I), collagen IV (Col IV), laminin 1 (LM 1) and laminin 5 (LM 5) as described in Fig. 1A. Compared with wild-type cells, there is nearly no adhesion of α_2-deficient keratinocytes to collagen I and collagen IV whereas adhesion of both strains of keratinocytes to laminin 1 and laminin 5 is similar. (E) Wild-type mouse keratinocytes were seeded on coats of collagen I (Col I), collagen (IV) and laminin 1 (LM 1) in the absence (– ab) or presence (+ ab) of monoclonal antibody against integrin α_2 subunit. The antibody inhibits keratinocyte adhesion to collagens but not laminin 1.

Skin wounds close normally in α_2-deficient mice

It has been suggested that $\alpha_2\beta_1$ integrin could be important for wound contraction and closure because it mediates contraction of collagen gels by fibroblasts and migration of keratinocytes on collagen (Schiro et al., 1991; Pilcher et al., 1997; Nguyen et al., 2001). We therefore compared closure of skin wounds in wild-type and α_2-deficent mice. Measurement of scar areas at successive time points did not reveal any differences between both strains of mice (Fig. 9). This confirms the results obtained with another strain of α_2-deficient mice (Chen et al., 2002).

Discussion

Ablation of $\alpha_2\beta_1$ integrin in mice does not impair normal development and lifespan, and has so far revealed only a subtle phenotype, with partially defective branching morphogenesis and haemostasis (Holtkötter et al., 2002; Chen et al., 2002; Grüner et al., 2004). Similarly, deletion of integrin α_1 permits normal murine development and connective tissue defects are apparent only after experimental injuries (Gardner et al., 1996; Gardner et al., 1999; Pozzi et al., 2000; Chen et al., 2004). This suggests that there is sufficient back-up between these integrins and other receptors in vivo. To address the existence of compensatory mechanisms, we subjected primary skin cells, fibroblasts and keratinocytes, from wild-type and α_2-deficient mice to a panel of functional assays that had previously suggested important roles for $\alpha_2\beta_1$ integrins. Cultivated keratinocytes and fibroblasts provide two excellent models of normal cells differing by their repertoire in collagen-binding integrins because keratinocytes express $\alpha_2\beta_1$ integrin only and fibroblasts express $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins. Moreover, although we cannot exclude the fact that culture conditions modulate expression levels, the qualitative integrin profiles on cultivated cells are consistent with the integrin mRNA profiles in dermis and epidermis (B.E., unpublished observation).

The $\alpha_2\beta_1$ integrin was initially identified as a cell-surface collagen-binding protein by ligand-affinity chromatography and by showing that antibodies against the integrin partially or completely inhibited adhesion of different cell types to native collagens (Wayner and Carter, 1987; Kramer and Marks, 1989; Elices and Hemler, 1989). The collagen-binding property of $\alpha_2\beta_1$ integrin was confirmed by in vitro affinity studies with purified proteins (Kern et al., 1993; Xu et al., 2000; Tiger et al., 2001; Tulla et al., 2001). Together these observations led to the current belief that $\alpha_2\beta_1$ integrin is required for cell adhesion and migration on collagens. Our results show that indeed this holds true for primary keratinocytes which express solely $\alpha_2\beta_1$ integrins as collagen receptors, however, only to some extent for primary skin fibroblasts with a broader repertoire of collagen-binding integrins. Indeed keratinocytes

from mutant mice lacking $\alpha_2\beta_1$ integrins are incapable of adhering to collagen I and IV and adhesion of wild-type keratinocytes to these collagens is completely inhibited by function-blocking antibodies against the integrin α_2 subunit. Thus, keratinocytes absolutely rely on $\alpha_2\beta_1$ integrins to adhere to collagens and, at least for this task, they lack a back-up mechanism to substitute for $\alpha_2\beta_1$ integrin deficiency. A different and more complex picture emerged for fibroblasts, where adhesion to collagen I was severely impaired in the initial steps and partially rescued at later time points. As blockade of β_1 integrins by antibodies abolished fibroblast adhesion to collagen I, either $\alpha_1\beta_1$ or $\alpha_{11}\beta_1$ integrins or both are compensating for the absence of $\alpha_2\beta_1$ integrins at late time points of adhesion. In contrast to complete inhibition of keratinocyte adhesion to collagens with a function-blocking antibody against the integrin α_2 subunit, this antibody did not inhibit adhesion of wild-type fibroblast to collagen I, although knockout or knockdown of the integrin α_2 subunit reduced fibroblast adhesion to collagen I by 30%. As shown recently for platelets and Chinese hamster ovary cells (Van de Walle et al., 2005), the conformation of $\alpha_2\beta_1$ integrin may differ on fibroblasts and keratinocytes, explaining different reactivity towards the antibody. Alternatively, although function-blocking antibodies directly or sterically hinder integrin binding to the ligand, outside-in signaling may still proceed as previously suggested (Chen et al., 2002), allowing intracellular cross-talk between collagen-binding integrins expressed by fibroblasts. By contrast, when the $\alpha_2\beta_1$ integrin is absent, as in α_2-deficient fibroblasts, no antibody-triggered outside-in signaling can proceed or when $\alpha_2\beta_1$ is the sole collagen-binding integrin, as in wild-type keratinocytes, antibody-triggered outside-in signaling cannot lead to cross-talk between collagen-binding integrins.

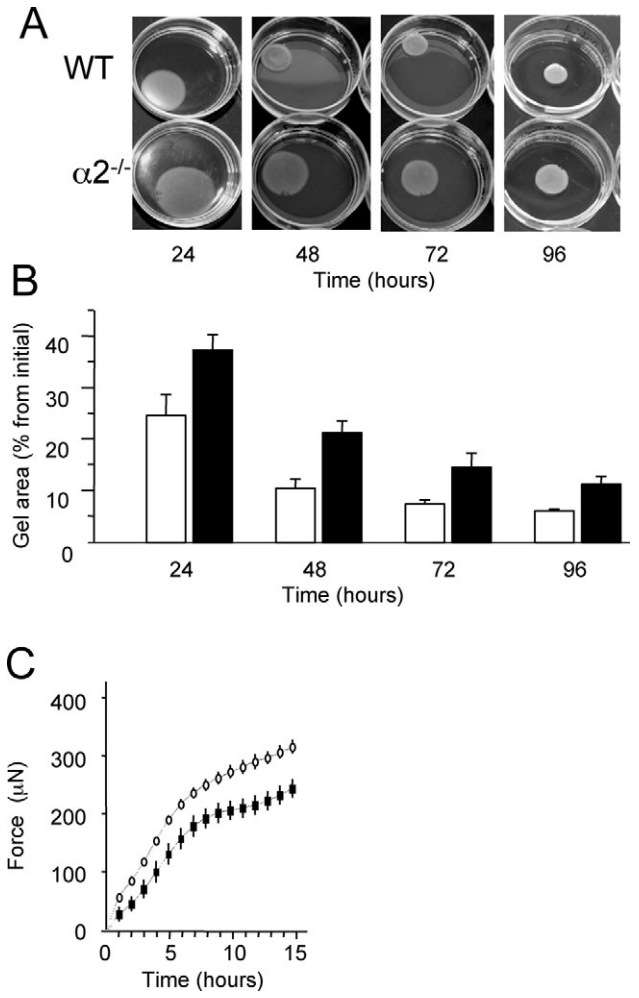

Fig. 6. The $\alpha_2\beta_1$ integrin is important for fibroblast force transmission within 3D collagen networks. (A) Equal numbers of wild type (WT) and integrin α_2-deficient ($\alpha2^{-/-}$) fibroblasts were seeded within triplicate gels of collagen I. The time course of collagen gel contraction was monitored by photographing the gels at successive time intervals as indicated. Note that gel contraction is delayed for integrin α_2-deficient fibroblasts. (B) The gel diameters were measured at successive time points and used to calculate the gel areas, which are plotted as a percentage of the original area at the onset of the experiment. Each point represents the mean ± s.d. of three independent experiments. (C) The isometric forces generated by wild-type (circles) and integrin α_2-deficient fibroblasts (squares) embedded in tethered collagen lattices were monitored over time as indicated. Each data point represents the mean ± s.d. of four measurements.

Partial inhibition of α_2-deficient fibroblast adhesion to collagen I by antibodies against the integrin α_1 subunit indicates that $\alpha_1\beta_1$ compensates, at least in part, the absence of $\alpha_2\beta_1$ integrins and we also anticipate similar functional compensation by $\alpha_{11}\beta_1$ integrins. However, the reverse is not true, i.e. compensation by $\alpha_2\beta_1$ integrin for the lack of $\alpha_1\beta_1$, because antibodies against integrin α_1 subunit completely inhibit both wild-type and α_2-deficient fibroblast adhesion to collagen IV. These results strongly suggest that neither $\alpha_2\beta_1$ nor $\alpha_{11}\beta_1$ integrins substitute for $\alpha_1\beta_1$-mediated adhesion of fibroblasts to collagen IV and laminin 1. This is in line with results obtained with embryonic fibroblasts from α_1-deficient mice that displayed defective spreading on collagen IV (Gardner et al., 1996) and with studies establishing that $\alpha_1\beta_1$ integrin binds better to collagen IV than to collagen I (Kern et al., 1993; Tiger et al., 2001; Tulla et al., 2001). It could be that the amounts of $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrins expressed by fibroblasts are not sufficient to substitute for $\alpha_1\beta_1$ integrin binding to collagen IV. By contrast, although binding of $\alpha_1\beta_1$ integrin to collagen I is lower than that of $\alpha_2\beta_1$ integrin (Kern et al., 1993; Tiger et al., 2001; Tulla et al., 2001), it does participate in α_2-deficient fibroblast adhesion to collagen I.

In addition to its contribution to fibroblast adhesion, the $\alpha_2\beta_1$ integrin is required to generate and counter-balance mechanical forces in 3D networks of collagen fibrils, but not for fibroblast migration on 2D collagen I coats. The ability of α_2-deficient fibroblasts to contract free-floating collagen gels was delayed and did not reach wild-type levels, indicating that $\alpha_2\beta_1$ integrins are required for exerting tractional forces. It agrees with previous observations showing that antibodies against $\alpha_2\beta_1$ integrin impede collagen gel contraction (Schiro et al., 1991; Klein et al., 1991; Langholz et al., 1995). Impairment of fibroblast mechanical properties is further underscored by the observation that α_2-deficient fibroblasts generate isometric forces of lower magnitude than wild-type fibroblasts in tethered collagen lattices. We had previously observed that expression of integrin α_2 subunit is upregulated in fibroblasts embedded in contracting collagen I lattices and reaches a plateau level 12-24 hours after seeding (Klein et al., 1991),

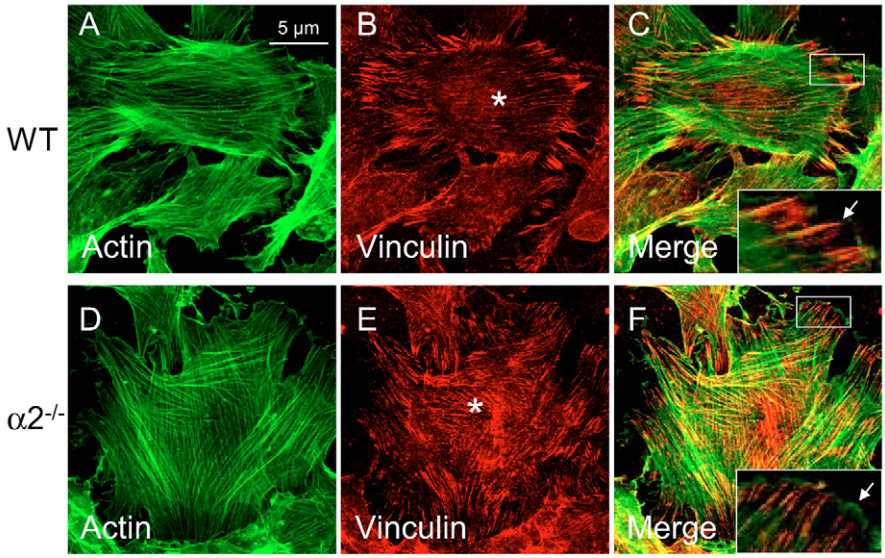

Fig. 7. The $\alpha_2\beta_1$ integrin plays a role in the distribution of focal adhesions in fibroblasts. Wild type (WT) and α_2-deficient ($\alpha2^{-/-}$) fibroblasts were seeded on glass coverslips coated with collagen I (40 μg/ml). After 60 minutes of adhesion, adherent cells were fixed and double-stained for fibrillar actin (A,D) and vinculin (B,E). The cells were observed by confocal microscopy and images were captured using single channel excitation. The two images were superimposed (C,F) using Photoshop (Adobe). Numerous vinculin-positive focal adhesions are present on the entire basal surface of α_2-deficient fibroblasts, but not of wild-type fibroblasts (asterisk). Inserts in C and F represent higher magnifications of focal adhesions at actin stress fibre termini (arrows).

suggesting that $\alpha_2\beta_1$ integrin is important in the initial phases of matrix remodeling. These results led us to surmise that no compensatory activity is exerted by $\alpha_1\beta_1$ or $\alpha_{11}\beta_1$ integrins for tractional force generation. We cannot, however, exclude expression or activation of discoidin domain receptors 1 and 2 at later time points after fibroblast seeding, as these receptors are characterized by an unusually long lag time between collagen binding and activation (Vogel et al., 1997; Shrivastava et al., 1997).

It may appear surprising that movement of α_2-deficient fibroblasts on collagen-I-coated surfaces was not altered in comparison to control cells because migration also requires transmission of forces. However, the mechanisms governing cell motility on 2D surfaces and remodeling of 3D networks are different in terms of activation of motor proteins (Meshel et al., 2005) and magnitude of forces (Eastwood et al., 1998). We anticipate that the decreased efficiency of α_2-deficient fibroblasts to develop tension relates to the atypical distribution of the force-bearing focal adhesions and de-synchronization of Rho-GTPase activation as both are involved in coordinating mechanotransduction (Bershadsky et al., 2003). Based on pharmacological approaches, previous works suggested involvement of Rho GTPases in collagen gel contraction by fibroblasts (Abe et al., 2003; Parizi et al., 2000). Our recent studies show more directly that a tight regulation of RhoA activation controls collagen gel contraction and distribution of focal adhesions in fibroblasts (Zhang et al., 2006). Locking RhoA in its active conformation results in a reduced ability of a clonal population of fibroblasts to contract collagen gels and to an altered distribution of focal adhesions (Zhang et al., 2006), two features closely resembling those of fibroblasts lacking α_2 expression and activating RhoA. This parallel strongly suggests that collagen gel contraction, activity of α_2 integrin and regulation of RhoA activation are linked together and that a precise control of the turnover between active and inactive RhoA is crucial for force transmission. This is particularly relevant for dermal fibroblasts which are embedded in 3D surroundings rich in collagen fibrils in vivo.

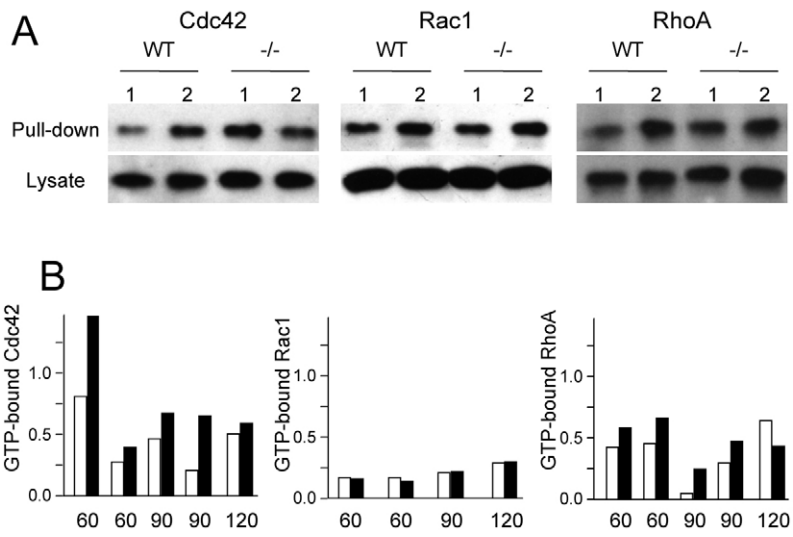

Fig. 8. Activation of Rho GTPases upon fibroblast adhesion to collagen I. Wild-type (WT) and α_2-deficient (–/–) fibroblasts were serum-starved for 24 hours and subsequently seeded at the same cell density on collagen I. After 60 to 120 minutes of adhesion the cells were lysed and active, GTP-bound RhoA, Rac1 and Cdc42 were pulled down as described in the Materials and Methods. (A) Representative blots of total (lysate) and GTP-bound RhoA, Rac1 and Cdc42 (pull-down) after 90 (lane 1) and 120 (lane 2) minutes of adhesion to collagen for wild-type (WT) and α_2-deficient (–/–) fibroblasts. (B) Band intensity of total and GTP-bound proteins were measured by densitometry on blots from different experiments and the relative amounts of active, GTP-bound proteins were normalized to those of the corresponding lysates (arbitrary units). The graphs show the results from different experiments performed for 60, 90 and 120 minutes as indicated below each set of columns. White columns, control fibroblasts; black columns, α_2-deficient fibroblasts.

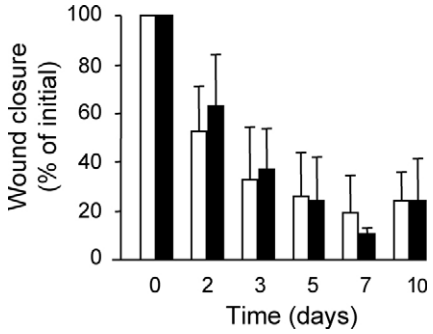

Fig. 9. Reduction of wound area is normal in integrin-α_2-null mice. Full-thickness wounds (6 mm in diameter) were created on the backs of α_2-deficient (black bars) and wild-type mice (white bars). Wounds ($n \geqslant 16$ per time point and genotype) were photographed at the indicated time points and each wound area was calculated using Adobe Photoshop software. Results are expressed as means ± s.d.

This is not the case for keratinocytes, which are polarized and at the most contact collagens in a 2D manner.

In conclusion, it holds true that the $\alpha_2\beta_1$ integrin has the ability to mediate cell adhesion to collagens but it fulfils this task in a cell-type-specific manner. For keratinocytes, it is the only receptor executing the task in vitro. Yet in vivo, the function of $\alpha_2\beta_1$ in epidermal cells remains elusive. It was suggested to be important for keratinocyte adhesion and migration on the provisional matrix during skin wound healing (Pilcher et al., 1997; Nguyen et al., 2001). However, it is apparently not needed as wounds heal normally in mice deficient in $\alpha_2\beta_1$ (Chen et al., 2002). Furthermore, using primary fibroblasts from α_2-knockout animals allowed us to establish that they are equipped with sufficient back-up by other collagen-binding integrins that gradually compensate cell adhesion and force transmission. Compensation is an important biological phenomenon that could not be assessed in previous works using cells with a restricted repertoire in collagen-binding integrins and it helps to explain the subtle phenotype of α_2-deficient mice. Whether compensation for the lack of $\alpha_2\beta_1$ integrin in fibroblasts is due to redundancy or to an increase in the functional capacity of $\alpha_1\beta_1$ and $\alpha_{11}\beta_1$ collagen-binding integrins may be addressed in future work by generating fibroblast models with double- and triple-integrin deficiency.

Materials and Methods

Isolation and culture of mouse skin primary cells

Mice deficient for integrin α_2 subunit (Holtkötter et al., 2002) and corresponding wild-type littermates were generated by heterozygous matings of animals that had been backcrossed into a C57Bl/6 background for more than six generations. Newborn animals were killed by decapitation. The entire trunk skin was removed and incubated overnight with 0.1% trypsin, 0.02% EDTA in PBS, followed by mechanical separation of epidermis from dermis. To isolate fibroblasts, the dermis was diced finely and incubated with 400 U/ml of collagenase I (Cell Systems, St Katharinen, Germany) for 1 hour at 37°C. Tissue debris was removed by centrifugation and collected fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS, PAA Laboratories, Cölbe, Germany), 2 mM glutamine and antibiotics (Seromed-Biochrom, Berlin, Germany), and 50 μg/ml sodium ascorbate. The fibroblasts were used between passage three and five for experiments. Keratinocytes were isolated according to Rheinwald and Green (Rheinwald and Green, 1975) with some modifications. Finely minced epidermis was incubated in DMEM/Ham's F12 (3:1 v/v; Seromed-Biochrom) supplemented with 50 μM Ca^{2+}, 1.8×10^{-4} M adenine, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 1×10^{-10} M cholera enterotoxin, 10 ng/ml EGF, antibiotics (all from Sigma-Aldrich, Schnelldorf, Germany) and 10% FCS (PAA Laboratories) depleted of calcium by treatment with Chelex (BioRad, Munich, Germany). Cells were dissociated by agitation and seeded onto rat plasma fibronectin-coated (30 μg/ml; Sigma) plates together with growth-arrested 3T3 J2 feeder cells. Cultures were maintained in DMEM/Ham's with supplements at 32°C and used for experiments at passage two to four. Mouse neonatal primary keratinocytes (CellnTec, Bern, Switzerland) were subcultured in serum-free mouse keratinocyte growth medium (CellnTec).

Cell adhesion assays

Tissue culture wells (96-well plates, Costar) were coated with collagen I (Seromed-Biochrom), collagen IV (kindly provided by K. Kühn, Max-Planck Institute for Biochemistry, Martinsried, Germany), laminin-nidogen complex, referred to hereafter as laminin 1 (kindly provided by R. Timpl, Max-Planck Institute for Biochemistry, Martinsried, Germany) and laminin 5 (Rousselle and Aumailley, 1994). After saturation of the wells with 1% BSA (Fraction V, Serva, Heidelberg, Germany), equal number of cells were seeded in triplicate wells for 30 minutes when not otherwise indicated. For inhibition experiments with integrin function-blocking antibodies, suspended cells were mixed with appropriate dilutions of antibodies before seeding on coated wells. We used hamster monoclonal antibodies against rat and cross-reacting with mouse integrin β_1 (Ha2/5), α_1 (Ha31/8) and α_2 (Ha1/29) subunits (PharMingen, Heidelberg, Germany). At the end of the experiments, adherent cells were quantified as previously reported (Aumailley et al., 1989) and photographed with a digital camera (PowerShot G5, Canon, Tokyo, Japan) mounted on a phase-contrast microscope (Axiovert 100, Carl Zeiss, Göttingen, Germany).

siRNA-mediated silencing of the α_2 subunit

Double-stranded RNA oligonucleotides specifically targeting mouse integrin α_2 subunit were obtained from Eurogentec (Sart-Tilman, Belgium) and the sense sequences were as follows: 5′-GGAGACAUCUCCAGUUCUUTT-3′ (siRNA1), 5′-GUCCAGACUUUCAGUUCUUTT-3′ (siRNA2), 5′-GCAUGGCAUUGGU-GACUAUTT-3′ (siRNA3), and 5′-AUACUUACGCACGCUCCAATT-3′ (control siRNA). Semi-confluent wild-type mouse fibroblasts were transfected by calcium phosphate co-precipitation of one or two siRNAs (100 nM each) and further grown up to 76 hours. In some experiments the fibroblasts were transfected a second time after 36 hours. Expression of integrin α_2 subunit was determined by SDS-PAGE fractionation of cell lysates and immunoblotting.

SDS-PAGE and immunoblotting

Confluent cell monolayers were lysed and homogenized in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM each $MgCl_2$, $MnCl_2$, $CaCl_2$, 1 μg/ml each leupeptin and pepstatin, 0.5 mM PMSF. Cell lysates were clarified by centrifugation and aliquots were fractionated by SDS-PAGE on 7% acrylamide gels under reducing conditions followed by immunoblotting with rabbit antibodies raised against the intracellular domain of integrin α_1 (AB1934, Chemicon Europe, Hofheim, Germany), α_2 and α_{11} subunits (peptide synthesis, conjugation to keyhole limpet hemocyanin and immunization were performed at Innovagen, Lund, Sweden) and secondary antibodies coupled to horseradish peroxidase (DakoCytomation). Reactive bands were visualized by ECL (Amersham Biosciences, Freiburg, Germany).

Cell migration assays

Cell migration was monitored by time-lapse video-microscopy. Briefly, equal numbers of cells were seeded in the centre of wells (10-μl drop/well, 24-well tissue culture plates), in DMEM supplemented with 10% fetal calf serum to allow rapid cell adhesion. After 1 hour, the cells were washed with PBS and the wells filled with fresh DMEM containing collagen I (40 μg/ml). Cell movement was recorded on a thermally controlled chamber (37°C, 5% CO_2) placed on an inverted microscope (Axiovert S100TV, Carl Zeiss) equipped with a digital CCD camera (Xillix MicroImager, Richmond, British Columbia, Canada). Phase-contrast photographs were automatically captured every 5 minutes for 800 minutes and stored with Openlab software system (Improvision, Coventry, England). The sequences of images were converted to Quick Time movies and migration tracks of at least 20 cells were analysed using Dynamic Image Analysis System software (Solltech, Oakdale, IA). Extracted migration parameters included cell velocity (cell speed in μm/minute) and processive index defined by the ratio between the linear and the absolute distances covered by a cell during the time of recording.

Contraction of collagen gels

Mouse fibroblasts were seeded at a density of 1.5×10^5 cells/ml into 32 mm bacteriological plates (2 ml/dish) in DMEM supplemented with 10% fetal calf serum, sodium ascorbate (50 μg/ml), antibiotics and containing 0.3 mg/ml of acid-extracted collagen I from newborn calf skin (Institut für Biomedizinische Forschung, Leipzig, Germany) as previously described (Kessler et al., 2001). The cultures were placed at 37°C to allow collagen polymerization and gradual lattice contraction was monitored by measuring gel diameter of triplicate setups at successive time points up to 96 hours.

Force measurement in tethered collagen lattices

Mouse fibroblasts were suspended in supplemented DMEM (1.8×10^5 cells/ml) containing collagen I (1.75 mg/ml; rat tail collagen; First Link, Brierley Hill, UK) and placed in rectangular moulds (17×22×7 mm) in which lattice shrinkage is mechanically prevented by gel attachment to polyethylene bars located at the long ends of the moulds. The polyethylene bars were connected to a culture force transducer allowing isometric tension recording (KG 7A with Bridge-Amplifier DUBAM 7C, Scientific Instruments, Heidelberg, Germany). Force output was digitized at 0.23 Hz (Analog to Digital Converter μMeter4 and Software Nextview light from BMC Systeme, Maisach, Germany) and recorded on a personal computer. Quadruplicate lattices were used in each experiment.

Wound healing analysis

Wild-type and α_2-deficient mice (females, 3-5 month-old) were anaesthetized by intraperitoneal injection of ketamine (10 g/l)/xylazine (8 g/l) solution (10 μl/g body weight). Two full-thickness wounds of 6 mm diameter excising the skin and panniculus carnosus were created using biopsy punches on both sides of the dorsal midline on the shaved back ($n\geq8$ mice per time point and genotype) and left uncovered. Wounds were digitally photographed at day 0, 2, 3, 5, 7 and 10 after injury. Wound areas were calculated using Photoshop (Adobe Systems, San Jose, CA) and expressed as percentage of initial (day 0) wound area.

Cell staining

Fibroblasts were seeded on glass coverslips coated with collagen I (40 μg/ml) for 60 minutes, fixed with 2% paraformaldehyde in PBS for 15 minutes, permeabilized with 0.2% Triton X-100 and processed for immunofluorescence staining. Mouse monoclonal antibody F-VII against vinculin (a gift from M. Glukhova, Institut Curie, Paris, France) was used as primary antibody followed by Cy3-conjugated secondary antibodies (Dianova, Hamburg, Germany) applied together with FITC-conjugated phalloidin (Sigma-Aldrich). The coverslips were mounted on histoslides in DAKO medium (DakoCytomation, Hamburg, Germany) and observed by laser-scanning confocal microscopy (Leica Instruments, Heidelberg, Germany). Confocal images were captured with single channel excitation, stored with the microscope internal software and merged using Photoshop.

GTPase pull-down assays

Fibroblasts were serum-starved for 24 hours, resuspended in serum-free medium and seeded on tissue culture plates coated with collagen I (40 μg/ml). After 60, 90 or 120 minutes, adherent cells were lysed in ice-cold buffer containing 1% Triton X-100, 25 mM HEPES pH 7.3, 150 mM NaCl, 4% glycerol, 4 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Lysates were cleared by centrifugation and supernatants (500 μl) were used to pull down Cdc42/Rac1 and RhoA with GST-PBD and GST-RBD fusion proteins, respectively, as previously described (Servotte et al., 2006; Zhang et al., 2006). Lysate (40 μl aliquots) and pull-down fractions were separated by SDS-PAGE on 15% acrylamide gels under reducing conditions and immunoblotted with mouse monoclonal primary antibodies against RhoA (clone 26C4; Santa Cruz Biotechnology, Heidelberg, Germany), Rac1 (clone 23A8; Biozol diagnostika, Eching, Germany) and Cdc42 (clone 44; Becton Dickinson, Heidelberg, Germany).

We are grateful to Ingo Haase and Semra Frimpong for assisting with keratinocyte culture, to Marina Glukhova, Klaus Kühn and Rupert Timp for gifts of reagents, to Monika Pesch and Kerstin Elias for excellent technical assistance and to Andreas Woeste for providing plenty of primary wild-type mouse fibroblasts to set up siRNA transfections. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 589), the Deutsche Zentrum für Luft- und Raumfahrt (50WB0321), the Center for Molecular Medicine Cologne (TV80) and the Medical Faculty of the University of Cologne. M.A. is a researcher from the Centre National de la Recherche Scientifique.

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