Akt1 promotes focal adhesion disassembly and cell motility through phosphorylation of FAK in growth factor-stimulated cells

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Summary

The crosstalk between spatial adhesion signals and temporal soluble signals is key in regulating cellular responses such as cell migration. Here we show that soluble growth factors enhance integrin signaling through Akt phosphorylation of FAK at Ser695 and Thr700. PDGF treatment or overexpression of active Akt1 in fibroblasts increased autophosphorylation of FAK at Tyr397, an essential event for integrin turnover and cell migration. Phosphorylation-defective mutants of FAK (S695A and T700A) underwent autophosphorylation at Tyr397 and promoted cell migration in response to the integrin ligand fibronectin, but importantly, not in response to PDGF. This study has unveiled a novel function of Akt as an 'ignition kinase' of FAK in growth factor signaling and may shed light on the mechanism by which growth factors regulate integrin signaling.

Key words: Akt, FAK, Growth factor, Integrin, Migration

Introduction

Cell migration is essential for many biological processes including embryonic development, wound healing, and immune responses, and aberrant motility can also facilitate disease progression in cancer. Cell migration is mediated by signals from adhesion systems in combination with soluble signals such as growth factors (GFs). Although integrins and growth factor receptors (GFRs) can be activated independently by their own ligands, integrin-mediated adhesion to the extracellular matrix (ECM) can induce GFR activation and vice versa (Trusolino et al., 1998; Woodard et al., 1998; Zheng and Clemmons, 1998; Adelsman et al., 1999; DeMali et al., 1999). Evidence from several model systems has demonstrated that integrins can physically associate with GFRs, thereby regulating the capacity of integrins and GFRs to propagate downstream signaling (Miyamoto et al., 1996; Schneller et al., 1997; Goel et al., 2004). However, an indirect crosstalk that does not require an association between these receptors has also been proposed (Sieg et al., 2000; Aplin and Juliano, 2001; Baron et al., 2003). This type of crosstalk between integrins and GFRs may take place at the level of reorganization of cytoskeleton or lipid raft, or more downstream signaling molecules such as focal adhesion kinase (FAK).

FAK is a central regulator of integrin-mediated signaling. FAK is present in focal adhesions and is essential for focal adhesion turnover (e.g. disassembly) and cell migration (Ilić et al., 1995; Gilmore and Romer, 1996; Owen et al., 1999; Sieg et al., 1999; Webb et al., 2004). Clustering of integrins activates FAK, which results in autophosphorylation of Tyr397, formation of a complex with Src, and increased phosphorylation of the targets of the FAK–Src complex, such as paxillin and p130Cas (Schaller et al., 1994; Calalb et al., 1995; Schaller and Parsons, 1995; Tachibana et al., 1997). In addition to being a component of focal adhesions, FAK can physically associate with GFRs including c-Met, epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Sieg et al., 2000; Chen and Chen, 2006). Although previous reports proposed that GFR binding to FAK leads to activation of FAK by changing its conformation (Sieg et al., 2000; Chen and Chen, 2006), this is unlikely to be the case since a FAK mutant unable to bind to c-Met still could promote hepatocyte growth factor (HGF)-induced cell migration (Chen and Chen, 2006). Therefore the precise mechanism by which GFRs activate FAK has remained unclear.

Akt, a downstream target of GFRs, is a key regulator of biological processes including cell migration (Higuchi et al., 2001; Kim et al., 2001). The Akt family of serine-threonine kinases consists of three members: Akt1, Akt2 and Akt3. Although all the Akt family members share a similar domain structure, accumulating evidence suggests that they each have unique roles in various biological processes (Chen et al., 2001; Cho et al., 2001b; Cho et al., 2001a; Garofalo et al., 2003; Tschopp et al., 2005). In terms of cell motility, the specific role of each individual Akt family member seems to depend, in large part, on the cell type studied. In fibroblasts, which we use in this study, Akt1 specifically is responsible for promoting cell motility (Zhou et al., 2006; Higuchi et al., 2008). Although several Akt substrates; such as ACAP1, Girdin and Skp2; have been implicated in the regulation of cell migration (Enomoto et al., 2005; Li et al., 2005; Lin et al., 2009), it remains to be determined whether phosphorylation of these proteins is indeed required for Akt-mediated cell motility in various cellular contexts.

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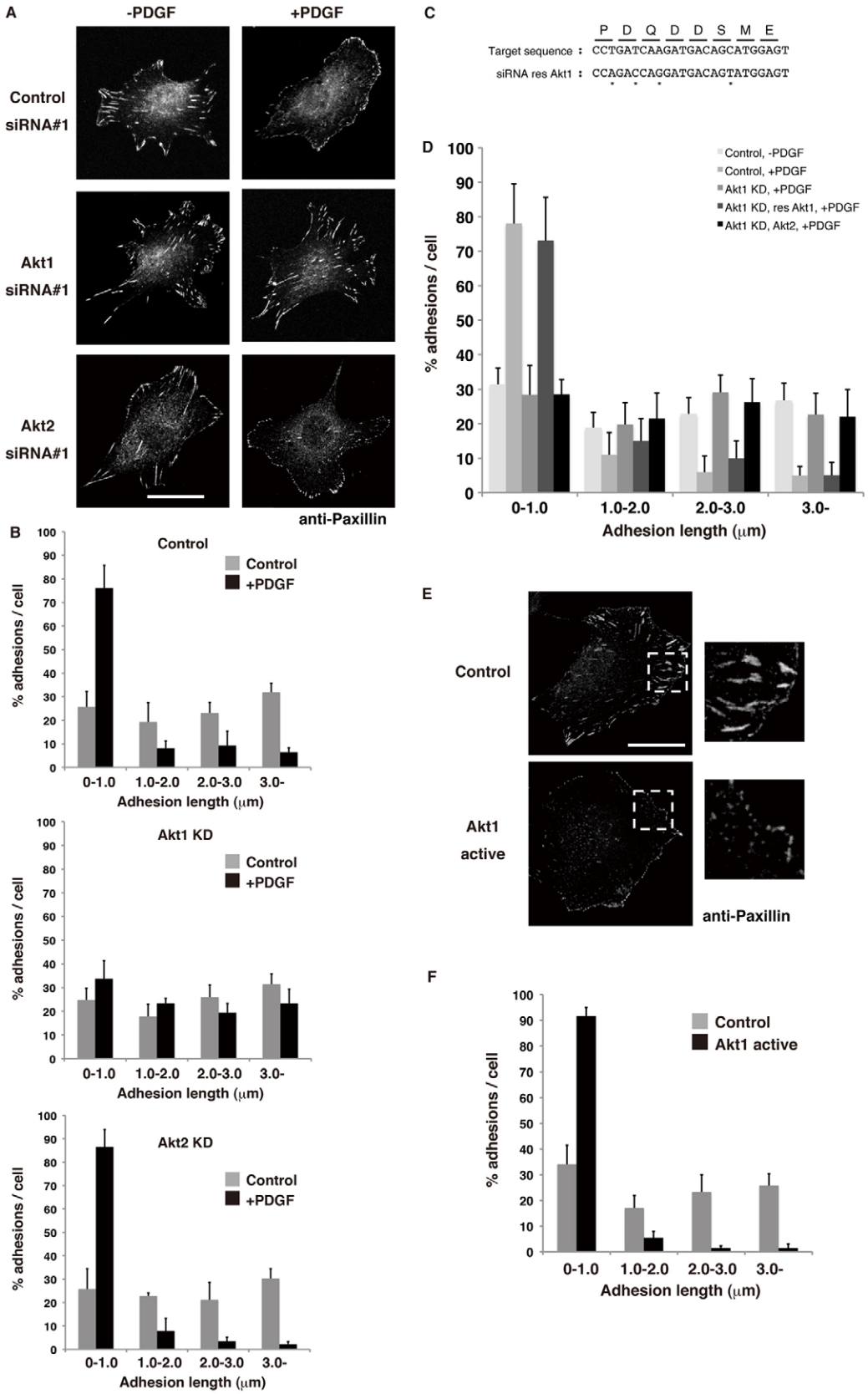

Fig. 1. See next page for legend.

In this study, we investigated the mechanisms by which Akt1 promotes cell motility, and found that in GF-stimulated cells Akt1, but not Akt2, regulates integrin-mediated cell adhesion through direct phosphorylation of FAK. Our results unveil a missing link between the integrin and GFR signaling pathways, which does not require physical association of receptor proteins.

Results

Akt1 mediates growth factor-induced focal adhesion disassembly

We have previously shown that Akt1, but not Akt2, plays an essential role in promoting cell motility in PDGF-stimulated fibroblasts (Higuchi et al., 2008), although the underlying mechanism has remained unclear. Since disassembly of focal adhesions (FAs) is important for promoting cell motility, and is observed after PDGFR activation (Heldin et al., 1998; Ruusala et al., 2008), we first examined whether Akt1 is involved in the disassembly of integrin-mediated cell adhesion. We stimulated NIH 3T3 cells with PDGF and performed immunocytochemistry using an antibody to the FA marker paxillin to visualize FAs. We found that FAs quickly disassembled after stimulation with PDGF (Fig. 1A,B), consistent with previous reports (Heldin et al., 1998; Ruusala et al., 2008). Importantly, we found that knockdown of Akt1, but not that of Akt2, suppressed PDGF-induced disassembly of FAs without overtly affecting the distribution of focal adhesions (Fig. 1A,B; supplementary material Fig. S1). To further examine the function of Akt1, we generated an Akt1 construct that is resistant to siRNA targeting by introducing silent mutations at the region targeted by siRNA (Fig. 1C and Fig. 2B). Expression of siRNA-resistant Akt1 (res Akt1), but not Akt2, restored PDGF-induced FA disassembly (Fig. 1D). These results confirm that Akt1, but not Akt2, is necessary for PDGF-induced disassembly of FAs in these cells.

Because we had previously found that active Akt1 can promote cell motility (Higuchi et al., 2001; Higuchi et al., 2008), we next asked whether expression of active Akt1 is sufficient for promoting FA disassembly. Expression of an active form of Akt1 (Akt1 mΔPH), a PH domain deletion mutant that contains a myristoylation site at the N-terminus, markedly decreased the size of FAs (Fig. 1E,F), a phenotype similar to that of PDGF-stimulated cells. In contrast, expression of a kinase-negative form of Akt1 did not decrease, but rather increased the size of FAs (data not shown). These results suggest that Akt1, but not Akt2, is essential for FA disassembly in PDGF-stimulated fibroblasts.

Fig. 1. Akt1 mediates GF-induced focal adhesion disassembly. (**A**) NIH 3T3 cells were plated on coverslips and transfected with the indicated siRNAs for 2 days. Cells were serum-starved for 4 h and then stimulated with 20 ng/ml PDGF for 15 min. After fixation with 4% formaldehyde, cells were stained with antibodies against paxillin. Scale bar: 20 μm. (**B**) Analysis of the length of paxillin-based adhesion structure in A. (**C**) The target sequence of siRNA for Akt1 (Akt1 no. 1) and the nucleotide substitution for the generation of the siRNA-resistant Akt1. (**D**) NIH 3T3 cells were transfected with the indicated siRNAs and plasmids as in C. Cells were then stained with antibodies against paxillin as in A. (**E**,**F**) NIH 3T3 cells were transfected with the indicated plasmids for 18 h. Cells were then stained with antibodies against paxillin as in A. Enlarged views of the indicated regions are shown on the right. Scale bar: 20 μm. Results are means ± s.d. from three independent experiments.

Akt1 mediates GF-induced, but not FN-induced, activation of FAK

It has been well documented that autophosphorylation of FAK at Tyr397 is an early and essential step for FA disassembly (Webb et al., 2004; Hamadi et al., 2005). Therefore, we next examined whether knockdown of Akt1 might affect autophosphorylation of FAK and found that it reduced PDGF-induced autophosphorylation of FAK (Fig. 2A). We also found that expression of siRNA-resistant Akt1 restored PDGF-induced autophosphorylation of FAK (Fig. 2B). Expression of Akt2, on the other hand, had little effect on the autophosphorylation of FAK (Fig. 2B). Moreover, expression of an active Akt1 promoted autophosphorylation of FAK at Tyr397 (Fig. 2C). These results suggest that Akt1 plays an important role in the activation of FAK.

Since FAK can be activated by either GFs or the extracellular matrix such as fibronectin (FN) (Matsumoto et al., 1994; Schaller et al., 1994; Abedi et al., 1995; Schlaepfer and Hunter, 1996), we next examined whether Akt1 mediates PDGF-induced and/or FN-induced activation of FAK. We first examined the effect of PDGF in suspended cells to eliminate the effect of integrin-mediated adhesion signals. NIH 3T3 cells were detached and incubated in suspension for 1.5 h. As shown in Fig. 2D, the addition of PDGF induced autophosphorylation of FAK at Tyr397 under this condition. We found that knockdown of Akt1 slightly but significantly reduced autophosphorylation of FAK (Fig. 2D). Knockdown of Akt1 had little effect on the level of tyrosine phosphorylation of PDGFR detected at ~190 kDa, suggesting that Akt1 is necessary for PDGF-induced activation of FAK without affecting the activation of GFRs.

We next examined the role of Akt1 in FN-induced activation of FAK. Serum-starved NIH 3T3 cells were detached and kept in suspension or replated on FN-coated tissue culture dishes. To our surprise, knockdown of Akt1 had no effect on FN-induced autophosphorylation of FAK (Fig. 2E). These results suggest that Akt1 mediates GF-induced, but not FN-induced, activation of FAK in NIH 3T3 cells.

Akt1 phosphorylates FAK at Ser695 and Thr700

To examine the mechanism by which Akt1 promotes phosphorylation of FAK at Tyr397, we asked whether Akt might directly phosphorylate FAK and thereby promote its catalytic activity. An *in vitro* kinase assay revealed that active Akt1 was capable of inducing phosphorylation of both FAK WT and a FAK mutant lacking the autophosphorylation site (FAK Y397F; Fig. 3B). In addition, kinase-negative Akt1 did not induce phosphorylation of FAK WT (Fig. 3B), indicating that Akt1 directly phosphorylates FAK *in vitro*.

To identify Akt1 phosphorylation site(s) on FAK, we have mutated potential phosphorylation sites on FAK, predicted based on the consensus sequence RxRxxS/T (and some RxxS/T) for Akt phosphorylation sites (Fig. 3A). We first focused on Ser517, Thr600 and Ser601 that are evolutionally conserved from chicken to human and located in the kinase domain (Fig. 3A). However, we found that mutation of Ser517, Thr600 and Ser601 into Ala (S517A or T600A, S601A) had little effect on the level of phosphorylation of the FAK fragment FAK(411–852 aa) by active Akt1 (Fig. 3C). We then focused on Ser695, because Ser695 is located in the proline-rich domain, which is required for promoting cell migration and is conserved among species (from chicken to human; Fig. 3A). Also, Ser695 was shown to be

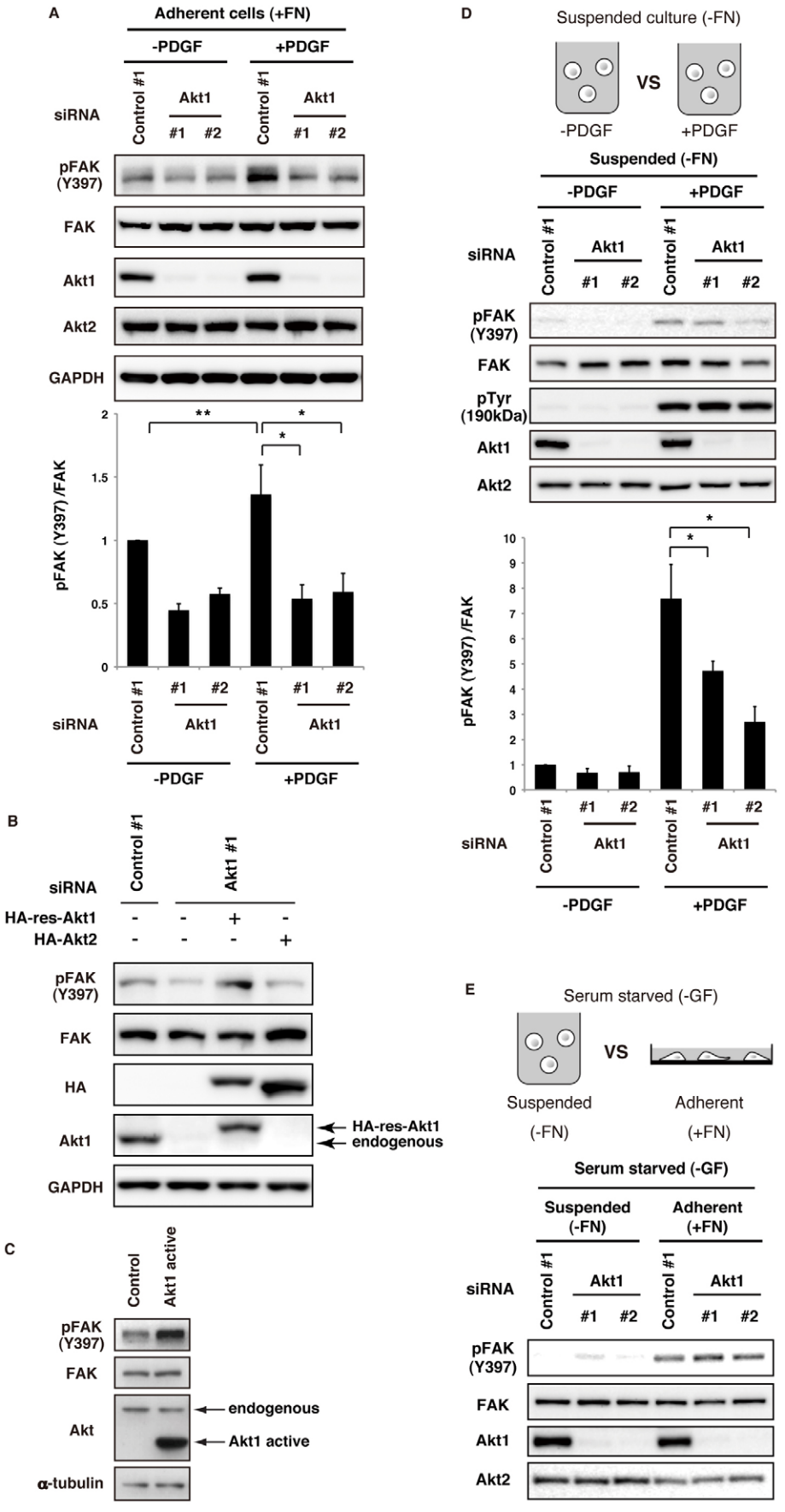

Fig. 2. Akt1 mediates GF-induced, but not FN-induced, activation of FAK. (**A**) NIH 3T3 cells were transfected with the indicated siRNAs for 3 days. Cells were then stimulated with 20 ng/ml PDGF for 15 min. Cell lysates were subjected to immunoblotting with the indicated antibodies. pFAK(Y397)/FAK ratios were then determined (*P<0.01, **P<0.05; t-test). (**B**) NIH 3T3 cells were transfected with the indicated siRNAs for 24 h. Cells were then transfected with the indicated plasmids for 18 h. Cells were serum-starved for 4 h and then stimulated with 20 ng/ml PDGF for 15 min. Cell lysates were subjected to immunoblotting with the indicated antibodies. (**C**) NIH 3T3 cells were transfected with the indicated plasmids for 18 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. (**D**) NIH 3T3 cells were transfected with the indicated siRNAs for 3 days. Serum-starved cells were detached and held in suspension for 1.5 h. Cells were then stimulated with or without 20 ng/ml of PDGF for 30 min. Cell lysates were subjected to immunoblotting with the indicated antibodies. pFAK(Y397)/FAK ratios were then determined (*P<0.05; t-test). (**E**) NIH 3T3 cells were transfected with the indicated siRNAs for 3 days. Serum-starved cells were detached and held in suspension for 1 h. Cells were then plated on FN-coated dishes or kept in suspension for 1 h. Cell lysates were then subjected to immunoblotting as in D. Note that knockdown of Akt1 suppressed PDGF-induced, but not FN-induced, phosphorylation of FAK (at Tyr397).

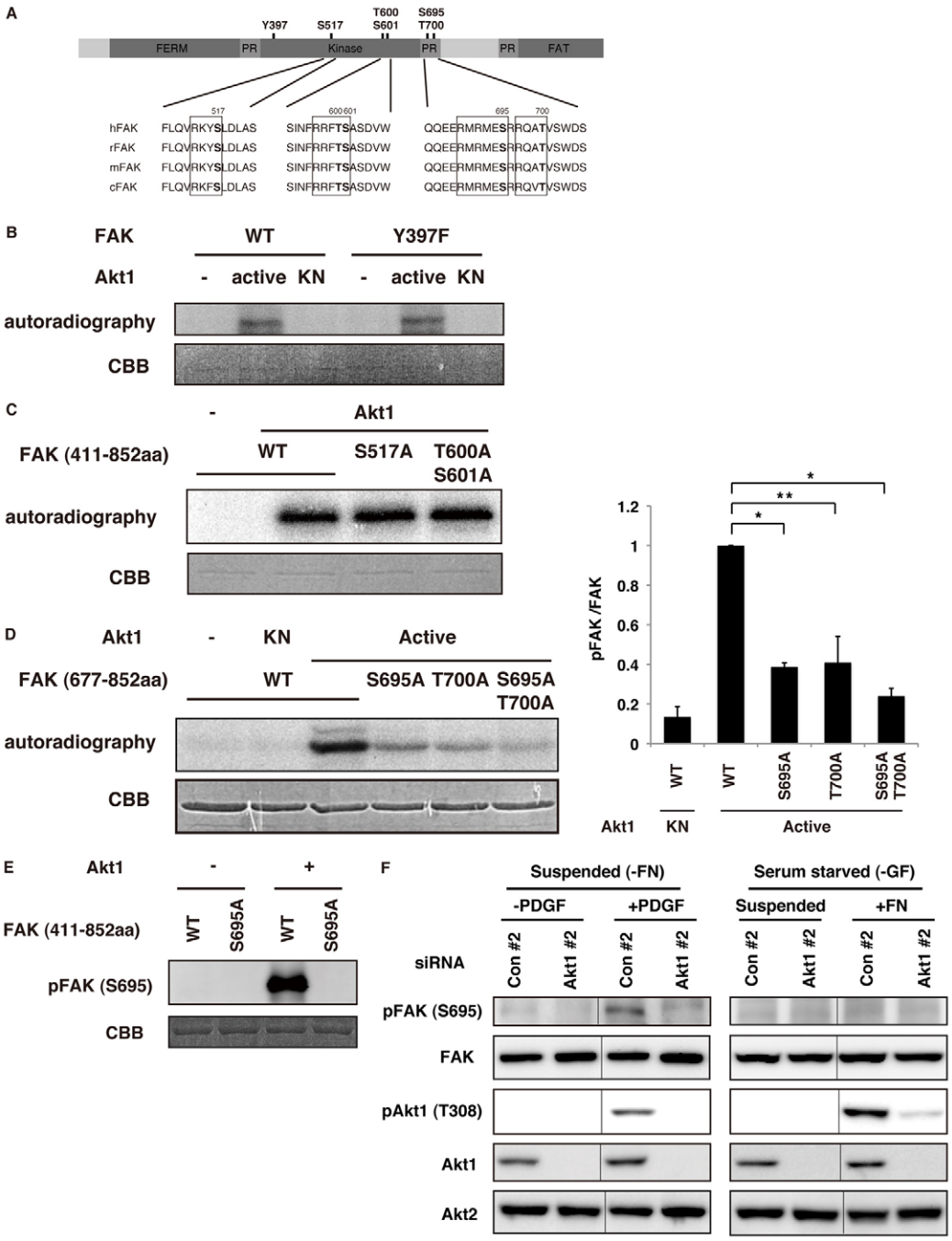

Fig. 3. Akt1 phosphorylates FAK both *in vitro* and *in vivo*. (**A**) Domain structure of FAK and the amino-acid sequences surrounding putative Akt1 phosphorylation sites [(Rx)RxxS/T, shown in bold]. (**B–D**) Akt1 phosphorylates FAK at Ser695 and Thr700 *in vitro*. Purified FAK full length (WT or Y397F; B), FAK(411–852 aa) (WT, S517A or T600A/S601A; C) or FAK(677–852 aa) (WT, S695A, T700A or S695A/T700A; D) were incubated with recombinant active or kinase negative (KN) Akt1 in the presence of [γ-^{32}P]ATP. Phosphorylated FAK was detected by autoradiography. The amount of FAK proteins or fragments were detected by CBB-R250 staining. (*P<0.005, **P<0.02; t-test). (**E**) Purified FAK fragment (411–852 aa) WT or S695A were phosphorylated *in vitro* in the presence or absence of active Akt1 and subjected to immunoblotting with anti-phosphorylated FAK (at Ser695). The amount of FAK fragments were detected by CBB-R250 staining. (**F**) NIH 3T3 cells were transfected with the indicated siRNAs for 3 days. Serum-starved cells were detached and held in suspension for 1.5 h (left panel) or 1 h (right panel). Cells were then stimulated with 20 ng/ml of PDGF or left unstimulated for 30 min (left panel) or plated on FN-coated dishes or kept in suspension for 1 h (right panel). Cell lysates were then subjected to immunoblotting with the indicated antibodies. All the lanes were run in the same gel and combined side by side.

essential for pressure-induced activation of FAK in a recent study (Wang and Basson, 2011). We then investigated T700 (Fig. 3A), which has recently been identified as a phosphorylation site in HEK293 cells by mass spectrometry (Grigera et al., 2005). An *in vitro* kinase assay revealed that mutations at Ser695 or Thr700 markedly reduced the level of FAK (full length or 677–852 aa)

phosphorylation (Fig. 3D; supplementary material Fig. S2). These results suggest that Akt1 directly phosphorylates FAK at Ser695 and Thr700 *in vitro*.

To understand the physiological role of phosphorylation at these amino-acid residues, we generated polyclonal antibodies that specifically recognize phosphorylated Ser695 or Thr700 of FAK (Fig. 3E; supplementary material Fig. S3) and examined the relationship between Ser695 and Thr700 phosphorylation. Mutation at either S695 or T700 reduced the level of phosphorylation at the other site (supplementary material Fig. S4), suggesting that phosphorylation at one site promotes phosphorylation at the other. By using this anti-phospho-FAK(Ser695) antibody, we further examined whether Akt1 mediates phosphorylation of FAK at Ser695 in NIH3T3 cells. We found that PDGF stimulation increased phosphorylation of FAK at Ser695 in these cells (Fig. 3F). Moreover, knockdown of Akt1 suppressed PDGF-induced phosphorylation of FAK at this site (Fig. 3F). Importantly, phosphorylation of FAK at Ser695 did not increase in FN-stimulated cells (Fig. 3F). These results strongly support the notion that Akt1 promotes phosphorylation of FAK at Ser695 in PDGF-stimulated, but not in FN-stimulated cells.

Ser695 and Thr700 are essential for GF-induced autophosphorylation of FAK

Given that Akt1 is necessary for PDGF-induced autophosphorylation of FAK, and that Akt1 can directly phosphorylate FAK at Ser695 and Thr700, it is possible that phosphorylation of FAK by Akt1 plays a role in activating FAK. To examine this possibility, either wild-type FAK (FAK WT) or FAK mutants lacking either of the phosphorylation sites (FAK S695A, T700A and S695A/T700A) were transiently expressed in FAK knockout (KO) mouse embryonic fibroblasts (MEFs), and tested their autophosphorylation levels in response to PDGF or FN stimulation in these cells. As shown in Fig. 4A, treatment with PDGF induced autophosphorylation of FAK WT expressed in adherent FAK KO MEFs, but cells with phosphorylation site mutants of FAK (S695A, T700A or S695A/T700A) showed less PDGF-induced autophosphorylation. To distinguish between PDGF-induced and FN-induced activation of FAK, we next examined the effect of PDGF in suspended cells. PDGF treatment induced autophosphorylation of FAK WT, but cells with FAK S695A or FAK T700A showed significantly less PDGF-induced autophosphorylation (Fig. 4B). In contrast, FAK S695A and FAK T700A had no effect on FN-induced autophosphorylation (Fig. 4C). The localization of FAK S695A and FAK T700A expressed in FAK KO MEFs were similar to that of FAK WT, which localized mainly at the FAs (Fig. 4D; supplementary material Fig. S5). These results suggest that Akt1 mediates GF-induced, but not FN-induced, activation of FAK in a Ser695- and Thr700-dependent manner without affecting the localization of FAK.

Ser695 and Thr700 of FAK are essential for GF-induced, but not FN-induced, cell migration

We next examined the effects of Akt-induced phosphorylation of FAK at Ser695 on the control of cell motility. To this end, we carried out a modified Boyden chamber migration assay with FAK KO MEFs. As shown in Fig. 5A,B,D, expression of FAK WT enhanced PDGF- and active Akt1-stimulated cell migration in FAK KO MEFs, whereas expression of FAK Y397F did not promote PDGF- and active Akt1-stimulated cell motility, indicating that Tyr397 of FAK is essential for PDGF- and active Akt1-stimulated cell motility in MEFs as expected. Importantly, FAK mutants lacking either of the Akt phosphorylation sites (FAK S695A and FAK T700A) were ineffective in promoting PDGF- and active Akt1-stimulated migration of FAK KO MEFs (Fig. 5A,B,D). In contrast, FAK S695A and FAK T700A, but not FAK Y397F, were capable of promoting FN-stimulated cell migration when transiently expressed in FAK KO MEFs (Fig. 5C). These results suggest that Ser695 and Thr700 of FAK is essential for GF-promoted, but not FN-promoted, cell motility.

Ser695 of FAK is essential for GF-induced focal adhesion turnover

To test whether the reduced cell motility of FAK S695A-expressing cells is a result of disregulation of the focal adhesion turnover, we next examined the effects of Akt1 on the focal adhesion turnover. To observe focal adhesion dynamics, GFP-paxillin was co-expressed with FAK WT or FAK S695A in FAK KO MEFs, and we analyzed the turnover of adhesions by live fluorescence imaging. FAK KO MEFs expressing a control vector showed large focal adhesions and impaired migration compared with WT MEFs (Fig. 6A; supplementary material Movie 1). The disassembly of focal adhesions was significantly impaired in FAK KO MEFs, consistent with previous reports (Fig. 6A,B; supplementary material Movie 1) (Ilić et al., 1995; Webb et al., 2004). As shown in Fig. 6A,B, the impaired migration and focal adhesion turnover in FAK KO MEFs were restored when FAK WT was expressed in these cells (Fig. 6A,B; supplementary material Movie 2). Importantly we found that FAK S695A was ineffective in promoting PDGF-stimulated focal adhesion turnover of FAK KO MEFs (Fig. 6A,B; supplementary material Movie 3). These results together support the notion that GF-activated Akt1 directly phosphorylates FAK at Ser695 (and Thr700), which results in activation of FAK, focal adhesion disassembly, and increased cell motility.

Discussion

Recently, we and other groups have demonstrated that Akt1, but not Akt2, plays a pivotal role in promoting the motility of fibroblasts (Zhou et al., 2006; Higuchi et al., 2008). However, how Akt1 promotes cell motility is not fully understood. In this study, we have shown that Akt1 plays an essential role in PDGF-induced FA disassembly in fibroblasts. Moreover, our results suggest that FAK is a major target of Akt1 in promoting cell motility in response to growth factors, based on several lines of evidence: (1) expression of active Akt1 induced phosphorylation of FAK at Tyr397; (2) knockdown of Akt1 suppressed phosphorylation of FAK at Tyr397 in response to PDGF; (3) Akt1 phosphorylated FAK at Ser695 and Thr700 *in vitro* and in NIH 3T3 cells in response to PDGF (Ser695); and (4) expression of phosphorylation site mutants of FAK (FAK S695A and T700A) inhibited PDGF- or active Akt1-induced cell migration. These results strongly suggest a novel connection between growth factor signaling and adhesion machinery.

It has been well documented that phosphorylation of FAK at Tyr397 is an early and essential step in the full activation of FAK that occurs in response to many extracellular stimuli including ECM and GFs (Webb et al., 2004; Hamadi et al., 2005). Phosphorylation of FAK at Tyr397 creates a motif that is

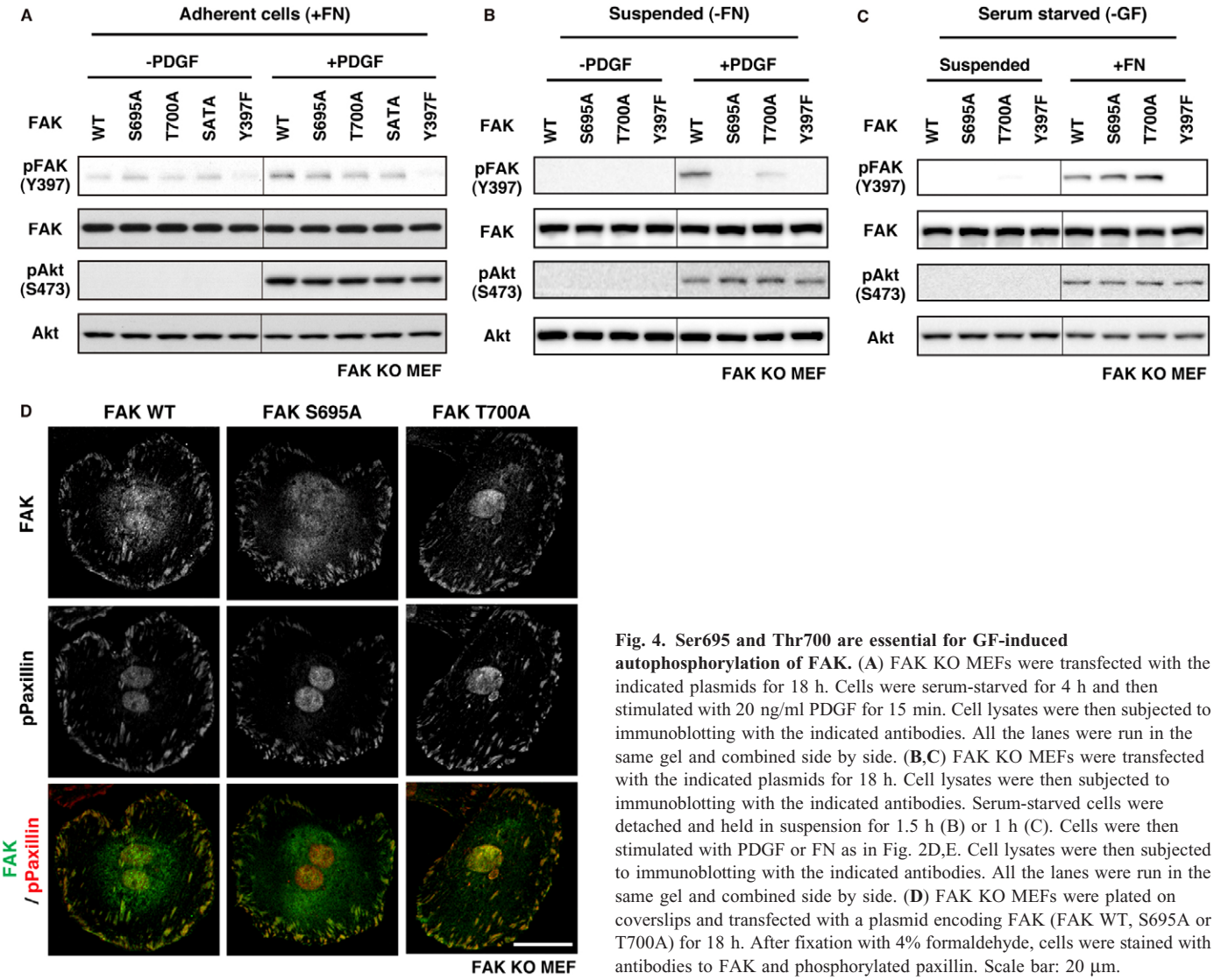

Fig. 4. Ser695 and Thr700 are essential for GF-induced autophosphorylation of FAK. (**A**) FAK KO MEFs were transfected with the indicated plasmids for 18 h. Cells were serum-starved for 4 h and then stimulated with 20 ng/ml PDGF for 15 min. Cell lysates were then subjected to immunoblotting with the indicated antibodies. All the lanes were run in the same gel and combined side by side. (**B**,**C**) FAK KO MEFs were transfected with the indicated plasmids for 18 h. Cell lysates were then subjected to immunoblotting with the indicated antibodies. Serum-starved cells were detached and held in suspension for 1.5 h (B) or 1 h (C). Cells were then stimulated with PDGF or FN as in Fig. 2D,E. Cell lysates were then subjected to immunoblotting with the indicated antibodies. All the lanes were run in the same gel and combined side by side. (**D**) FAK KO MEFs were plated on coverslips and transfected with a plasmid encoding FAK (FAK WT, S695A or T700A) for 18 h. After fixation with 4% formaldehyde, cells were stained with antibodies to FAK and phosphorylated paxillin. Scale bar: 20 µm.

recognized by various SH2 domain-containing proteins such as Src (Cobb et al., 1994; Xing et al., 1994). Within the FAK–Src complex, Src phosphorylates FAK at several tyrosine residues, resulting in the full activation of FAK (Calalb et al., 1995). A FAK mutant lacking the phosphorylation site (FAK Y397F) is ineffective in promoting FN- and PDGF-stimulated cell migration when transiently expressed in FAK KO MEFs (Sieg et al., 2000), indicating that phosphorylation of FAK at Tyr397 is a crucial step for both FN- and PDGF-stimulated cell migration. Interestingly, it has been shown in previous studies that the kinase activity of FAK is required for FN-stimulated, but not PDGF- or EGF-stimulated, cell migration in fibroblasts (Sieg et al., 1999; Sieg et al., 2000). These results suggest that the molecular mechanisms of FAK-mediated cell migration differ depending on whether the cell is activated by integrins or GFRs.

It has been well documented that phosphorylation of FAK at Tyr397 is mediated by autophosphorylation in FN-stimulated cells (Sieg et al., 1999; Lim et al., 2010). For GF-stimulated cells, however, Tyr397 may also be transphosphorylated by Src-family protein tyrosine kinases (PTKs), since expression of CSK, a negative regulator of the Src-family PTK, inhibited PDGF-stimulated cell migration (Sieg et al., 2000). This raises the question of how Src-family PTKs are recruited to FAK in GF-stimulated cells. The results in this study show that in fibroblasts phosphorylation at Ser695 and Thr700 is important for PDGF-induced phosphorylation of FAK at Tyr397 and for cell migration. We assume that phosphorylation of FAK at Ser695 and Thr700 by Akt1 leads to FAK activation through transphosphorylation by Src, which in turn results in FA disassembly, and increased cell motility. The idea that Akt1 functions as an ‘ignition kinase’ in activating FAK is further supported by the observation that Akt1 facilitates the formation of the FAK–Src complex in a kinase activity-dependent manner (data not shown).

With respect to the mechanism by which Akt1 regulates FAK activation and cell motility, we found, surprisingly, that Akt1 was required for PDGF-stimulated FAK activation and cell migration in fibroblasts, even though the same cells did not require Akt1 to

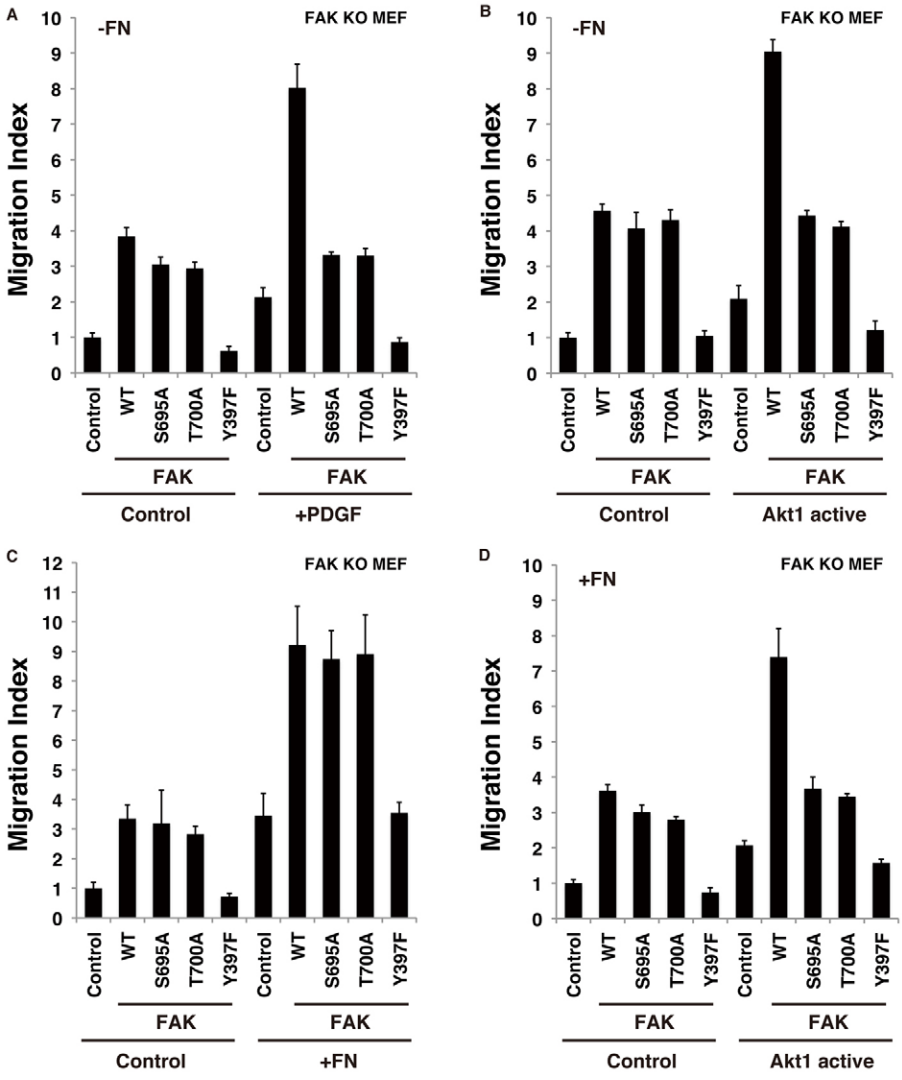

Fig. 5. Ser695 and Thr700 are essential for GF-induced, but not FN-induced, cell migration. (**A**) FAK KO MEFs were transfected with the indicated plasmids together with a plasmid encoding EGFP for 18 h. Cells were then transferred to the upper compartment of a modified Boyden chamber in the presence or absence of 20 ng/ml of PDGF. (**B**) FAK KO MEFs were transfected with the indicated plasmids as in A. Cells were then transferred to the upper compartment of a modified Boyden chamber. (**C**) FAK KO MEFs were transfected with the indicated plasmids as in A. Cells were then transferred to the upper compartment of a modified Boyden chamber. Both sides of the membrane of the chamber were coated with or without FN. (**D**) FAK KO MEFs were transfected with the indicated plasmids as in A. Cells were then transferred to the upper compartment of a modified Boyden chamber. Both sides of the membrane of the chamber were coated with FN. After 2 h, the number of GFP-positive cells that had migrated through the membranes was determined for each treatment. Results are presented as means ± s.d. from three independent experiments. Note that FAK promoted PDGF-induced cell migration in a manner dependent on S695 and T700.

promote FN stimulation of the same events. These results suggest either that in FN-stimulated cells there is a mechanism that bypasses the function of Akt1 or that only GF-activated Akt1 phosphorylates and activates FAK. In fact, we found that Akt1 can phosphorylate FAK at Ser695 in PDGF-stimulated cells but not in FN-stimulated cells (Fig. 3F). These results indicate that although Akt1 is activated by both FN and PDGF stimulation, the molecular mechanisms of Akt1-mediated signaling differ depending on extracellular stimuli. So, how does GF signaling induce a specific function of Akt1? We have previously shown that GF-activated PAK serves as a scaffold protein of the Akt1 pathway that regulates the target specificity of Akt1 (Higuchi et al., 2008). Since it has been reported that PAK is localized at FAs (Stoletov et al., 2001; Brown et al., 2002; Chan et al., 2008; Delorme-Walker et al., 2011) and that PAK forms a complex with FAK (Stoletov et al., 2001), it is possible that PAK may also serve as a scaffold protein that facilitate the association between Akt1 and FAK at FAs, resulting in Akt1-mediated phosphorylation and activation of FAK in GF-stimulated cells. Since it has been shown in a recent study that Ser695 is necessary for pressure-induced activation of FAK in Caco-2 cells (Wang and Basson, 2011), it would also be worth examining whether the PAK–Akt1 pathway is also involved in pressure-induced activation of FAK in these cells.

In a Boyden chamber migration assay, expression of FAK S695A or FAK T700A almost completely suppressed PDGF-stimulated cell migration (Fig. 5), although each of the mutations only partially reduced the Akt1-mediated phosphorylation (Fig. 3). These results suggest that phosphorylation at both sites (Ser695 and Thr700) is necessary for maximal activation of FAK. It would be worth examining whether Akt1 phosphorylation at these sites can induce the conformational change in FAK which leads to antophosphorylation of FAK, and if Akt1 phosphorylation of FAK can facilitate the recruitment of Src that activates FAK (Sieg et al., 2000).

In conclusion, we have demonstrated that Akt1 promotes FAK-mediated cell migration through direct phosphorylation of FAK. This finding may account for an indirect crosstalk between integrins and GFRs. Since Akt1 and FAK are overexpressed in a variety of invasive tumors, it will be interesting to examine the importance of the interaction between Akt1 and FAK in tumor progression.

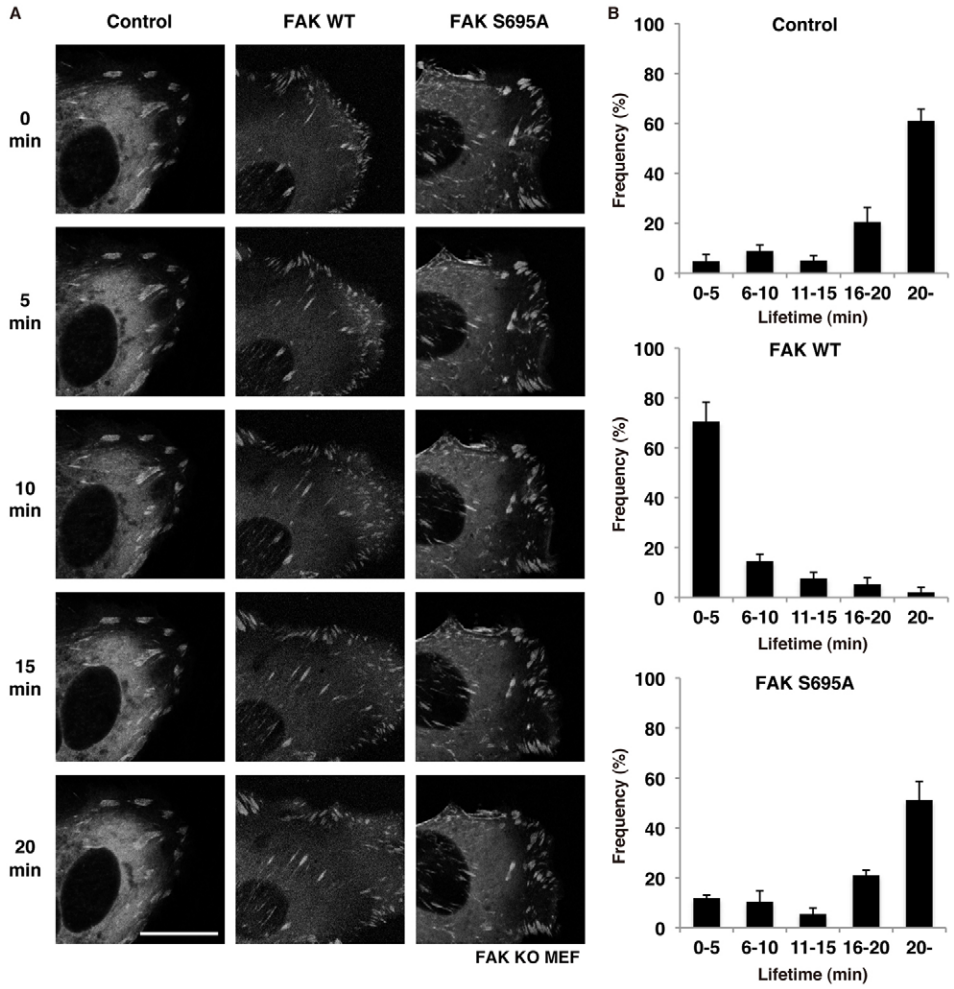

Fig. 6. Ser695 is essential for GF-induced focal adhesion turnover. (**A**) FAK KO MEFs were transfected with the indicated plasmids together with GFP-paxillin for 18 h. Cells were replated on glass-bottomed dish and imaged by time-lapse fluorescence microscopy (see supplementary material Movies 1–3). Scale bar: 20 μm. (**B**) Quantification of focal adhesion turnover rate. Note that FAK WT, but not S695A, could rescue the FA turnover defect in FAK KO MEFs. Results are presented as means ± s.d. from three independent experiments.

Materials and Methods

Plasmids, reagents and antibodies

The constructs encoding Akt1 was described previously (Higuchi et al., 2001; Higuchi et al., 2008). HA-tagged wild-type and Y397F mutant of FAK were kindly provided by Dr S. Hanks. The full-length mutants and the fragments of FAK (residue 677–852 and 411–852) were amplified by the polymerase chain reaction and cloned into the *Bam*HI site of pcDNA3.1. Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) to generate the Ser517 to Ala, Thr600 to Ala, Ser601 to Ala, Ser695 to Ala and Thr700 to Ala mutants of FAK. The antibodies used in this study include anti-FAK (Millipore), anti-phospho-FAK (Tyr397; Invitrogen), anti-phospho-FAK (Ser695), anti-paxillin (BD), anti-phospho-paxillin (Tyr 118; Invitrogen), anti-Akt ('pan' Akt; Cell Signaling), anti-Akt1 (Cell Signaling), anti-Akt2 (Cell Signaling), anti-phospho-Akt (Ser473; Cell Signaling), anti-phospho-Akt1 (Thr308; Millipore), anti-α-tubulin (Sigma), anti-pTyr (Millipore), anti-GAPDH (Chemicon), anti-Src (Cell Signaling) and anti-GFP (MBL).

Cell lines and transfection

NIH 3T3 cells and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). FAK KO MEFs were maintained in DMEM containing 10% fetal bovine serum (FBS) and 1% (v/v) NEAA (non essential amino acids). NIH 3T3 cells and FAK KO MEFs were transfected with plasmids by the use of Lipofectamine Plus (Invitrogen), and COS-1 cells were transfected with FuGENE6 (Roche) according to the manufacturer's instruction.

RNA interference

We used Akt1 Stealth siRNA (Invitrogen, Akt1-MSS235872) and Akt2 Stealth siRNA (Invitrogen, Akt2-MSS235874) to knockdown endogenous Akt1 and Akt2, respectively. As a second set of siRNAs for Akt1 or Akt2, we used Akt1 Stealth siRNA (Invitrogen, Akt1-MSS235873) and Akt2 Stealth siRNA (Invitrogen, Akt2-MSS235876). Transfection of siRNA duplexes was performed using the Lipofectamine RNAiMAX Reagent (Invitrogen). Cells were analyzed 3 days after transfection.

Immunoblotting

Cells were washed with PBS and lysed with a cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM β-glycerophosphate, 5 mM EGTA, 1 mM $Na_4P_2O_7$, 5 mM NaF, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM dithiothreitol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Cell lysates were subjected to immunoblotting with the indicated antibodies and the immune complexes were detected with chemiluminescence reagent (PerkinElmer).

Protein purification

The expression of GST-FAK was induced in *Escherichia coli* BL21 cells by culture in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside, and the recombinant protein was purified using of glutathione–Sepharose 4B (Amersham Biosciences). The glutathione-Sepharose was further incubated with PreScission protease to remove the GST tag from the fusion proteins.

In vitro kinase assay

COS-1 cells (4.0×10^5) were transfected with a plasmid encoding HA-tagged Akt1 (mΔPH or KN) for 18 h. Cells were then lysed in extraction buffer supplemented with protease inhibitors, and subjected to immunoprecipitation with antibodies to HA and used in an *in vitro* kinase assay. The kinase assay was performed in the presence of reaction mixture containing Akt1 immunoprecipitate, purified FAK proteins (fragments), 5 μCi of [γ-^{32}P]ATP and 100 μM unlabeled ATP, and incubated for 30 min at 30℃. Proteins were then resolved by SDS-PAGE and subjected to autoradiography. Recombinant Akt1 was used as the source of kinase activity in some experiments (Fig. 3C).

Cell staining

NIH 3T3 cells were transfected with siRNAs for either Akt1, Akt2 or their corresponding control siRNAs for 3 days. Cells were serum-starved in 2% FBS for 4 h and then stimulated with or without 20 ng/ml PDGF for 15 min. Cells were then fixed with 4% formaldehyde for 10 min at room temperature, permeabilized

with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min, and incubated with 2% fetal bovine serum (FBS) and 2% BSA in PBS for 30 min to block nonspecific antibody binding. Focal adhesions (FAs) were visualized by antibody to paxillin. After washing with PBS, the cells were stained with Alexa-Fluor-488-conjugated anti-mouse IgG. FAK KO MEFs were transfected with a plasmid encoding FAK (WT, S695A or T700A) or GFP-tagged FAK (WT, S695A or T700A) for 18 h for the experiments in Fig. 4C and supplementary material Fig. S5. Cells were then subjected to immunostaining as in Fig. 1A.

Migration assay

To assess the motility of cells, we performed the 'modified' Boyden chamber migration assay as described previously (Higuchi et al., 2001; Higuchi et al., 2008). Both sides of the membrane were coated with or without 10 μg/ml of fibronectin for 20 min at 37℃ and washed with PBS. Then the chambers were placed in 24-well dishes filled with DMEM containing 10% FBS. Transfected cells (1×10^5) were plated in the upper compartment and allowed to migrate through the pores of the membrane for 2 h. Cell motility was quantified by counting the GFP-positive cells that had migrated through the membranes by fluorescence microscope.

Analysis of focal adhesion dynamics

FAK KO MEFs were transfected with the indicated plasmids together with GFP–paxillin for 18 h and then replated on glass-bottomed dishes. Cells on the microscope stage were kept at 37℃ with a humid CO_2 atmosphere. Time-lapse images were captured at 1-minute intervals for 2 h. Focal adhesion turnover rate was assessed in at least five cells by determining the time period between formation and dissolution of the GFP–paxillin spot.

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