Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics

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Introduction

Integrins are heterodimeric transmembrane adhesion receptors composed of α and β subunits, which serve, often in combination with receptor tyrosine kinases, to control cell adhesion, migration, proliferation, differentiation, and survival (Hynes, 2002; Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002; Giancotti and Tarone, 2003). Together, these functions govern morphogenesis and tissue homeostasis and, when deregulated, contribute to tumorigenesis and cancers (Avizienyte et al., 2002; Hsia et al., 2003; Hannigan et al., 2005; McLean et al., 2005; Owens et al., 2005; Wu et al., 2005; Janes and Watt, 2006; Wilhelmsen et al., 2006).

αβ1, α2β1, α5β1, αvβ6, and α6β4 are all found in skin epidermis, where they bind to their ligands in the basement membrane and serve a diverse array of functions (Fuchs et al., 1997; Watt, 2002; Wilhelmsen et al., 2006). Conditional targeting of the genes encoding the hemidesmosomal integrins α6 and β4 results in epidermal–dermal detachment, skin blistering, and defects in cell survival (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996), whereas targeted deletions of α3 and β6 result in microblistering and wound defects, respectively (DiPersio et al., 1997). In contrast, β1 ablation causes defects in epidermal–dermal attachment, basement membrane organization/assembly, hair follicle downgrowth/morphogenesis, and epidermal wound closure (DiPersio et al., 1997; Brakebusch et al., 2000; Raghavan et al., 2000; Grose et al., 2002).

αβ1 integrins form focal adhesions (FAs), composed of a complex group of proteins and signaling molecules that associate with the actin and microtubule cytoskeletons and orchestrate these diverse functions. Primary β1-knockout (KO) epidermal keratinocytes can be cultured in vitro under conditions where they proliferate similarly to their wild-type (WT) counterparts (Raghavan et al., 2003). These keratinocytes display alterations in cell-matrix adhesion, enlarged, peripheral FAs, and robust actin stress fibers, suggestive of properties that might contribute to the perturbations in hair follicles and epidermal wound closure seen in vivo. However, the molecular mechanisms by which αβ1 integrins control the diverse set of cell biological processes in epidermal keratinocytes remain largely unknown.

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Abbreviations used in this paper: cKO, conditional KO; FA, focal adhesion; FN, fibronectin; GAP, GTPase-activating protein; ILK, integrin-linked kinase; KO, knockout; MK, mouse keratinocyte; MLCl, myosin light chain; MYP, myosin phosphatase target; PAK, p21-activated kinase; PIX, PAK-interacting exchange factor; PKL, p95 paxillin kinase linker; PXN, paxillin; PYK2, proline-rich tyrosine kinase 2; ROCK, Rho kinase; VIN, vinculin; WT, wild-type.

The online version of this article contains supplemental material of epidermal explants. Fak-null keratinocytes display an aberrant actin cytoskeleton, which is tightly associated with robust, peripheral focal adhesions and microtubules. We find that without FAK, Src, p190RhoGAP, and PKL–PIX–PAK, localization and/or activation at focal adhesions are impaired, leading to elevated Rho activity, phosphorylation of myosin light chain kinase, and enhanced tensile stress fibers. We show that, together, these FAK-dependent activities are critical to control the turnover of focal adhesions, which is perturbed in the absence of FAK.

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Integrins lack endogenous enzymatic activity and are thus believed to depend on signal transducers such as the nonreceptor kinases FAK, its sequence homologue proline-rich tyrosine kinase 2 (PYK2), or integrin-linked kinase (ILK), as well as a variety of scaffolding proteins that link integrins to the actin cytoskeleton to unfold their functions (Hynes, 2002). Upon integrin engagement with the extracellular matrix, FAK becomes activated and physically interacts with β1’s cytoplasmic tail as well as with various signaling molecules at FAs (Schaller et al., 1995; Chen et al., 2000). In cultured β1 integrin–null epidermal cells, FAK activity is markedly reduced, revealing FAK as a potential transducer of β1 integrin’s diverse activities in skin (Raghavan et al., 2003).

The notion that FAK-mediated β1 integrin signaling controls these processes in the skin epidermis is suggested from Fak conditional gene targetings, which have revealed defects in basement membrane assembly and/or remodeling in developing dorsal forebrain (Beggs et al., 2003). However, although Fak-deficient fibroblasts exhibit restricted migration, endothelial cells and HeLa cells show increased motility (Ilic et al., 1995; Yano et al., 2004; Tilghman et al., 2005; Braren et al., 2006). Additionally, loss of FAK results in apoptosis in embryonic fibroblasts and endothelial cells, but not in meningeal fibroblasts (Ilic et al., 1995; Beggs et al., 2003; Shen et al., 2005; Braren et al., 2006). Whether cellular context accounts for these seemingly opposing findings is not yet clear.

Recently, Fak was conditionally targeted in postnatal mouse skin epithelium, revealing defects in hair follicles and sebaceous glands (Essayem et al., 2006) and resistance to tumorigenesis (McLean et al., 2004). However, the molecular and cellular mechanisms responsible for these phenotypes have yet to be elucidated, and the differences in KO strategies and genetic backgrounds preclude comparisons between Fak and β1 conditional targetings. To unravel the relative importance of epidermal FAK in mediating downstream signaling functions of β1 integrins, we have mated Fak(fl/fl) mice with the same K14-Cre recombinase mice that we used previously to target β1 integrin. For these comparisons, we also derived primary epidermal keratinocytes from both Fak-null and β1-null skins.

We show that, in contrast to β1 and distinct from some prior functions attributed to FAK, FAK is largely dispensable for epidermal adhesion, basement membrane organization, proliferation, survival, and terminal differentiation. Rather, FAK controls cytoskeletal dynamics and FA disassembly, and without it, keratinocytes are perturbed in their motility and their ability to polarize migration out of skin explants. Mechanistically, our data suggest that these defects are in part due to decreased phosphorylation of Src and p190RhoGAP (GTPase-activating protein), yielding increased Rho/Rho kinase (ROCK) signaling and inefficient recruitment of the p95 paxillin kinase linker–PAK-interacting exchange factor–p21-activated kinase (PKL–PIX–PAK) complex to FAs. Finally, we substantiate the biological significance of these activities downstream of αβ1 integrin and FAK by inhibiting tension signaling with small molecule inhibitors and explore the consequences to FA dynamics.

Results

FAK is not required for integrin expression, basement membrane formation, or cell-substratum adhesion in skin epidermis

FAK activity has been found to depend on αβ1 integrin signaling in the skin epidermis (Raghavan et al., 2003). To test the hypothesis that FAK functions as a key transducer of αβ1 signaling in epidermis, we mated K14-Cre mice (Vasioukhin et al., 1999) to mice where the second kinase domain of Fak was flanked by loxP sites. Upon excision of floxed exons by Cre recombinase, an early stop codon is generated, ablating FAK protein expression (Beggs et al., 2003). Immunofluorescence microscopy documented specific loss of FAK in skin epithelium and not dermis, and this was further confirmed by immunoblot analyses (Fig. 1, A and B).

Although, at birth, Fak conditional KO (cKO) mice were phenotypically indistinguishable from WT littermates, their hair coat did not emerge on time (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1; Essayem et al., 2006). Histological analysis further revealed a defect in follicle downgrowth (Fig. S1 C). This defect appeared to be rooted in a failure of follicles to migrate downward, rather than alterations in proliferation and/or apoptosis (Fig. S1, D and E). Detection of such early neonatal defects was uniquely possible with K14-Cre mice, which embryonically ablate floxed genes in epidermis (Vasioukhin et al., 1999).

To determine whether FAK is required for integrin expression or basement membrane formation in skin, we first conducted immunofluorescence microscopy using antibodies against β1 integrin, expressed in dermal fibroblasts and basal epidermal cells, and laminin 5, the primary ECM ligand for epidermal integrins and a major component of the basement membrane at the dermal–epidermal boundary. These labeling patterns were indistinguishable between WT and FAK-deficient skin (Fig. 1 C). Ultrastructural analyses further revealed an intact basement membrane, accompanied by structurally normal αβ4 integrin–containing hemidesmosomes of comparable length and density (Fig. 1, D and E). An explanation of the box-and-whisker diagram (Tukey, 1977) used to graphically display the spread of these data can be found in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1).

We next used FACS analyses to quantify integrin surface levels on freshly isolated epidermal keratinocytes (Fig. 1 F). Normal surface levels of β1, β4, α2, α6, αv, and β6 integrins were observed, indicating that loss of FAK did not affect integrin expression. Moreover, as judged by FACS with an antibody specific for activated β1 integrin, no differences were noted in the activity of αβ1 integrins in the absence of FAK. Consistent with these observations, WT and Fak-null epidermal cells adhered similarly to a variety of purified ECM substrates of the underlying basement membrane (Fig. 1 G). Collectively, these studies suggest that FAK functions in the subset of αβ1 integrin functions required for hair follicle morphogenesis, but it is dispensable for basement membrane assembly, integrin expression, adhesion, and tissue homeostasis.
Primary Fak-null keratinocytes grow in culture but exhibit enhanced sensitivity to serum starvation

To probe for molecular and biological functions of FAK in epidermal keratinocytes, we cultured primary keratinocytes on fibroblast feeder layers (Fig. 2). Fak KO keratinocytes were propagated over multiple passages without major growth defects, and feeder cells could be withdrawn upon passaging. However, these cells appeared to be more sensitive to serum starvation than WT keratinocytes. Thus, on fibronectin (FN)-coated dishes, MAPK activity, as measured by p42/44 phosphorylation, was reduced in serum-starved Fak KO, as compared with either starved WT or serum-stimulated KO or WT keratinocytes. In contrast, the phosphorylation of other signaling molecules such as AKT appeared unchanged.

FAK- and β1-deficient epidermal cultures differ in their PYK2 and ILK activities, but both display alterations in cell shape

We next examined whether Fak ablation affected the activity and/or subcellular localization of the two other β1 signal transducing, nonreceptor kinases, PYK2 and ILK (Hannigan et al., 1996; Astier et al., 1997; Gismondi et al., 1997). In the absence of FAK, Pyk2 was slightly hyperphosphorylated, but phospho-Pyk2 still localized to Fak-deficient FAs. ILK levels and localization were similar between WT and Fak KO.
keratinocytes (Fig. 3 A). In contrast, activated FAK and PYK2 were markedly reduced in β1-null keratinocytes, where some delocalization of ILK from FAs was also noted (Fig. 3 B). PYK2/ILK signaling was still at least partially maintained in Fak KO cells; however, rhodamine-phalloidin labeling revealed marked defects in actin organization and cell spreading, resembling those seen in β1-null keratinocytes.

To investigate these defects in greater detail, we plated keratinocytes on FN-coated glass dishes, fixed and stained them at multiple intervals, and quantified the contact area between keratinocytes on FN-coated glass dishes, fixed and stained them (A and B). Although peripheral FAs exhibited markedly enhanced staining intensity and area in FAK-deficient cells, the smaller and more weakly stained central FAs were largely absent (Fig. 5, C and D). Defects in cell shape; actin organization; and FA size, distribution, and intensity were largely restored after reexpressing FAK in Fak KO keratinocytes (Fig. 5, E–G). These data underscore the importance of FAK in FA regulation and actin organization.

FAK promotes cytoskeletal dynamics and FA turnover

To examine actin–FA dynamics in real time, we first mated K14-Cre, Fak(fl/fl) mice on the background of previously generated K14-GFPactin transgenic mice (Vaezi et al., 2002) and then transfected Fak-null and WT K14-GFPactin keratinocytes with an RFPzyxin expression vector (Bhatt et al., 2002; Raghavan et al., 2003). Video microscopy revealed a considerably more static actin cytoskeleton and associated FAs in Fak-null keratinocytes relative to their WT counterparts (Fig. 6, A and B and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1). Most WT keratinocytes extended lamellipodia uniformly around their circumference, and as they formed, lamellipodia seeded numerous focal complexes connected by short, thin actin bundles (Fig. 6 A). A few of these focal complexes slowly developed into more robust FAs, which then served as convergence sites for larger stress fibers. In contrast, Fak-null keratinocytes formed lamellipodia primarily around existing robust FAs, with new focal complexes maturing rapidly into atypically large peripheral FAs associated with unusually thick actin cables (Fig. 6 A and Videos 1 and 2).

WT keratinocytes displayed dynamic FAs, which were continually drawn toward the cell center (Fig. 6 B and Video 1). In contrast, the large FAK-deficient FAs accumulated at cell edges for extended times (Fig. 6 B and Video 2). After prolonged periods of cellular tugging, retractive forces often caused abrupt release of associated cellular contacts with the substratum, resulting in rapid, uncoordinated changes in FAK KO morphology (Videos 1 and 2).

To assess differences in the kinetics of FA dynamics, we monitored individual FAs in keratinocytes transfected with GFP-PXN and calculated the rate constants for both FA assembly and disassembly (Webb et al., 2004). Although FA assembly rates were only slightly lower in Fak KO versus WT keratinocytes, disassembly rates were significantly decreased (Fig. 6 C). This was further substantiated by FRAP experiments, where a significant increase was noted in the half-times of fluorescence recovery after photobleaching (Fig. 6 D). In contrast, the mobile fraction of GFP-PXN appeared unaffected more pronounced over time, as did the range of effects observed (Fig. 4 B and Fig. S2).

To further investigate the root of FAK-dependent changes in cytoskeletal architecture and cell shape/spreading, we counterstained rhodamine-phalloidin labeled keratinocytes with antibodies against the FA molecules vinculin (VIN) and paxillin (PXN). VIN and PXN localized at the sharp, constricted cellular apexes that were closely associated with massive bundles of actin fibers lining the periphery of aberrantly shaped Fak-deficient keratinocytes (Fig. 5, A and B). Although peripheral FAs exhibited markedly enhanced staining intensity and area in FAK-deficient cells, the smaller and more weakly stained central FAs were largely absent (Fig. 5, C and D). Defects in cell shape; actin organization; and FA size, distribution, and intensity were largely restored after reexpressing FAK in Fak KO keratinocytes (Fig. 5, E–G). These data underscore the importance of FAK in FA regulation and actin organization.
by the status of FAK (Fig. 6 E). These data provide compelling evidence that the large FAs in FAK-deficient keratinocytes arise from a defect in FA disassembly.

**FAK functions in organizing and polarizing actin networks to facilitate efficient, coordinated cell migration**

The observed defects in FA disassembly and cytoskeletal organization led us to posit that FAK may function in promoting directed cell migration. We tested this possibility by first generating tissue explants from K14-GFPactin, WT, and Fak-null skins placed on FN-coated glass dishes and then monitoring the outgrowth of interconnected epithelial sheets of GFPactin-expressing keratinocytes from these explants (Vaezi et al., 2002). Quantification revealed a marked delay in the outgrowth from Fak-null explants compared with WT explants (Fig. 7, A and B), whereas lamellipodial activity appeared similar between WT and KO explants (Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1).

WT explants polarized their actin cytoskeleton, exhibiting parallel actin bundles that were oriented perpendicularly to the leading edge of the outgrowing cells (Fig. 7, C and D). Antibodies against E-cadherin, which mark stable cell–cell contacts, underscored the elongated, polarized character of these epidermal sheets. Actin fibers associated with PXN-labeled FAs were also distributed evenly and unidirectionally oriented toward the leading edges of the outgrowing WT explants.

In contrast, Fak-null epidermal explants exhibited a seemingly random orientation of actin bundles, often crossing at multiple places, whereas enlarged FAs pointed in different directions in disoriented cells at the leading edge (Fig. 7, C and D).

A priori, the differences in explant outgrowth could be due solely to a failure to coordinate cytoskeletal dynamics across the outgrowing epithelial sheet. Alternatively, it could be that the defects in outgrowth stem from intrinsic defects in the migration of individual cells, which subsequently fail to coordinate directed movements within the outgrowing sheet. To assess the motility of individual keratinocytes, equal numbers of WT and KO keratinocytes were seeded in the top compartment of Boyden chambers and assayed for the number of cells that migrated through the filter to the FN matrix located in the chamber below. Quantification revealed that the number of WT cells migrating through the filter was approximately five times higher than the number of KO cells (Fig. 7 E). These data suggest that FAK promotes FA dynamics and directed migration in keratinocytes and supports the polarized, unidirectional migration of epithelial sheets at the wound edge.

**FAK is not required for microtubule targeting to FAs**

The migration defects arising from loss-of-function FAK mutations pointed to a major role for FAK in regulating FA dynamics for the purpose of orchestrating cytoskeletal organization...
Fak-null FAs fail to recruit and activate p190RhoGAP and PAK

The main characteristic of Fak KO keratinocytes is robust FAs, which are tightly associated with prominent stress fibers. Elevated Rho activity can result in elevated stress fiber formation and stabilization of FAs (Chrzanowska-Wodnicka and Burridge, 1996), whereas RhoA inactivation by p190RhoGAP can promote cell spreading and migration (Arthur and Burridge, 2001). Furthermore, FAK has been associated with the transient suppression of Rho activity during cell spreading (Ren et al., 2000) and it has been speculated that FAK may cooperate with β1 integrin and Src tyrosine kinase to phosphorylate and activate p190RhoGAP at FAs (Fincham et al., 1999; Arthur et al., 2000; Dumenil et al., 2000; Brouns et al., 2001).

To test this hypothesis, we first assessed Src activity in FN-stimulated keratinocytes grown in the presence or absence of serum. Immunoprecipitation of Src followed by anti–pY418-Src immunoblot analysis revealed a marked reduction of phosphorylated (active) Src in Fak-null keratinocytes grown under serum-free conditions where Src activation is likely to emanate primarily from integrin activation (Fig. 8 A). As shown by its faster electrophoretic mobility, Src was mostly unphosphorylated (inactive). In contrast, no considerable changes in the status of Src were noted in cells cultured in the presence of serum, where Src activation can additionally occur through ligand-mediated engagement of transmembrane tyrosine kinase receptors. These results agree with the previously established requirement of FAK autophosphorylation for efficient recruitment of Src to FAs (Schaller et al., 1994).

We assessed the status of p190RhoGAP by immunoprecipitating it from total cell lysates and conducting an antiphosphotyrosine (p-Tyr) immunoblot analysis. p190RhoGAP was hypophosphorylated in Fak KO cells grown without serum, whereas comparable amounts of tyrosine-phosphorylated p190RhoGAP were detected when serum was present (Fig. 8 B). The requirement for FAK in phosphorylating and activating both Src and p190RhoGAP at integrin activation sites provides an underlying mechanism for the convergence of robust actin fibers at sites of FAs in Fak KO keratinocytes.

RhoGTP can activate ROCK, resulting in the phosphorylation and inactivation of the regulatory subunit of myosin light chain (MLC) phosphatase (myosin phosphatase target [MYPT]), leading in turn to increased phosphorylation and activation of MLC and stress fiber formation (Matsumura, 2005). To test whether this tension-signaling pathway is hyperactivated in Fak-null keratinocytes, we examined the levels and phosphorylation status of MYPT and MLC (Fig. 8 C). Although total

and cellular movements. Increasing evidence has implicated microtubule targeting to FAs as an initial step in FA turnover (Bershadsky et al., 1996; Kaverina et al., 1998, 1999; Small et al., 2002; Ezratty et al., 2005). To assess whether microtubule targeting to FAs might be altered by FAK deficiency, we used immunofluorescence and video microscopy to examine microtubule networks. In WT keratinocytes, microtubules formed a regular array around the nucleus and projected toward the cell periphery (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1). In Fak-null keratinocytes, this organization was perturbed, and the majority of microtubules pointed toward the robust FAs that were associated with massive stress fibers. Time-lapse analyses of keratinocytes transfected with either GFP-tubulin (Video 4) or a GFP-tagged plus-end microtubule binding protein (EB1; Video 5) further indicated that dynamic microtubules target robust, peripheral FAs, resulting in an altered distribution of the microtubule cytoskeleton in the absence of FAK.

Collectively, our data suggest that FAK modulates FA dynamics in mouse epidermal keratinocytes but it is not essential for the targeting of dynamic microtubules to FAs. Additionally, as microtubule targeting is thought to promote FA turnover, the presence of microtubules at FAK-deficient FAs implies that, whatever the mechanism involved in microtubule-mediated FA turnover, it is dysfunctional in the absence of FAK. This result supports previous observations where FAK-deficient embryonic fibroblasts do not disassemble FAs when microtubules repolymerize in response to washout of the microtubule depolymerizing drug nocodazole (Ezratty et al., 2005).
MYPT levels appeared to be slightly reduced, MYPT was hyperphosphorylated (inactive) at its ROCK-sensitive threonine residue. The outcome of these changes appeared to be a small but statistically significant increase in the total levels of active pMLC, as judged not only by immunoblot analysis but also quantitative immunofluorescence microscopy (Fig. 8, C–E). Moreover, in WT cells, anti-pMLC decorated the central actin fibers as well as the cortical actin network, whereas in Fak KO cells, anti-pMLC displayed a more robust decoration of the massive stress fiber network.

To determine whether this increase in Rho/pMLC-mediated tension is attributable to the spreading defects of Fak KO keratinocytes and/or their defective FA dynamics, we treated keratinocytes with the ROCK inhibitor Y-27632, the myosin inhibitor blebbistatin, and the MLC kinase (MLCK) inhibitor ML7, and then conducted immunofluorescence microscopy and quantitative relative integrated fluorescence intensity analyses to evaluate the effects on cell spreading, FA size, and FA localization. As shown in Fig. 9 (A and B), the differences in WT versus KO keratinocyte spreading were largely ameliorated by Y-27632 and blebbistatin, and to a lesser extent by ML7.

Quantitative analyses of FA staining intensity are shown in Fig. 9 C (see also Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1). These data further revealed that the statistical distribution of FAs in KO keratinocytes, which was highly skewed toward larger, more intensely stained FAs (mock), was significantly restored to normal upon treatment with these RhoA/tension-relieving drugs. In contrast, treatment with nocodazole, which depolymerizes microtubules and enhances Rho activity, resulted in increased FA size and VIN staining intensity even in KO cells. Quantitative analyses further revealed a clear shift in the distribution of FAs to a more peripheral location (Fig. S4).

**Fak-null focal contacts are defective at activating PAK**

MLCK can be phosphorylated and inhibited by PAK (Sanders et al., 1999), which has recently been proposed to form a complex with PKL, βPIX, and PXN at FAs (Turner et al., 1999; West et al., 2001; Nayal et al., 2006). To test whether the function of this complex might require FAK activity, we first examined the relative levels of PKL, βPIX, and PAK. As judged
by immunoprecipitation and immunoblot analyses, PKL was markedly reduced in KO keratinocytes (Fig. 10 A). This was confirmed by immunofluorescence microscopy where anti-PKL labeled WT FAs but was only weakly detectable at KO FAs (Fig. 10 B). This difference was substantial, as KO FAs are considerably larger and thus would have otherwise been expected to label more strongly with PKL.

By immunoblot analyses, overall levels of βPIX and PAK appeared to be largely unaffected by Fak deficiency (Fig. 10 A). However, even though βPIX localization appeared normal, PAK localization was markedly altered in Fak KO keratinocytes (Fig. 10 B). The epidermal outgrowth from FAK-deficient explants (cKO) is severely decreased compared with WT explants. White dotted lines mark leading edges; yellow arrows denote distance between explant and its leading edge. (B) Quantification of epidermal outgrowth from skin explants. Mean of three independent experiments ± SD. (C and D) Immunostaining for actin (red) and either E-cadherin (green) or PXN (green) reveals that cells, actin fibers, and FAs are highly polarized and oriented toward the leading edge in WT but not cKO explants. Bars, 20 μm. (E) Fak KO MKs exhibit a markedly reduced ability to migrate from the top to the bottom compartment of a Boyden chamber containing fibroblast-conditioned medium. Mean of three independent experiments ± SD. P = 0.0003, two-tailed t test.
Discussion

FAK versus αβ1 signaling in epidermal keratinocytes and skin explants

Of central importance to most cells is the ability to balance proliferation and differentiation, control adhesion to an underlying substratum, and when necessary, remodel this substratum and migrate in a directed fashion. These functions are particularly central to the mitotically active epidermal cell, which must continually undergo proliferation, detachment, and differentiation in the course of homeostasis, and upon injury, proliferate and migrate toward and repair the wound. Such movements are also important in hair follicle morphogenesis and cycling. The αβ1 integrins and their orchestrated association with the actin and microtubule cytoskeletons play a crucial role in these processes, and their downstream signal transducers are thought to contribute heavily to these events.

For the present study, we established a system that allowed us to directly compare the relative contributions of FAK to the many diverse functions of αβ1 integrins in the epidermis. Our studies provide compelling evidence that although αβ1 integrins are known to function broadly in cell adhesion, basement membrane assembly and organization, tissue homeostasis, and balancing growth and differentiation, FAK is selectively involved in efficient cell spreading, regulation of FA and cytoskeletal dynamics, and directed migration (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1). When our present data are coupled with our previous observation that FAK is hypophosphorylated in β1 KO keratinocytes (Raghavan et al., 2003), we could position FAK activation downstream of αβ1 signaling and upstream of this subset of cellular functions.

PYK2 localized to the FAK-deficient FAs. However, in contrast to the β1-deficient FAs, PYK2 was still phosphorylated in the absence of FAK. Hence, we surmise that some of the more global alterations seen in β1 KO cells may reflect the loss of overlapping but discrete subsets of signaling functions mediated through FAK and PYK2. Additionally, although ILK was still expressed comparably, its localization in β1-null keratinocytes was diffuse, suggesting that one or more of its functions (Legate et al., 2006) may be selectively compromised in the β1-null but not Fak-null state.

FAK-related activation, signal transduction, small GTPase regulation, and tension signaling

Our loss-of-function studies in keratinocytes revealed that Rho/ROCK signaling was hyperactive in the absence of FAK, as reflected by increased MLC phosphorylation and massive cortical stress fiber bundles, which converged on large, peripheral FAs. The enhanced Rho activity in Fak-null keratinocytes implies that FAK indirectly or directly must either activate RhoGEFs (guanine nucleotide exchange factors) or repress RhoGAPs (Etienne-Manneville and Hall, 2002). Consistent with this notion, p53/Fak-null fibroblasts are not able to suppress Rho activity during cell spreading (Ren et al., 2000).

Our data unveil an essential role for FAK in activating Src and p190RhoGAP upon integrin engagement. Thus, in keratinocytes, FAK is required for integrin/Src-dependent p190RhoGAP phosphorylation, suggesting a model where FAK/Src-induced phosphorylation of p190RhoGAP at FAs might locally promote RhoGTP hydrolysis to suppress Rho-induced stress fiber formation and FA stabilization (Fig. S5 B). Although we were unable to establish a physical association between endogenous p190RhoGAP and FAK.

Figure 8. FAK regulates p190RhoGAP and Rho/ROCK signaling. (A–C) Immunoprecipitation and immunoblot analyses of tyrosine phosphorylation status of Src (p1418-Src) in MKs cultured in the presence or absence of serum/growth factors [A]; tyrosine phosphorylation of p190RhoGAP in serum-starved Fak KO MKs [B]; and phosphorylation of the regulatory subunit of MLC phosphatase (MYPT) and MLC (pMLC), reflective of increased Rho/ROCK mediated tension [C]. (D and E) Enhanced pMLC in Fak KO versus WT MKs. Immunofluorescence microscopy of representative cells labeled with phosphorylated MLC antibodies (pMLC; pSer19 MLC; green), MLC antibodies (MLC; red), and nuclear chromatin [DAPI; blue]. Quantitative ratio imaging of pMLC and MLC indicates elevated pMLC levels in WT versus KO keratinocytes. (P = 1.59E-05, two-tailed t test; n ≥ 52 cells). Error bars indicate SEM.
FAK has been shown to bind p190RhoGAP and phosphorylate it in vitro (Holinstat et al., 2006). When coupled with the biochemical interactions between these molecules, the FAK, Src, and integrin loss-of-function analyses provide a tight molecular link between Fak/Src-mediated integrin signaling and the regulation of Rho activity via p190RhoGAP.

Rho activity can result in elevated stress fiber formation and stabilization of FAs (Chrzanowska-Wodnicka and Burridge, 1996), whereas RhoA inactivation by p190RhoGAP promotes cell spreading and migration (Arthur and Burridge, 2001). Consistent with the notion that elevated Rho is responsible for the defects in FA dynamics and cell spreading seen in our Fak-null keratinocytes, these defects could be largely suppressed by inhibitors of ROCK or myosin II. MLCK inhibition also had an effect, suggesting that multiple pathways are involved in regulating FA-mediated tension.

In fibroblasts, MLCK can be inactivated by PAK (Sanders et al., 1999), and a constitutively active form of PAK causes dissolution of stress fibers and FA reorganization (Manser et al., 1997). Our finding that endogenous phosphorylated (active) PAK is diminished at FAs in Fak-null keratinocytes extends these earlier inverse connections between activated PAK and MLCK-mediated tension. Moreover, when considered with the knowledge that PKL, βPIX, and PAK form a complex that binds to PXN at FAs (Frost et al., 1998; Manser et al., 1998; Turner et al., 1999; Zhao et al., 2000; West et al., 2001; Webb et al., 2004; Naylor et al., 2006), our data provide compelling evidence that inactivation of the PXN–PAK–MLCK pathway in the absence of FAK activity might lead to increased tension and larger, less dynamic FAs.

Our findings point to a function for FAK in localizing PKL–PIX–PAK to FAs to ease MLCK activity. How FAK regulates PKL–PIX–PAK localization remains unclear, but we do see a reduction in PKL at Fak-null FAs, which could account for the markedly reduced PAK activity at these sites. It was recently shown that PKL can be cooperatively phosphorylated on multiple tyrosine residues by FAK and Src to promote PKL localization to FAs (Brown et al., 2005). Overall, these data support a model whereby FAK promotes the recruitment and activation of PAK at FAs to suppress MLCK activity. This adds an additional way in which activation of FAK tips the balance in favor of the unphosphorylated (inactive) form of MLC to reduce tensile stress fibers associated with FA dynamics (Fig. S5 B).

**FAK, FA dynamics, directed cell migration, and cellular context**

The migratory behavior of cells is complex and can be altered by a variety of parameters. Providing a structural link between the matrix and the cytoskeleton are integrins and their associated scaffolding and signaling molecules. This machinery couples matrix stiffness to cellular tension and is a crucial determinant of cell motility (Gupton and Waterman-Storer, 2006). Our studies provide functional evidence to reinforce the notion that FAK is an essential component of FA distribution and dynamics.
within cells. In the absence of FAK, the small central FAs diminished dramatically in number and were replaced by large peripheral focal contacts. Kinetic studies on FA assembly and disassembly indicate that FAK’s major requirement resides in promoting FA disassembly, although moderate alterations in assembly rates were observed (this study; Webb et al., 2004).

The defects in directed migration of cells exiting from Fak-null skin explants were striking and graphically revealed the physiological consequences to tension-induced perturbations in actin organization. The reduced FA dynamics also appeared to contribute, as robust FAs were misoriented in FAK-deficient explants. The pronounced targeting of microtubules to these FAs is consistent with prior studies showing that microtubules grow along actin stress fibers (Kodama et al., 2003; Rodriguez et al., 2003). This finding suggests that despite their robust appearance, these FAs are still able to undergo the microtubule-mediated turnover first described by Small et al. (2002) (for review see Kaverina et al., 2002). Future studies will be necessary to explore these connections in depth.

The grossly perturbed epidermal migration that we observed is consistent with both the abnormalities seen in hair follicle morphogenesis and the resistance of Fak-null skin to tumorigenesis and metastasis (McLean et al., 2004; Essayem et al., 2006). Although postnatal ablation of FAK in the skin epithelium resulted in no significant wound healing defects (McLean et al., 2004; Essayem et al., 2006; unpublished data), delays in epidermal wound closure were noted in β1-null skin (Grose et al., 2002). We surmise that our enhanced ability to detect and monitor migration differences in skin explants arises from an increase in keratinocyte mobilization in vitro, where growth conditions are optimal and hemidesmosomal adhesion to ECM is reduced (Fuchs and Raghavan, 2002).

It is also noteworthy that FAK’s specific functions may vary both with changes in microenvironment and inherent differences in cell types. The importance of these contexts is underscored by Fak-null-mediated basement membrane assembly defects, which were observed in cerebellum (Beggs et al., 2003) but not in embryonic fibroblasts (Ilic et al., 1995) or skin epidermis (this study). Furthermore, although both Fak-null primary keratinocytes and immortalized Fak embryonic fibroblasts exhibit elaborate arrays of stress fibers and FAs that impair migration, FAK-deficient HeLa cells display elevated Rac rather than Rho activity and show enhanced rather than impaired migration (Ilic et al., 1995; Webb et al., 2004; Yano et al., 2004; Braren et al., 2006). In contrast, Fak-null endothelial cells spread poorly and display aberrant lamellipodial extensions and altered actin cytoskeleton, and yet surprisingly, they exhibit no obvious perturbations in polarized migration during vascularigenesis in tissue explants (Braren et al., 2006). Finally, Fak mutant fruit flies, which do not express a Pyk2-like gene, are surprisingly viable and fertile and show no defects in either integrin function or cell migration (Grabbe et al., 2004).

Summary

In closing, our analysis of FA dynamics in primary keratinocytes lacking FAK function has provided new insights and strengthened prior notions as to how FAK activity converges on the constellation of pathways that regulate cytoskeletal dynamics and balance FA assembly and disassembly in cells. Our findings are particularly interesting in the context of recent in vivo studies, which showed that oncogenic transformation is severely reduced in skin with reduced or abrogated FAK function (McLean et al., 2001, 2004). In this regard, FA signaling has recently been implicated in oncogenic transformation and activated FAK is a well-established marker for both transformation and metastasis. The identification of FAK-mediated alterations in several specific FA-associated proteins, cytoskeletal changes, and FA dynamics now illuminates several potential molecular pathways by which FA signaling might result in oncogenic transformation. These new findings provide fertile ground for future investigations in this arena.
Materials and methods

Mice and tissue culture

FAK floxed mice (Beggs et al., 2003), K14-Cre mice (Vasioukhin et al., 1999), and K14-GFPActin mice (Vaezi et al., 2002) have been described. Homozygous FAK floxed; K14-Cre and FAK floxed; and K14-cre; K14-GFPActin animals were generated by mating, and their genotypes were determined by PCR. Primary mouse keratinocytes (MKs) were isolated from the epidermis of newborn mice using trypsin, after prior separation of the epidermis from the dermis by a 2-h dispase treatment. MKs were plated on either mitomycin C-treated 3T3 fibroblast feeder cells or onto different ECM components. Cells were cultured in E-media supplemented with 15% serum and a final concentration of 0.05 mM Ca2+ different ECM components. Cells were cultured in E-media supplemented with 0.05 mM Ca2+ and incubated for 1 h. After 1 h, the medium was exchanged for medium containing 0.05 mM Ca2+. MKs were plated on FN-coated dishes for 6 h in E-media containing 0.05 mM Ca2+. After 6 h, the medium was exchanged for medium containing 5 μM ML7 (BIOREX Research Laboratories, Inc.) or 5 μM Y27632 (Calbiochem) and incubated for an additional 12 or 24 h before fixation. For ectopic expression and rescue experiments, MKs were transfected with RFP-zyxin (M. Beckerle, University of Utah, Salt Lake City, UT), pEGFP-PXN (E. Marcantonio, Columbia University Medical Center, New York, NY), and pBABE-FAK (Y. Pylevova and F. Giancotta, Memorial Sloan-Kettering Cancer Center, New York, NY) using Fugenex (Roche Applied Science).

FRAP and FA assembly/disassembly measurements

Kinetics of FA assembly and disassembly were performed as previously described (Webb et al., 2004). MKs were plated on FN-coated dishes (MatTek) in media containing 0.05 mM Ca2+ and transfected with GFP-PXN. FRAP experiments were performed on a microscope (DeltaVision; Stress Photonics) equipped with a 60× α-plane fluor (1.45 oil) lens and an EM charge-coupled device camera (Hamamatsu). The rate constants for FA assembly and disassembly were obtained by calculating the slope of relative fluorescence intensity increases or decreases of individual FAs on a semilogarithmic scale against time.

For FRAP experiments, MKs were plated on FN-coated 3.5-cm dishes in media containing 0.05 mM Ca2+ and transfected with GFP-PXN. FRAP experiments were performed on a microscope (DeltaVision; Stress Photonics) equipped with a 60× Plan APO N (1.42 oil) objective. The refractive index of the oil was 1.514 (Applied Precision). Five pre-bleach images were acquired followed by a 1-s bleach event. Fluorescence recovery was recorded for 120 s after photobleaching (71 frames), and data from these photokinetic experiments were analyzed using DeltaVision software.

Quantification of FA size, relative staining intensity, and distance from the cell periphery

MKs were fixed and stained with mouse anti-VIN and FITC anti-mouse antibodies to label FAs, TRIC-phalloidin to determine the cell boundaries, and DAPI. Pictures were acquired on a confocal microscope (510 META; Carl Zeiss Microlmaging, Inc.) at 63× C-Apochromat (1.2 W Korr) objective on a 12-bit scale. FA characteristics were quantified using MetaMorph 7 (Universal Imaging Corp.). An integrated morphometric analysis was performed on thresholded images to select classified objects of a size range of 0.1 × 0.1 to 1.0 × 1.0 μm. FAs were classified based on the anti-VIN staining intensity.

The distance between all FAs and the cell periphery was determined by selecting the cell boundaries based on the rhodamine-phalloidin staining for individual cells, conversion to a binary image, and inversion of the binary image. Subsequently, an euclidean distance map was generated from the inverted image. The euclidean distance map displays the closest distance at any point within the object (foreground) and the area surrounding the object (background) as an intensity value. Regions demarcating individual FAs of the given cell were transferred on the euclidean distance map where the minimum intensity of each individual FA corresponds to its closest distance to the cell edge.

Migration assays

Explant outgrowth migration assays were performed as described previously (Raghavan et al., 2003), with minor modifications. In brief, explants were cut using a 3-mm dermal biopsy punch (Millex), placed on FN-coated 35-mm, glass-bottomed plates (MatTek), and submerged in E-media containing 0.6 mM Ca2+. For video microscopy, explant cultures were incubated with E-media containing 0.6 mM Ca2+ and 50 mM HEPes buffer, pH 7. Transwell migration assays were performed on 24-well plates. The underside of each well was coated with 10 μg/mL FN and placed atop fibroblast-conditioned E-media containing 0.05 mM Ca2+. Primary MKs were freshly isolated, and a total of 50,000 cells/well were plated in 100 μl E-medium containing 0.05 mM Ca2+. 12 h later, cells were washed off the top membrane and fixed on the bottom membrane. Cells were stained using hematoxylin and eosin and counted under the microscope.

Immunofluorescence and immunoprecipitations

Tissues or cells were subjected to immunofluorescence microscopy and analyzed using a LSM 510 confocal microscope or a spinning-disc confocal microscope (PerkinElmer). mAbs used were as follows: rat anti-p1 (1:100; Chemicon), CD29 (REG7; BD Biosciences), α2 (1:50; BD Biosciences), αβ (1:50; BD Biosciences), αββ (1:100; Chemicon), β4 (1:50; BD Biosciences), β-tubulin (1:200; Sigma-Aldrich), mouse anti-VIN (Sigma-Aldrich), FAK (Upstate Biotechnology), PXN (Upstate Biotechnology), ILK (Upstate Biotechnology), Src (Upstate Biotechnology), PKL (BD Biosciences), FAK (Sigma-Aldrich), phosphotyrosine (4G10; Upstate Biotechnology), E-cadherin (Developmental Studies Hybridoma Bank), Erk1/2 (Sigma-Aldrich), and actin (Sigma-Aldrich). Rabbit polyclonal antibodies used were as follows: laminin 5 (1:200; a gift from R. Burgeson, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA), AKT (Cell Signaling Technology, phospho-AKT (Cell Signaling), pY414-38 (Biogenesis International), pY389-FAK (BioSource International), PYK2 (BD Bioscience), pY402-PYK2 (Abcam), p42/44 (Cell Signaling), MYPT1 (Upstate Biotechnology), phospho-MYPT1 (Upstate Biotechnology), and p190RhoGAP (Sigma-Aldrich). Primary antibodies were used at 1:100 unless specified otherwise. Fluorescent-conjugated secondary antibodies rhodamine RedX (Jackson ImmunoResearch Laboratories) and Alexa Fluor (Invitrogen) were used at 1:500. Additional reagents used were TRITC/FITC phalloidin (1:1,000; Sigma-Aldrich), DAPI (1:5,000; Sigma-Aldrich), and pEGFP-tubulin (CLONTECH Laboratories, Inc.). Protein concentration was determined using the protein assay (Bio-Rad Laboratories, Inc.). Immunoprecipitations were performed from cell lysates containing 1 mg of total protein. Cell lysates were precleared for 1 h using protein G–Sepharose beads before incubation with the immunoprecipitating antibodies. Primary antibodies were used at a concentration of 2–4 μg/ml lg according to manufacturers’ recommendations and incubated for 4 h at 4°C. Protein complexes were collected by incubation with protein G–Sepharose beads before incubation with the immunoprecipitating antibodies. Membranes were blocked for 30 min with 5% nonfat milk in PBS containing 0.1% Tween 20, except for phosphoproteins and antibodies to α and β tubulin, where membranes were blocked for 30 min with 3% BSA in PBS containing 0.1% Tween 20. Primary antibodies were generally used at a concentration of 1:1,000, and HRP-conjugated secondary antibodies were used at 1:3,000. Immunobots were developed using standard ECL (GE Healthcare) or Super Signal West Pico substrate (Pierce Chemical Co.).

Immunobots

Proteins were separated by electrophoresis on 6–10% PAGE gels or 4–12% gradient gels (Invitrogen), transferred to nitrocellulose membrane, and subjected to immunoblotting. Membranes were blocked for 30 min with 5% nonfat milk in PBS containing 0.1% Tween 20, except for phosphoproteins and antibodies to α and β tubulin, where membranes were blocked for 30 min with 3% BSA in PBS containing 0.1% Tween 20. Primary antibodies were generally used at a concentration of 1:1,000, and HRP-conjugated secondary antibodies were used at 1:3,000. Immunobots were developed using standard ECL (GE Healthcare) or Super Signal West Pico substrate (Pierce Chemical Co.).

FACS analyses

Freshly isolated primary MKs were washed twice with PBS blocking solution containing 2% FCS at 4°C. Cells were resuspended and incubated in primary antibody (1:50) in blocking solution for 30 min at 4°C, washed three times with blocking solution, and incubated with PE-conjugated secondary antibodies (1:100) for 30 min at 4°C. Cells were washed three times in blocking solution and resuspended in blocking solution containing propidium iodide. FACS analyses were performed on a FACSscan (Becton
Dickinson). BrdU incorporation was quantified by FACS using a FITC BrdU FACS Flow kit (BD Biosciences) following the manufacturer's instructions.

**EM**
Tissues were fixed for >1 h in 2% glutaraldehyde, 4% formaldehyde, and 2 mM CaCl₂ in 0.05 M sodium cacodylate buffer and then processed for Epon embedding. Samples were visualized with a transmission electron microscope (Hercules 12-G2; FEI). For hemidesmosome quantification, EM images were taken with a digital camera (model XR60; Advanced Microscopy Techniques Corp.) at a magnification of 49,000×. A total of 150 images were randomly taken at sites of the dermal–epidermal boundary for each experimental model. Total continuous membrane length and individual hemidesmosomes’ lengths along the plasma membrane were measured using ImageJ (NIH).

**Statistical analysis**
Statistical analysis was performed using OriginLab 7.5 software. Box-and-whisker plots are used to describe the entire population without assumptions on the statistical distribution. A t test was used to assess the statistical significance of differences between two experimental conditions, and analysis of variance in combination with a Tukey post hoc test was used to compare multiple experimental conditions.

**Online supplemental material**
Fig. S1 describes defects in hair follicle morphogenesis in mice conditionally mutant for FAK in the skin epithelium. Fig. S2 is a schematic representation of a box-and-whisker diagram. Fig. S3 shows the convergence of stress fibers and microtubules at FAs. Fig. S4 illustrates the distribution, relative VIN staining intensity, and size of FAs after treatment with small molecule inhibitors of tension-signaling components. Fig. S5 summarizes the presented data in a schematic model. Videos 1 and 2 are time-lapse videos illustrating the dynamics of FAs (RFPzyxin) and the actin cytoskeleton (GFPactin) in WT and KO keratinocytes. Video 3 is a time-lapse video of GFPtubulin. Video 4 is a time-lapse video of GFPtubulin in WT and KO keratinocytes. Video 5 is a time-lapse video of EB1-GFP in WT and KO keratinocytes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200608010/DC1.

**References**


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