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ORIGINAL ARTICLE

Phosphorylation of focal adhesion kinase on tyrosine 194 by Met leads to its activation through relief of autoinhibition

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Focal adhesion kinase (FAK) has a crucial role in integration of signals from integrins and growth factor receptors. In this study, we demonstrate that growth factor receptors including hepatocyte growth factor receptor Met, epidermal growth factor receptor, and platelet-derived growth factor receptor directly phosphorylate FAK on Tyr194 in the FERM domain (band 4.1 and ezrin/radixin/moesin homology domain). Upon binding to Met or phosphoinositides, FAK may undergo conformational changes, which renders Tyr194 accessible for phosphorylation. Substitution of Tyr194 with Phe significantly suppresses the activation of FAK by Met. In contrast, substitution of Tyr194 with Glu (Y194E substitution) leads to constitutive activation of FAK. The phosphorylation of FAK on Tyr194 may cause conformational changes in the FERM domain, which disrupts the intramolecular inhibitory interaction between the FERM and kinase domains of FAK. Moreover, substitution of the basic residues in the ^{216}KAKTLRK^{222} patch in the FERM domain with Ala antagonizes the effect of the Y194E substitution on FAK activation, thus suggesting that the interactions between the phosphorylated Tyr194 and the basic resides in the ^{216}KAKTLRK^{222} patch may allow FAK to be activated through relief of its autoinhibition. Collectively, this study provides the first example to explain how FAK is activated by receptor tyrosine kinases.
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Introduction

Focal adhesion kinase (FAK), a 125 kDa non-receptor tyrosine kinase localized in focal adhesions, is known for its pivotal role in the control of a wide variety of cell functions, including cell migration, cell cycle progression, cell survival and tumor progression (reviewed by McLean *et al*., 2005; Mitra *et al*., 2005). FAK was originally identified as a substrate of Src and subsequently found to be activated upon cell adhesion to extracellular matrix proteins (Guan and Shalloway, 1992; Hanks *et al*., 1992; Schaller *et al*., 1992). Later, it was also found to be activated by a number of growth factors (Matsumoto *et al*., 1994; Rankin and Rozengurt, 1994; Chen *et al*., 1998; Sieg *et al*., 2000). It is generally believed that autophosphorylation of FAK on a particular tyrosine residue, Y397, is an early, essential step for the full activation of FAK in response to many extracellular stimuli. The Y397 is the major site of FAK autophosphorylation, which creates a high affinity binding site for the Src-homology 2 domain of several proteins including the Src family kinases (Cobb *et al*., 1994; Schaller *et al*., 1994; Xing *et al*., 1994; Eide *et al*., 1995). Activated Src phosphorylates FAK on multiple sites, including Y576 and Y577, both of which are located in the activation loop within the kinase domain (Calalb *et al*., 1995; Lietha *et al*., 2007). The ensuring phosphorylation of FAK by Src on Y576 and Y577 is required for the full enzymatic activity of FAK (Calalb *et al*., 1995; Lietha *et al*., 2007).

FAK contains a central tyrosine kinase domain flanked by large NH2- and COOH-terminal regions. The COOH-terminus contains a focal adhesion targeting domain responsible for FAK localization in focal adhesions (Hildebrand *et al*., 1993). The NH2-terminus contains a region of sequence homology with FERM domain (band 4.1 and ezrin/radixin/moesin proteins). The FERM domain of FAK has been described to involve in interactions with other proteins including the cytoplasmic region of integrins (Schaller *et al*., 1995; Chen *et al*., 2000), the FERM domain of ezrin (Poullet *et al*., 2001), the pleckstrin homology domain of the Tec-family kinase Etk (Chen *et al*., 2001), the Arp2/3 complex (Serrels *et al*., 2007) and receptor tyrosine kinases (RTKs), including platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) and hepatocyte growth factor (HGF) receptor (Sieg *et al*., 2000; Chen and Chen, 2006). It has been proposed that an intramolecular inhibitory interaction between the FERM and kinase domains of FAK suppresses its catalytic activity (Cooper *et al*., 2003; Dunty *et al*., 2004). Truncation of the FERM domain increases FAK tyrosine phosphorylation and/or activity (Cooper *et al*., 2003; Dunty *et al*., 2004; Jácamo and Rozengurt, 2005). Mutation of K38 within the FERM domain also leads to FAK activation (Cohen

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and Guan, 2005). Crystal structure analysis reveals that the FERM domain of FAK directly binds to its own kinase domain, blocking access to the catalytic cleft and protecting the FAK activation loop from Src phosphorylation (Lietha *et al*., 2007). At the center of the autoinhibitory interaction between the FERM and kinase domains of FAK, F596 in the kinase domain inserts into a hydrophobic pocket in the FERM domain formed by Y180, M183, V196 and L197 (Lietha *et al*., 2007). More recently, dynamic changes of FAK from autoinhibited to active conformation were verified in intact cells (Cai *et al*., 2008).

We have previously described that FAK is phosphorylated and activated upon HGF stimulation (Chen *et al*., 1998), which thereby contributes to HGF-induced cell scattering (Lai *et al*., 2000). In addition, elevated expression of FAK renders epithelial cells susceptible to cellular transformation by HGF stimulation (Chan *et al*., 2002). Recently, we demonstrated that FAK directly interacts with the HGF receptor (that is, Met) upon HGF stimulation (Chen and Chen, 2006). This interaction occurs through the FERM domain of FAK and phosphorylated Y1349 and Y1356 of c-Met. In particular, a basic patch (^{216}KAKTLRK^{222}) in the FERM domain is crucial for the interaction (Chen and Chen, 2006). In this study, we aim to examine whether the FAK FERM domain is phosphorylated by Met and investigate the significance of this phosphorylation event.

Results

Met, EGFR and PDGFR phosphorylate the NH2 domain of FAK on Y5 and Y194

As activated Met physically interacts with the FERM domain of FAK (Chen and Chen, 2006), we speculated that Met may phosphorylate the FERM domain of FAK and thereby activate its catalytic activity. As shown in Figure 1a, the constitutively active form of c-Met (Tpr-Met), but not oncogenic Src or FAK itself, induced tyrosine phosphorylation of the NH2 domain (aa. 1–391) of FAK. In contrast, the NH2 domain (aa. 1–397) of PYK2, another member in the FAK family, could not be phosphorylated by Tpr-Met (Figure 1b). To identify the phosphorylation sites in the NH2 domain of FAK, every tyrosine residue in this region was substituted with Phe (Supplementary Figure S1). Of total 13 tyrosine residues in this region, mutation at Y5 or Y194 caused a partial decrease in the tyrosine phosphorylation of FAK NH2 domain induced by Tpr-Met (Figure 1c). Mutation of both Y5 and Y194 eliminated the phosphorylation of FAK NH2 domain by Tpr-Met both in intact cells (Figure 1c) and *in vitro* (Figure 1d). Interestingly, Y5 and Y194 are conserved in FAK, but not in PYK2 (Figure 1e). Moreover, like Tpr-Met, c-Met, EGFR and PDGFR were able to phosphorylate FAK NH2 domain, but not its Y5F/Y194F mutant in intact cells (Figure 2a). *In vitro*, c-Met and EGFR failed to phosphorylate the Y5F/Y194F mutant, whereas PDGFR phosphorylated the Y5F/Y194F mutant to some extent (Figure 2b), indicating that PDGFR could phosphorylate FAK NH2 domain at other sites in addition to Y5 and Y194 *in vitro*. These results indicate that RTKs including c-Met, EGFR and PDGFR directly phosphorylate FAK NH2 domain on Y5 and Y194.

The phosphorylation of FAK on Y194 by Met was further demonstrated by a phospho-specific antibody (referred as anti-FAK pY194), which recognizes FAK, but not the Y194F mutant, in the presence of Tpr-Met (Figures 3a and b). Moreover, dephosphorylation of FAK by the tyrosine phosphatase SHP2 prevented it from detection by anti-FAK pY194 (Figure 3c), indicating that this antibody truly recognizes the phosphorylated Y194 of FAK rather than dephosphorylated Y194. Although the Y194F mutant remained phosphorylated at other tyrosine residues (Figure 3b), it was not detected by anti-FAK pY194 antibody, supporting the specificity of this antibody to the phosphorylated Y194 in FAK. Unfortunately, generation of an antibody specific to the phosphorylated Y5 of FAK was not successful.

An initial conformational change in FAK may render Y194 accessible for phosphorylation

The crystal structure of FAK reveals that Y194 is mostly buried in the structure and is not a good substrate for a kinase (Lietha *et al*., 2007; Supplementary Figure S2). Thus, it can be assumed that initial conformational changes in FAK would be necessary in order to render this residue accessible for phosphorylation. To address this question, we first examined whether Met binding has an impact on the Y194 phosphorylation. We have previously demonstrated that phosphorylation of Met on Y1349 and Y1356 are required for it to bind the FERM domain of FAK (Chen and Chen, 2006). Mutation of Met at both sites did not affect its catalytic activity, but decreased its capability to phosphorylate FAK Y194 in intact cells (Figure 4a) and *in vitro* (Figure 4b), thus suggesting that Met binding may have an impact to the conformation of the FERM domain, which thereby render Y194 accessible for phosphorylation.

Phosphatidylinositol 4,5-P_2 has been shown to interact with FAK FERM domain and induce conformational changes in FAK, leading to FAK activation (Cai *et al*., 2008). We found that phosphatidylinositol 4,5-P_2 and, to a lesser extent, phosphatidylinositol 3,4,5-P_3 facilitated Y194 phosphorylation of the FAK-NK fragment (containing both the NH2 domain and kinase domain of FAK), but not the NH2 domain alone, *in vitro* (Figures 4c and d). These results suggest that phosphoinositide binding to the FERM domain may have an impact to the intramolecular interaction between the FERM and kinase domains of FAK rather than the conformation of the FERM domain itself, which leads to accessibility of Y194 for phosphorylation. Together, our results suggest that upon binding to Met or phosphoinositides, Y194 of FAK may become accessible for phosphorylation.

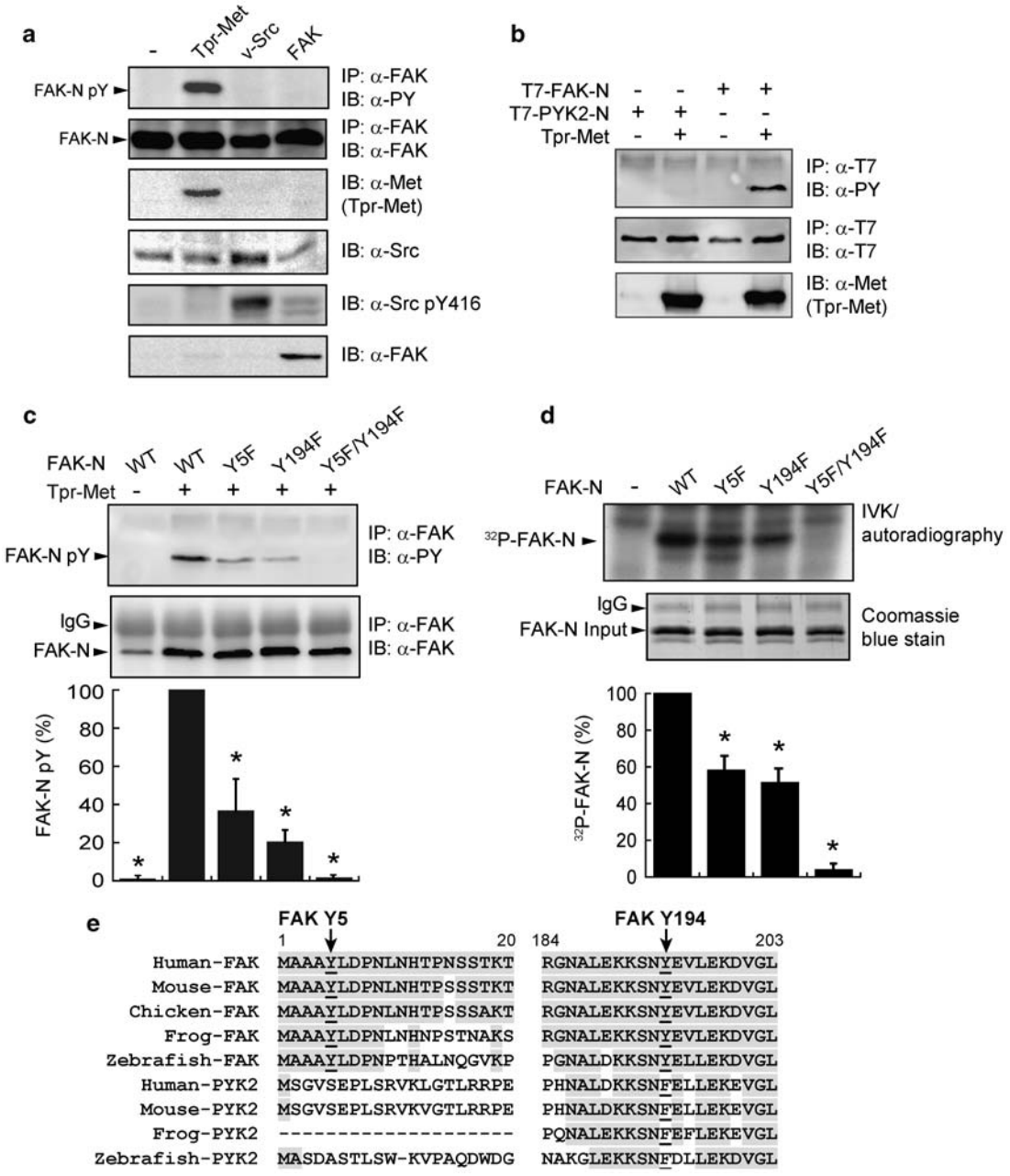

Figure 1 Tpr-Met phosphorylates the NH2 domain of FAK on Y5 and Y194. (**a**) FAK-NH2 domain (FAK-N) was transiently co-expressed with Tpr-Met, v-Src or FAK in HEK293 cells. FAK-NH2 domain was immunoprecipitated by anti-FAK and the washed immunocomplexes were analyzed by immunoblotting with anti-PY or anti-FAK. An equal amount of whole cell lysates was analyzed by immunoblotting with antibodies as indicated. (**b**) T7-tagged FAK-NH2 domain (T7-FAK-N) and T7-tagged PYK2-NH2 domain (T7-PYK2-N) were co-expressed with (+) or without (−) Tpr-Met in HEK293 cells. T7-tagged proteins were immunoprecipitated by anti-T7 and their tyrosine phosphorylation were analyzed. An equal amount of whole cell lysates was analyzed by immunoblotting with anti-Met. (**c**) FAK-NH2 domain and its mutants were transiently co-expressed with (+) or without (−) Tpr-Met in HEK293 cells. The tyrosine phosphorylation of FAK-NH2 domain was analyzed as described in panel (**a**). The tyrosine phosphorylation of the FAK-NH2 domain proteins was quantified and expressed as the percentage relative to the level of the wt FAK-NH2 domain, which is defined as 100%. Values (means ± s.d.) are from three independent experiments. $*P<0.05$. The position of immunoglobulin G (IgG) is indicated. (**d**) Tpr-Met was transiently expressed in HEK293 cells, immobilized on protein A beads with anti-Met, and subjected to an *in vitro* kinase assay using purified FAK-NH2 domain as substrates. Aliquots of purified proteins same as those used in *in vitro* kinase assays were fractionated by SDS–PAGE and visualized by Coomassie blue stain. The ^{32}P-incorporation of FAK-NH2 domain was quantified and expressed as the percentage relative to the level of the wt FAK-NH2 domain, which is defined as 100%. Values (means ± s.d.) are from three independent experiments. $*P<0.05$. IVK, *in vitro* kinase assay. (**e**) Two segments of the amino acid sequences (aa. 1–20 and aa. 184–203) of FAK from different species were aligned. The corresponding sequences of PYK2 were also included in the alignment. Note that Y5 and Y194 are highly conserved in FAK, but no tyrosine residue is found in the corresponding positions in PYK2.

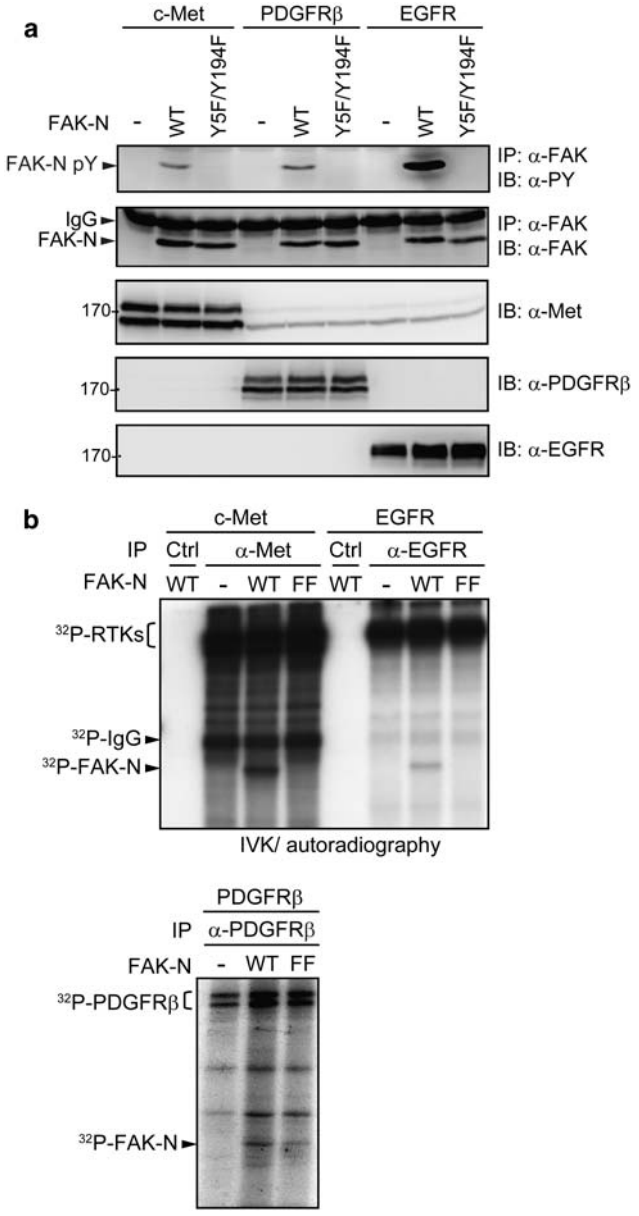

Figure 2 Y5 and Y194 of FAK are the phosphorylation sites for c-Met, EGFR and PDGFRβ. (**a**) FAK-NH2 domain (FAK-N) and its Y5F/Y194F mutants were transiently co-expressed with c-Met, EGFR, or PDGFRβ in HEK293 cells. The tyrosine phosphorylation of FAK-NH2 domain was analyzed as described in Figure 1a. An equal amount of whole cell lysates was analyzed by immunoblotting to monitor the expression of c-Met, EGFR, and PDGFRβ. (**b**) c-Met, EGFR and PDGFRβ was transiently expressed in HEK293 cells, immobilized on protein A beads with specific antibodies, and subjected to *in vitro* kinase assays using purified FAK-NH2 domain as substrates in the presence of [γ-^{32}P] ATP. Pre-immune control immunoglobulin was used as a control (Ctrl). Representative autoradiograph from three experiments is shown. WT, wild type; FF, Y5F/Y194F mutant; RTKs, receptor tyrosine kinases. IVK, *in vitro* kinase assay.

Phosphorylation of FAK on both Y194 and Y397 is essential for its maximal activation by Met

As Y194 is adjacent to the interface between the FERM and kinase domains of FAK (Supplementary Figure S2), we speculated that phosphorylation on this residue may have an impact to the FERM–kinase interaction and thereby affect the catalytic activity of FAK. To examine this possibility, FAK and its YF mutants were transiently co-expressed with Met and the phosphorylation of FAK on Y577 was measured (Figure 5a). The Y577 is located within the catalytic domain of FAK and its phosphorylation level has been used to reflect the catalytic activity of FAK (Calalb *et al*., 1995; Lietha *et al*., 2007). The Y577 phosphorylation of wild type (wt) FAK was apparently increased by Met. Substitution of Y5 did not affect the activation of FAK by Met (Supplementary Figure S3), indicating that Y5 is not involved in Met-induced activation of FAK. However, substitution of Y194 partially (~50%) suppressed FAK activation by Met. Substitutions of both Y194 and Y397 completely abolished the activation of FAK by Met (Figure 5a). These results indicate that the phosphorylation of Y194 plays a critical role in FAK activation and, more importantly, the phosphorylation of FAK on both Y194 and Y397 is essential for its maximal activation by Met. Notably, Met was able to increase the Y577 phosphorylation of the Y397F mutant to a level approximately 50% of that of wt FAK (Figure 5a), indicating that Met could cause FAK phosphorylation on Y577 through an Y397-independent mode.

We have previously demonstrated that FAK overexpression promotes the effect of HGF on cell migration (Lai *et al*., 2000) and renders epithelial cells susceptible to cellular transformation by HGF (Chan *et al*., 2002). In this study, we found that unlike the wt FAK, overexpression of Y194F or Y397F mutant in Madin–Darby canine kidney (MDCK) cells failed to promote their migration towards HGF (Figure 5c). Additionally, overexpression of FAK, but not Y194F or Y397F mutant, promoted HGF-induced anchorage-independent growth of MDCK cells (Figure 5d). These results together indicate that the phosphorylation of FAK on Y194 by Met is crucial not only for FAK activation but also for FAK to promote HGF-elicited cell functions.

Substitution of Y194 with Glu leads to constitutive activation of FAK

To mimic their phosphorylation states, Y5 and/or Y194 of FAK were substituted with Glu. Our results showed that the Y577 phosphorylation of the Y5E mutant remained at a level same as that of the wt FAK (Figure 6a), consistent with our notion that Y5 is not involved in the regulation of FAK's catalytic activity. In contrast, the Y194E mutant and the V196E mutant had much higher phosphorylation on Y397 and Y577 than the wt did (Figure 6a). As V196 of FAK is one of the residues that constitute the hydrophobic pocket essential for the FERM–kinase interaction (Lietha *et al*., 2007), substitution of V196 with a negatively charged residue may be sufficient to disrupt the hydrophobic pocket and thereby prevent the FERM–kinase interaction. In accordance with an increase in the Y577 phosphorylation, both Y194E and V196E mutants possessed much higher catalytic activity than the wt did (Figure 6b), correlated with a robust increase in the tyrosine phosphorylation of paxillin, a physiological substrate

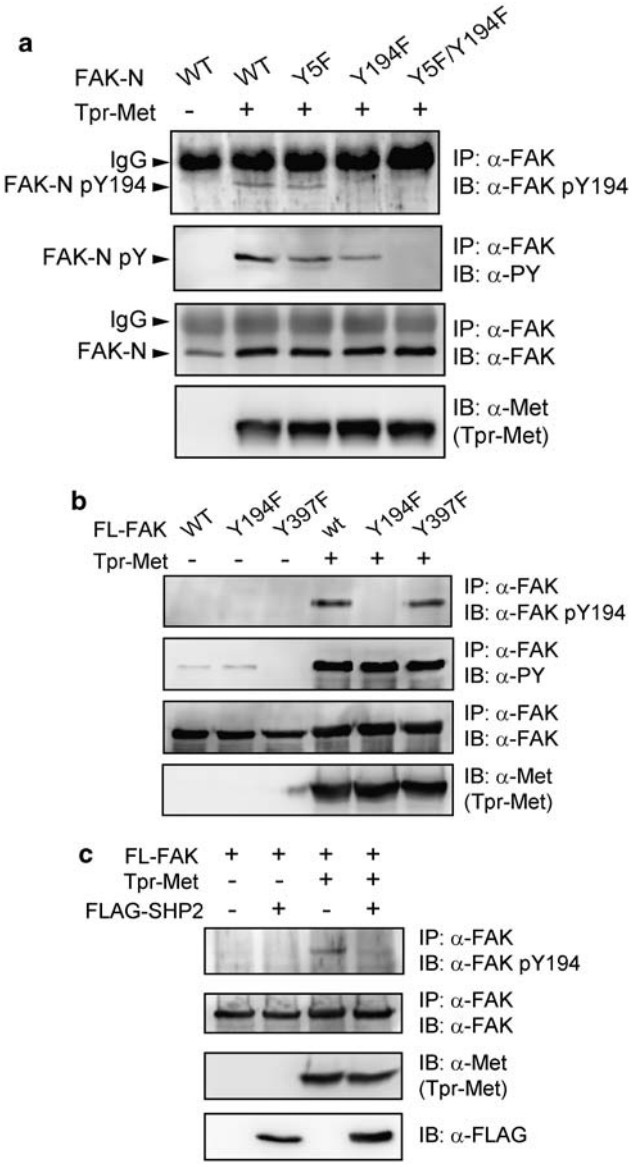

Figure 3 Validation of FAK Y194 phosphorylation by an antibody specific to pY194. (**a**) FAK-NH2 domain (FAK-N) and their YF mutants were transiently co-expressed with (+) or without (−) Tpr-Met in HEK293 cells. FAK proteins were immunoprecipitated by anti-FAK and the washed immunocomplexes were analyzed by immunoblotting with anti-FAK pY194, anti-phosphotyrosine (PY), and anti-FAK. (**b**) Full-length FAK (FL-FAK) and their YF mutants were transiently co-expressed with (+) or without (−) Tpr-Met in HEK293 cells. FAK proteins were immunoprecipitated and analyzed as described in panel (**a**). (**c**) Full-length FAK (FL-FAK) was co-expressed with (+) or without (−) Tpr-Met and FLAG-tagged SHP2 in HEK293 cells. FAK was immunoprecipitated by anti-FAK and the washed immunocomplexes were analyzed by immunoblotting with anti-FAK pY194 or anti-FAK. An equal amount of whole cell lysates was analyzed by immunoblotting with anti-Met or anti-FLAG.

of FAK (Figure 6b). To examine whether the increased Y577 phosphorylation of the Y194E mutant relies on Y397 phosphorylation, Y397 in the Y194E mutant was substituted with Phe. As shown in Figure 6c, no Y577 phosphorylation could be detected in the Y194E/Y397F mutant, indicating that increased Y577 phopshorylation of FAK by the Y194E substitution is Y397-dependent. In other words, Y577 phosphorylation of FAK is totally Src-dependent in the absence of activated Met. As the Y194E mutant is constitutively active, its overexpression is sufficient to support anchorage-independent cell growth in the absence of HGF stimulation (Figure 6d).

Phosphorylation of Y194 may cause conformational changes in the FERM domain and interfere with the intramolecular FERM–kinase interaction of FAK

To examine whether Y194 phosphorylation has an impact to the conformation of the FERM domain, FAK NH2 domain was purified and subjected to digestion by trypsin (Figure 7a) or chymotrypsin (Figure 7b). Our results showed that substitution of Y194 with Glu renders FAK NH2 domain more sensitive to proteolytic digestion. In particular, at the ratio of 1:500 (chymotrypsin: substrate), Y194E mutant was completely digested by chymotrypsin, whereas one-third of the wt FAK NH2 domain retained intact (Figure 7b). These results suggest that phosphorylation on Y194 may cause conformational changes in the FERM domain, which thereby alters its sensitivity to proteases.

To examine whether Y194 phosphorylation of FAK has an impact to the intramolecular FERM–kinase interaction, the co-immunoprecipitation of T7 epitope-tagged-FAK-NH2 domain and FLAG epitope-tagged-FAK-NK fragment (containing both the NH2 domain and kinase domain of FAK) was performed. Our results showed that Tpr-Met, but not its kinase-deficient mutant, potentiated the interaction between T7-FAK-NH2 domain and FLAG-FAK-NK fragment (Figure 8a). Additionally, the Y194E mutant of FLAG-FAK-NK bound more T7-FAK-NH2 domain than the wt did (Figure 8b). These results together suggest that upon Y194 phosphorylation, the intramolecular FERM–kinase interaction of FAK may be relieved, which thereby allows the kinase domain to interact *in trans* with another FERM domain. To examine if the Y194 phosphorylation could cause a conformational change in FAK, FLAG-FAK-NK fragment and its Y194E mutant were purified and subjected to partial digestion by trypsin (Figure 8c) or chymotrypsin (Figure 8d). The results showed that the FAK-NK fragment with a substitution of Y194 with Glu was more sensitive to trypsin and chymotrypsin, which thus suggest that the conformation of the Y194E mutant may not be as compact as the wt FAK, rendering it more sensitive to digestion by proteases. Notably, at the ratio of 1:500 (trypsin: substrate), an extra fragment from the Y194E mutant was detected (Figure 8c), supporting that changes in the tertiary structure of the Y194E mutant may allow exposure of previously buried regions to proteases.

Phosphorylated Y194 may interact with the basic residues in the ^{216}KAKTLRK^{222} patch in the FERM domain, which disfavors the intramolecular FERM–kinase interaction

All our experiments performed up to this point suggest that a negative charge at the residue 194 may interfere

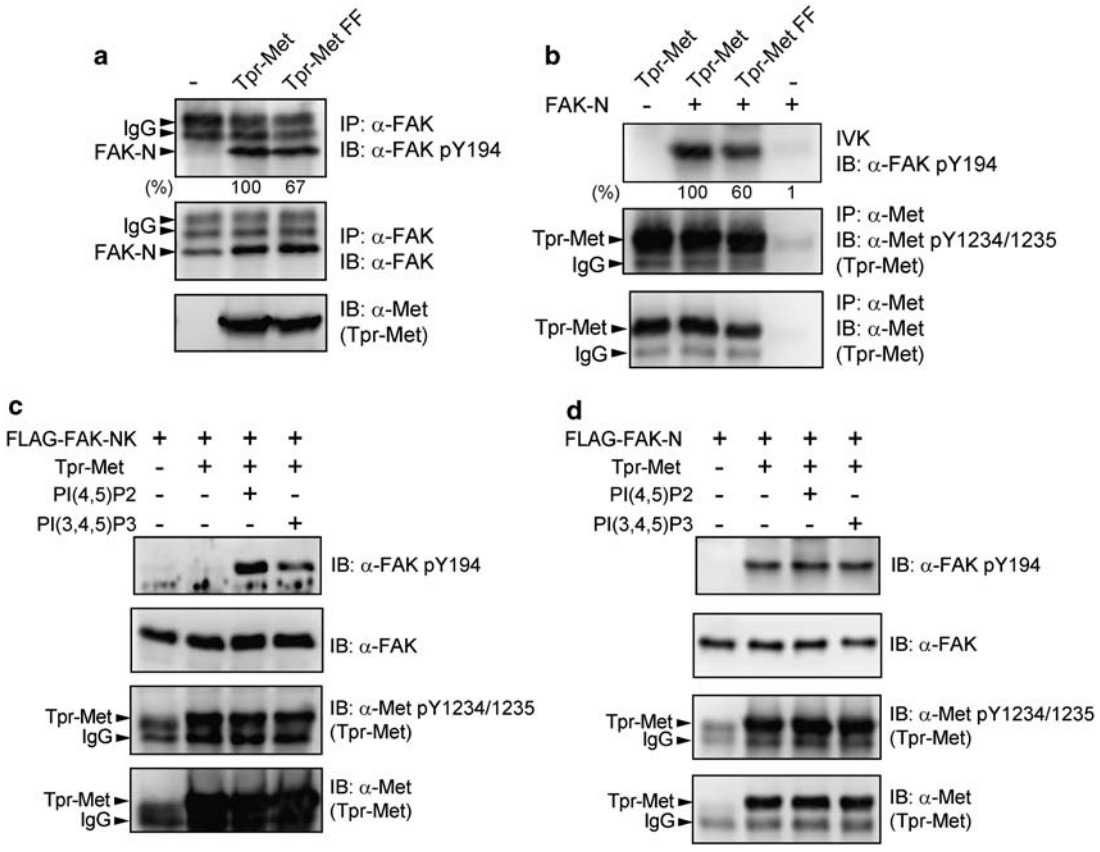

Figure 4 FAK may undergo conformational changes upon binding to Met or phosphoinositides, which renders Y194 accessible for phosphorylation. (**a**) FAK-NH2 domain (FAK-N) was transiently expressed with Tpr-Met or its Y482F/Y489F mutant (FF) in HEK293 cells. Y482 and Y489 in Tpr-Met are equivalent to Y1349 and Y1356 in c-Met, both of which are required for FAK binding. Y194 phosphorylation in FAK-NH2 domain was analyzed by immunoblotting with anti-FAK pY194, which was quantified and expressed as percentage relative to the level in the presence of Tpr-Met. (**b**) Tpr-Met and its Y482F/Y489F mutant (FF) were immobilized on protein A beads with anti-Met and subjected to an *in vitro* kinase assay using purified FAK-N as a substrate in the presence of 400 μM ATP. The phosphorylation of FAK-NH2 domain on Y194 was analyzed by immunoblotting with anti-FAK pY194, which was quantified and expressed as percentage relative to the level in the presence of Tpr-Met. The phosphorylation of Met on Y1234 and Y1235 was measured, which reflects the catalytic activity of Met. IVK, *in vitro* kinase assay. (**c**) Tpr-Met was immobilized on protein A beads with anti-Met and subjected to an *in vitro* kinase assay using purified FLAG-tagged FAK-NK fragment (FLAG-FAK-NK; containing both NH2 domain and kinase domain) as substrates in the presence of 400 μM ATP and 20 μM phosphatidylinositol 4,5-P_2 or phosphatidylinositol 3,4,5-P_3. The phosphorylation of FAK-NK fragment on Y194 was analyzed by immunoblotting with anti-FAK pY194. (**d**) Tpr-Met was immobilized on protein A beads with anti-Met and subjected to an *in vitro* kinase assay using purified FLAG-tagged FAK-NH2 domain (FLAG-FAK-N) as substrates in the presence of ATP and 20 μM phosphatidylinositol 4,5-P_2 or phosphatidylinositol 3,4,5-P_3. The phosphorylation of FAK-NH2 domain on Y194 was analyzed by immunoblotting with anti-FAK pY194.

with the FERM–kinase interaction of FAK. We hypothesized that the phosphorylated Y194 may interact with adjacent basic residues, which thereby affects the FERM–kinase interaction of FAK. Indeed, Y194 is adjacent to the ^{216}KAKTLRK^{222} patch in the FERM domain (Figure 9a). Substitution of one or two basic residues in the ^{216}KAKTLRK^{222} patch did not affect the Y577 phosphorylation in wt FAK (Chen and Chen, 2006; Figure 9d). However, substitution of any of the basic residues in the patch partially suppressed the Y577 phosphorylation of the Y194E mutant (Figure 9b). In the control experiment, mutation at both R204 and R205 did not affect the Y577 phosphorylation of the Y194E mutant (Figure 9c). These results suggest that the interactions between the phosphorylated Y194 and basic residues in the ^{216}KAKTLRK^{222} patch may interfere with the intramolecular FERM–kinase interaction, resulting in FAK autophosphorylation on Y397 and subsequently Y576/Y577 phosphorylation by Src. However, when Tpr-Met was expressed, the Y577 phosphorylation of FAK was significantly increased regardless of substitutions in the ^{216}KAKTLRK^{222} patch (Figure 9d). A possible explanation for this is that once FAK Y194 is phosphorylated, Met is able to access and directly phosphorylate Y576 and Y577 of FAK independently of Y397 and Src, as we proposed previously (Chen and Chen, 2006). Finally, we found that the Y194E substitution enhanced the interaction of FAK with Met, but on the other hand, the Y194F substitution suppressed it (Figure 9e), thus suggesting that the phosphorylation state of FAK Y194 may also modulate the Met–FAK interaction.

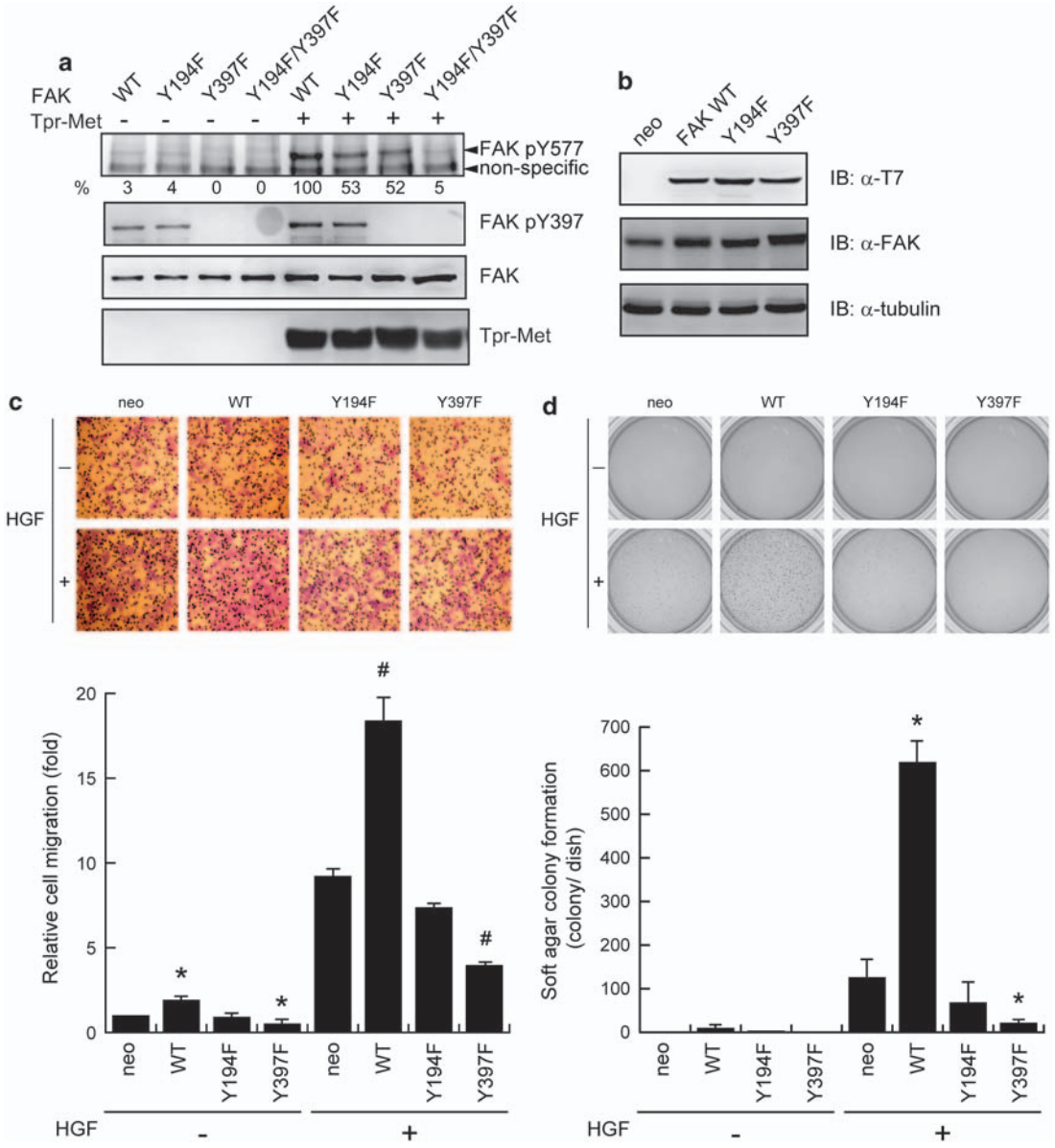

Figure 5 Phosphorylation of FAK on Y194 is critical for its activation and capability to promote HGF-elicited cell functions. (**a**) FAK and its YF mutants were transiently co-expressed with (+) or without (−) Tpr-Met in HEK293 cells. FAK proteins were immunoprecipitated with anti-FAK and the immunocomplexes were analyzed by immunoblotting with antibodies as indicated. The Y577 phosphorylation of FAK was measured and expressed as the percentage relative to wt FAK in the presence of Tpr-Met. Data shown are representative of three independent experiments. (**b**) An equal amount of cell lysates from control neomycin-resistant MDCK cells (neo) and those stably overexpressing T7-tagged FAK or its mutants were analyzed by immunoblotting with anti-T7, anti-FAK or anti-tubulin. (**c**) MDCK cells as described in panel **b** were subjected to a migration assay in the presence (+) or absence (−) of 20 ng/ml HGF, as described in the Materials and methods. Values (means ± s.d.) are from three independent experiments. $^{*}P<0.05$ (compared with the neo cells in the absence of HGF). $^{\#}P<0.05$ (compared with the neo cells in the presence of HGF). (**d**) MDCK cells as described in panel **b** were subjected to a soft agar-colony formation assay in the presence (+) or absence (−) of 20 ng/ml HGF, as described in the Materials and methods. Values (means ± s.d.) are from three independent experiments. $^{*}P<0.05$ (compared with the neo cells in the presence of HGF).

Discussion

FAK is physically associated with and activated by growth factor receptors including Met, EGFR and PDGFR (Sieg *et al*., 2000; Chen and Chen, 2006). However, the mechanism how growth factor receptors activate FAK remains unclear. It is generally believed that the binding of the FAK FERM domain to the growth factor receptors causes conformational changes in FAK, which leads to FAK activation. In this study, we demonstrate that Met, EGFR and PDGFR are able to phosphorylate the NH2 domain of FAK on Y5 and Y194. Although the significance of the Y5 phosphorylation is currently unknown, the Y194 phosphorylation is a crucial step for FAK activation by Met. Substitution of either Y194 or Y397 (autophosphorylation site) of

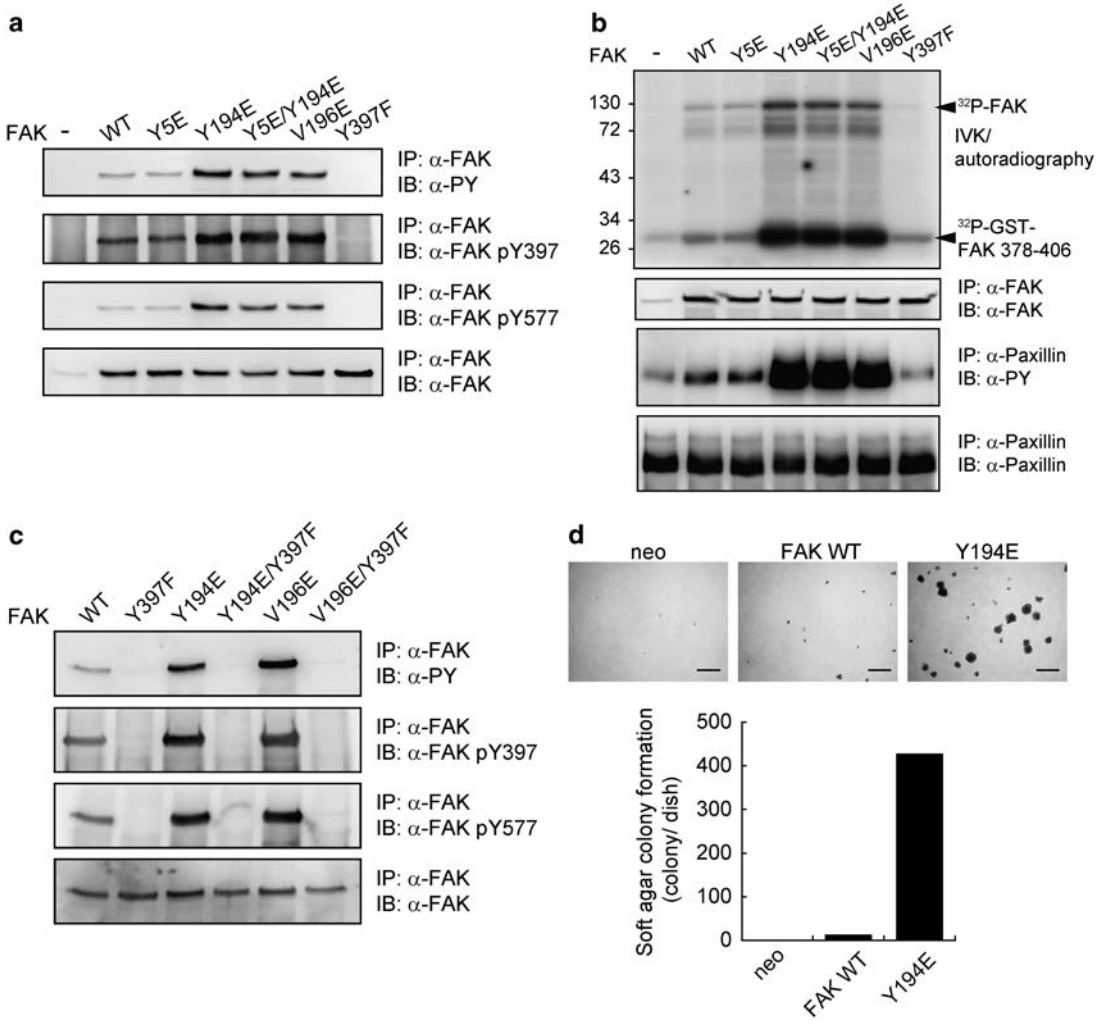

Figure 6 Substitution of Y194 with Glu leads to constitutive activation of FAK. (**a**) FAK and its mutants were transiently expressed in HEK293 cells and their phosphorylation on Y397 and Y577 was analyzed. (**b**) FAK and its mutants were transiently expressed in HEK293 cells. FAK proteins were immunoprecipitated with anti-FAK and the immunocomplexes were subjected to an *in vitro* kinase assay using purified GST-FAK (aa 378–406) as a substrate. An aliquot of the immunocomplexes was analyzed by immunoblotting with anti-FAK to monitor the amount of FAK in the immunocomplexes. IVK, *in vitro* kinase assay. Paxillin was immunoprecipitated by anti-paxillin and the immunocomplexes were analyzed by immunoblotting with anti-PY or anti-paxillin. (**c**) FAK and its mutants were transiently expressed in HEK293 cells and their phosphorylation on Y397 and Y577 was analyzed. (**d**) Control neomycin-resistant MDCK cells (neo) and those stably overexpressing FAK or its Y194E mutant were subjected to a soft agar-colony formation assay, as described in Materials and methods. Representative micrographs are shown. Values (means ± s.d.) are average from two experiments. Scale bars, 500 μm.

FAK with Phe partially (~50%) suppressed Met-induced activation of FAK (Figure 5a). Substitution of both Y194 and Y397 totally inhibited Met-induced activation of FAK (Figure 5a), thus suggesting that the maximal activation of FAK by Met requires its phosphorylation on both residues.

Our results support that events such as Met binding and phosphoinositide binding may first induce conformational changes in FAK, which thus renders Y194 accessible for phosphorylation (Figure 4). In the case of Met binding, it may be sufficient to cause a conformational change in the FERM domain, which thereby enhances the accessibility of Y194 for phosphorylation. However, in the case of phosphoinositide binding, it could have an impact to the FERM–kinase interaction of FAK, which allows Y194 to be phosphorylated more efficiently. Therefore, a possible scenario for Met to activate FAK (Figure 10) may be initiated by conformational changes in FAK through its binding to Met and/or phosphoinositides, which renders Y194 accessible for phosphorylation by Met. Phosphorylation of FAK on Y194 leads to partial activation of FAK and allows it to phosphorylate itself on Y397. The phosphorylated Y397 subsequently recruits the binding of Src, finally leading to full activation of FAK by phosphorylating Y576 and Y577 in the activating loop within the catalytic domain. Alternatively, once FAK Y194 is phosphorylated, Met is able to phosphorylate FAK Y577 through an Y397-independent manner (Figure 5a). In fact, we have previously demonstrated

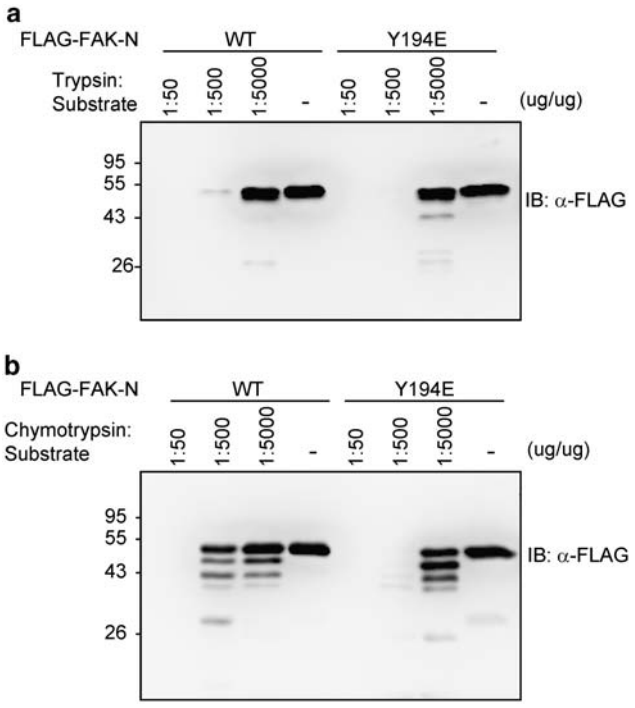

Figure 7 Substitution of Y194 with Glu may cause conformational changes in FAK-NH2 domain. (**a**) FLAG-FAK-NH2 domain (FLAG-FAK-N) and its Y194E mutant were expressed in HEK293 cells and purified as described in the Materials and methods. An equal amount of purified FAK-NH2 proteins was incubated with or without (−) trypsin at various ratios (trypsin : substrate) as indicated. The digested products were analyzed by immunoblotting with anti-FLAG. (**b**) An equal amount of purified FAK-NH2 proteins was incubated with or without (−) chymotrypsin at various ratios (chymotrypsin: substrate) as indicated. The digested products were analyzed by immunoblotting with anti-FLAG.

that Met induces FAK phosphorylation on Y576 and Y577 in $src^{-/-}$ $yes^{-/-}$ $fyn^{-/-}$ cells and directly phosphorylates FAK on both residues *in vitro* (Chen and Chen, 2006). Therefore, it is possible that Met-mediated phosphorylation of FAK on Y194 may induce a conformational change, which allows Met to access and phosphorylate Y576 and Y577 of FAK independently of Src.

The positive impact of Y194 phosphorylation to FAK activation is also supported by the phosphorylation-mimetic mutant Y194E, which possesses much higher catalytic activity than the wt FAK does (Figures 6a and b). But how does the phosphorylation on Y194 lead to FAK activation? Our results suggest that the phosphorylated Y194 may disfavor the intramolecular FERM–kinase interaction of FAK, which allows FAK to be activated through relief of its autoinhibition. This notion is mainly supported by our result that Y194-phosphorylated FAK or the Y194E mutant was more accessible to another FAK FERM domain than wt FAK (Figures 8a and b). In addition, compared with the wt FAK, the purified Y194E mutant was more sensitive to protease digestion (Figures 7a and b), suggesting that the conformation of the Y194E mutant may be altered, rendering it more accessible to be digested by proteases.

At the center of the intramolecular FERM–kinase interface of FAK, F596 in the kinase domain inserts into a hydrophobic pocket constituted by Y180, M183, V196 and L197 in the FERM domain (Lietha *et al*., 2007). The activation of FAK by substitution of V196 with Glu (Figures 6a and b) can be interpreted by disruption of the hydrophobic pocket. As Y194 is not one of those residues forming the hydrophobic pocket, how does the phosphorylation state of Y194 affect the FERM–kinase interaction? Our results (Figure 9b) suggest that phosphorylated Y194 may interact with the basic residues in the ^{216}KAKTLRK^{222} patch in the FERM domain, which may pull the hydrophobic pocket away from F596 in the kinase domain and thereby prevent the FERM–kinase interaction. Moreover, as the basic patch ^{216}KAKTLRK^{222} is crucial for FAK to interact with Met (Chen and Chen, 2006), it is possible that the interaction between the phosphorylated Y194 and the ^{216}KAKTLRK^{222} patch may affect the interaction of FAK with Met. In fact, the Y194E substitution enhances the interaction of FAK with Met, but on the other hand, the Y194F substitution suppresses it (Figure 9e). Our results together suggest that the phosphorylation of Y194 affects not only the catalytic activity of FAK but also its interaction with Met. However, further structure-based studies are required for interpreting the impacts of Y194 phosphorylation to both events.

In this study, we found that Met, EGFR and PDGFR phosphorylate the NH2 domain of FAK on same residues—Y5 and Y194. In fact, there are precedents showing different RTKs can have a similar cohort of phosphorylation sites in a given protein. For example, EGFR and insulin receptor phosphorylate the docking protein Gab1 with a similar cohort of phosphorylation sites, but display distinct preference towards those sites (Lehr *et al*., 1999, 2000). Thus, it is possible that Met, EGFR and PDGFR may have distinct preference for Y5 and Y194 of FAK. However, it is not clear whether the phosphorylation of Y5 and/or Y194 affect interactions of FAK with other cellular proteins, in particular, those known to interact with the FERM domain of FAK such as the Arp2/3 complex (Serrels *et al*., 2007). In addition, it remains possible that phosphorylated Y5 and/or Y194 may serve as binding sites for other Src-homology 2 domain-containing proteins. In conclusion, this study provides the first example to illustrate how FAK is activated by RTKs.

Materials and methods

Materials

Polyclonal anti-Met (C-12), anti-FAK (A-17) and anti-EGFR (1005) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal anti-paxillin, anti-FAK (clone 77), anti-phosphotyrosine (PY20) and polyclonal anti-PDGFRβ were from BD Transduction Laboratories (San Jose, CA, USA). Polyclonal anti-FAK pY397 and anti-FAK pY577 were purchased from BioSource International Inc. (Camarillo, CA, USA). Polyclonal anti-FAK pY194 was

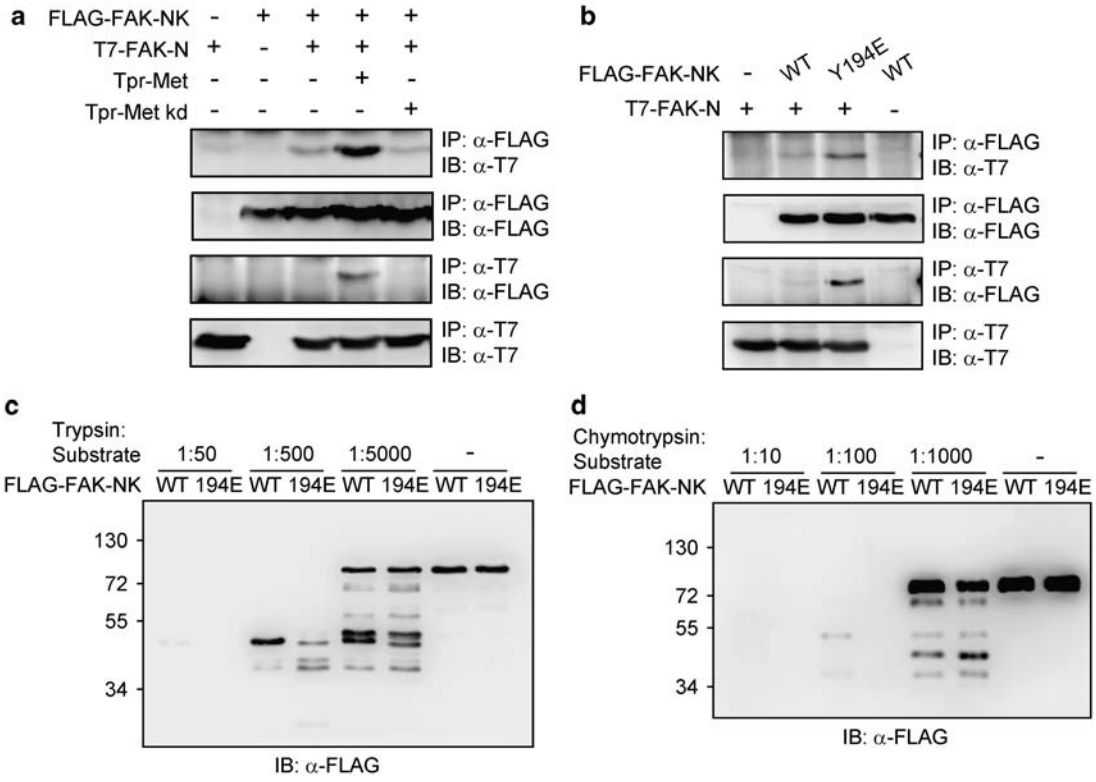

Figure 8 Phosphorylation of FAK on Y194 interferes with the intramolecular FERM–kinase interaction of FAK. (**a**) FLAG-tagged FAK-NK fragment (FLAG-FAK-NK) and T7-tagged FAK NH2 domain (T7-FAK-N) were transiently co-expressed with (+) or without (−) Tpr-Met or its kinase-deficient (kd) mutant in HEK293 cells. Co-immunoprecipitation of FLAG-FAK-NK and T7-FAK-N was analyzed as indicated. (**b**) FLAG-FAK-NK and its Y194E mutant were co-expressed with (+) or without (−) T7-FAK-N in HEK293 cells. Co-immunoprecipitation of FLAG-FAK-NK and T7-FAK-N was analyzed as indicated. (**c**) FLAG-FAK-NK and its Y194E mutant were expressed in HEK293 cells and purified as described in the Materials and methods. An equal amount of purified FAK-NK proteins was incubated with or without (−) trypsin at various ratios (trypsin: substrate) as indicated. The digested products were analyzed by immunoblotting with anti-FLAG. (**d**) An equal amount of purified FAK-NK proteins was incubated with or without (−) chymotrypsin at various ratios (chymotrypsin: substrate) as indicated. The digested products were analyzed by immunoblotting with anti-FLAG.

generated by Quality Controlled Biochemicals (Hopkinton, MA, USA). Monoclonal anti-Src (clone 327) was purchased from Oncogene Research Products (La Jolla, CA, USA). Polyclonal anti-Src pY416 was purchased from Cell Signaling Technology (Beverly, MA, USA). Monoclonal anti-T7 was purchased from Novagen (Madison, WI, USA). Protein A-Sepharose beads, FLAG peptides, monoclonal anti-FLAG (M2), and anti-FLAG (M2)-conjugated beads were purchased from Sigma-Aldrich (St Louis, MO, USA). Phosphatidylinositol 4,5-P_2 and phosphatidylinositol 3,4,5-P_3 were purchased from Echelon Biosciences Inc. (Salt Lake City, UT, USA).

Plasmids and mutagenesis

The cDNAs for FAK, PYK2, and oncogenic Src were kindly provided by JL Guan (University of Michigan, MI, USA). The plasmid pcDNA3.1-EGFR was kindly provided by MC Hung (University of Texas MD Anderson Cancer Center, TX, USA). The plasmid pcDNA3.1-PDGFRβ was kindly provided by CH Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The plasmid pCMV-FLAG-SHP2 was kindly provided by DL Wang (Academia Sinica, Taipei, Taiwan). Plasmids pMT2-cMet and pMT2-Tpr-Met were described previously (Chen and Chen, 2006). The following plasmids were constructed in our laboratory: the pcDNA3-FAK (amino acids [aa] 1–1053), pcDNA3-T7-FAK (aa 1–1053), pcDNA3-FAK-NH2 (aa 1–391), pcDNA3.1-T7-FAK-NH2 (aa 1–391), pcDNA3.1-T7-PYK2-NH2 (aa 1–397), pCMV-FLAG-FAK-NK (aa 31–686), pCMV-FLAG-FAK-N (aa 1–391) and pGEX1-FAK (aa 378–406). Mutagenesis was carried out using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Mutagenic primers for making FAK mutants used in this study are listed in Supplementary Table S1. All desired mutations were confirmed by dideoxy DNA sequencing.

Cell culture and transfections

Human embryonic kidney 293 (HEK293) cells and MDCK cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transient transfections, HEK293 cells (5×10^5) were seeded on a 6 cm culture dish. 18 h later, the cells were incubated with the mixture of plasmid (1 ~ 3 μg) and LipofectAMINE (Life Technologies-Invitrogen, Grand Island, NY, USA) for 5 h and allowed to grow for another 36 h. To generate MDCK cells stably overexpressing T7-tagged FAK or its mutants, MDCK cells were transfected with plasmids encoding desired proteins using LipofectAMINE. Two days after transfection, the cells were selected in the medium containing 0.5 mg/ml G418 for one week. The neomycin-resistant cells were collected and analyzed by immunoblotting with anti-T7 and anti-FAK.

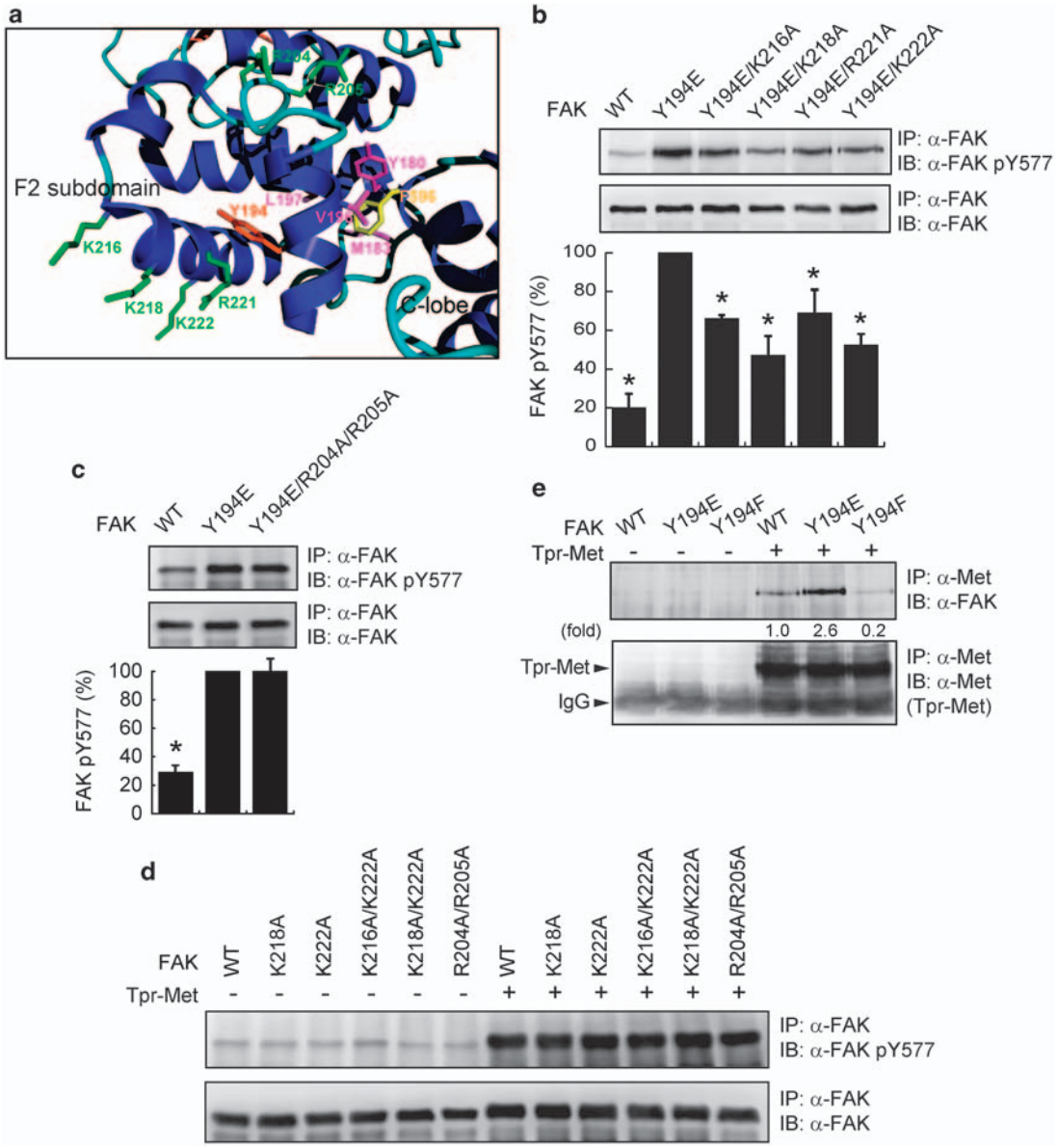

Figure 9 Phosphorylated Y194 of FAK may interact with the basic residues in the ^{216}KAKTLRK^{222} patch. (**a**) Diagram showing the relative position of Y194 to the ^{216}KAKTLRK^{222} patch and the FERM–kinase interface in FAK. At the center of the FERM–kinase interface, F596 in the kinase domain inserts into a hydrophobic pocket in the FERM domain formed by Y180, M183, V196 and L197. (**b**) FAK and its mutants were transiently expressed in HEK293 cells and their phosphorylation on Y577 was measured. The Y577 phosphorylation of FAK was quantified and expressed as the percentage relative to the level of the Y194E mutant, which is defined as 100%. Values (means ± s.d.) are from three independent experiments. *$P<0.05$. (**c**) FAK and its mutants were transiently expressed in HEK293 cells and their phosphorylation on Y577 was analyzed. The Y577 phosphorylation of the Y194E mutant is defined as 100%. Values (means ± s.d.) are from three independent experiments. *$P<0.05$. (**d**) FAK and its mutants were transiently co-expressed with (+) or without (−) Tpr-Met in HEK293 cells. The phosphorylation of FAK Y577 was analyzed. (**e**) FAK and its mutants were transiently co-expressed with (+) or without (−) Tpr-Met in HEK293 cells. The co-immunoprecipitation of FAK and Tpr-Met was performed. The level of co-precipitated FAK is measured and expressed as fold change relative to wt FAK. Similar results were observed in two independent experiments.

Immunoprecipitations and immunoblotting

Immunoprecipitation and immunoblotting were carried out as described previously (Chen and Chen, 2006). Chemiluminescent signals were detected and quantified using the Fuji LAS-3000 luminescence image system.

Protein purification

To obtain purified FAK-NH2 domain (aa 1–391) for *in vitro* kinase assays, HEK293 cells were transiently transfected with pcDNA3-FAK-NH2 or its mutants for 48 h and lyzed in 1% Nonidet P-40 lysis buffer (Chen and Chen, 2006). Cell lysates (1 mg) were incubated with protein A-Sepharose beads covalently linked with polyclonal anti-FAK for 2 h at 4 °C. Polyclonal anti-FAK antibodies were covalently linked on protein A-Sepharose beads by dimethyl pimelimidate dihydrochloride (Sigma-Aldrich). Beads were washed six times with 1% Nonidet P-40 lysis buffer. The bound proteins were eluted in 150 µl of 0.1 M glycine buffer (pH 2.8)

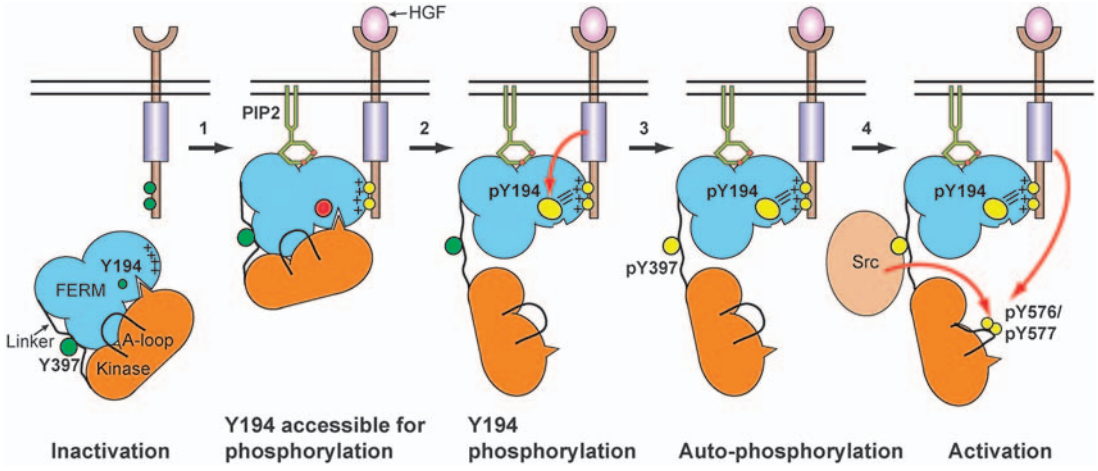

Figure 10 Diagram illustrating the activation of FAK by Met. Step 1, FAK binds to Met through its FERM domain upon HGF stimulation. Upon binding to Met and/or phosphatidylinositol 4,5-P_2 (PIP2), FAK may undergo conformational changes, which renders Y194 accessible for phosphorylation. The ^{216}KAKTLRK^{222} patch (+ + + +) in the FERM domain is critical for Met binding (Chen and Chen, 2006). Step 2, Met phosphorylates FAK on Y194, which leads to partially activation of FAK. The phosphorylated Y194 may interact with the basic residues in the ^{216}KAKTLRK^{222} patch, causing relief of autoinhibition. Step 3, FAK undergoes autophosphorylation on Y397. Step 4, the phosphorylated Y397 recruits the binding of Src, finally leading to full activation of FAK by phosphorylating Y576 and Y577 in the activation loop. Alternatively, Met is able to directly phosphorylate Y576 and Y577 upon Y194 phosphorylation. Note that for simplicity only the FERM and kinase domains of FAK are depicted. A-loop, activation loop in the kinase domain.

and neutralized with 1 µl of 1 M Tris–HCl (pH 9.0). To obtain purified FLAG-tagged FAK-NK (aa 31–686) and FLAG-tagged FAK-N (aa 1–391) for protease digestion, HEK293 cells were transiently transfected with pCMV-FLAG-FAK-NK or pCMV-FLAG-FAK-N for 48 h and lyzed in 1% Nonidet P-40 lysis buffer. Cell lysates (1 mg) were incubated with beads covalently linked with monoclonal anti-FLAG for 2 h at 4 °C. Beads were washed three times with 1% Nonidet P-40 lysis buffer and one time with Tris–buffered saline (50 mM Tris–HCl, 150 mM NaCl, pH 7.4). The bound proteins were eluted in 150 µl of 300 ng/ml FLAG peptides. The purified proteins were fractionated by SDS–polyacrylamide gel electrophoresis and visualized by Coomassie blue stain.

In vitro *kinase assay*

For *in vitro* kinase assays, Tpr-Met, c-Met, EGFR or PDFGR were transiently overexpressed in HEK293 cells and immobilized on protein A-Sepharose beads with specific antibodies. The kinase reactions were carried out in 40 µl of kinase buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MnCl_2$) containing 10 µCi of [γ-^{32}P] ATP (3000 Ci mmol^{-1}; PerkinElmer Life Sciences) and 0.5 µg of purified FAK-NH2 (aa 1–391) proteins for 15 min at 25 °C. Reactions were terminated by addition of SDS sample buffer, and the ^{32}P-incorporated proteins were fractionated by SDS–polyacrylamide gel electrophoresis and visualized by autoradiography. The radioisotope activity was quantified using a phosphoimager system (Pharmacia, Piscataway, NJ, USA). In some cases, *in vitro* kinase assays were carried out in the presence of regular ATP (400 µM) rather than [γ-^{32}P] ATP and the *in vitro* phosphorylated FAK proteins were analyzed by immunoblotting with anti-FAK pY194.

Protease digestion

Trypsin digestion reactions were carried out in 30 µl of Tris–buffered saline (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) containing 250 ng of purified FLAG-tagged FAK proteins and various amount of trypsin (Promega, Madison, WI, USA) for 15 min at 37 °C. Chymotrypsin digestion reactions were carried out in 30 µl of chymotrypsin digestion buffer (100 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.8) containing 250 ng of purified proteins and various amount of chymotrypsin (Sigma-Aldrich) for 15 min at 30 °C. The reactions were terminated by addition of SDS sample buffer, and the digested products were fractionated by SDS–polyacrylamide gel electrophoresis and detected by immunoblotting with anti-FLAG.

Cell migration assay

MDCK cells were collected by trypsinization and suspended in serum-free medium at 2×10^4 cells/ml. Migration assays were carried out in a Neuro Probe 48-well chemotaxis chamber (Cabin John, MD, USA). Briefly, the medium containing type I collagen (10 µg/ml) ± HGF (20 ng/ml) was added to the lower chamber. The lower and upper chambers were separated by a polycarbonate membrane (8 µm pore size, Poretics, Livermore, CA, USA). Cells were allowed to migrate for 7 h at 37 °C in a humidified atmosphere containing 5% CO_2. The membrane was fixed in methanol for 10 min and stained with modified Giemsa stain (Sigma-Aldrich) for 1 h. Cells on the upper side of the membrane were removed by cotton swabs. Cells on the lower side of the membrane were counted under a light microscope. Each experiment was performed in triplicate.

Soft agar-colony formation assay

MDCK cells (5×10^3) were suspended in 1 ml Dulbecco's modified eagle's medium containing 0.3% agar and 10% serum and added onto a layer of medium containing 0.5% agar and 10% serum in a six-well plate. One ml of medium containing 0.3% agar and 10% serum ± HGF (20 ng/ml) was added to the dish every other day. After 14 days, cell colonies were stained with 0.5 mg/ml *p*-Iodonitrotetrazolium violet for 1 day at room temperature. To quantify the number of colonies in a whole dish, the photographs of the stained dishes were taken by a digital camera and the number of colonies larger than 40 pixel^2 in size was measured using NIH Image J software (http://rsb.info.nih.gov.ij/), a free software provided by NIH (Bethesda, MD, USA).

Structural illustration
Protein structure data of the FAK fragment containing the FERM and kinase domains (PDB ID: 2J0J) were obtained from Protein Data Bank (http://www.pdb.org) and illustrated in ribbon style using YASARA view software provided by YASARA Biosciences GmbH (Vienna, Austria).

Statistical analysis
Student's *t*-test was used to determine whether there was a significant difference between two means. *P*-values of <0.05 were considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)