We have previously described regulation of focal adhesion kinase (FAK) by its amino-terminal FERM-like domain through an autoinhibitory interaction with its kinase domain (Cooper, L. A., Shen, T. L., and Guan, J. L. (2003) Mol. Cell. Biol. 23, 8030–8041). Here we show that the first two subdomains of the FERM-like domain are independently capable of inhibiting phosphorylation of FAK in trans. We characterized several point mutations within the first subdomain of the FERM-like domain and find that mutation of Lys-38 to alanine results in a FAK mutant that is strongly hyperphosphorylated when expressed in mammalian cells, and promotes increased phosphorylation of the FAK substrate paxillin. A second mutation of Lys-78 to alanine results in a FAK mutant that is underphosphorylated, but can be activated by extracellular matrix stimuli. Like deletion of the amino terminus itself the K38A mutation is phosphorylated in suspension. The Δ375 truncation mutant of FAK is strongly phosphorylated both when Tyr-397 is mutated to phenylalanine, and in the presence of the Src inhibitor, PP2, suggesting that removal of the amino terminus can render FAK Src independent. This is in contrast to the K38A mutant that is not phosphorylated in the Y397F background, and which shows decreased phosphorylation in the presence of the Src inhibitor PP2, suggesting that regulation of FAK by Src is a secondary step in its activation. The K38A mutation weakens the interaction between the amino terminus of FAK and its own kinase domain, and disrupts the ability of the amino terminus to inhibit the phosphorylation of FAK in trans. The K38A mutation also increases the ability of FAK to promote cell cycle progression and cell migration, suggesting that hyperphosphorylation of this mutant can positively affect FAK function in cells. Together, these data strongly suggest a role for the first FAK subdomain of the FERM domain in its normal regulation and function in the cell.

Focal adhesion kinase (FAK) is a 125-kDa tyrosine kinase that participates in signal transduction cascades stimulated by adhesion through integrin receptors to the extracellular matrix. Clustering of integrins into forming focal adhesions upon attachment of cells to the extracellular matrix leads to the localization of FAK to these structures where it becomes strongly phosphorylated and interacts with other signaling molecules like Src, the p85 subunit of phosphatidylinositol 3-kinase, paxillin, p130 Cas, and Grb7. Phosphorylation of FAK is important in promoting FAK-dependent signal transduction because its phosphorylation state regulates the ability of these signaling molecules to interact with FAK (Src and phosphatidylinositol 3-kinase), and/or it regulates the catalytic activity of FAK that influences the ability of these molecules to become phosphorylated themselves (paxillin and p130 Cas for example). The phosphorylation and activity of FAK are strictly dependent on cell adhesion through integrin receptors, thus it is not surprising that FAK plays an important role in regulating events that are dependent on adhesion like cell cycle progression, and cell survival, or events, like cell migration where continual modulation of the interaction between the cell and the underlying extracellular matrix are necessary (1–6).

The regulation of the activity of FAK by cell adhesion is complicated and involves a number of different factors. Localization of FAK to focal adhesions through its carboxyl-terminal region is essential for its normal regulation as most FAK mutants that do not localize properly to focal adhesions fail to become highly phosphorylated (7, 8). Likewise artificially clustering integrins at the cell surface can mimic cell adhesion and allow the phosphorylation of FAK, suggesting that aggregation of β integrin cytoplasmic domains and their associated proteins is sufficient to allow phosphorylation of FAK (9). FAK can also become phosphorylated in suspension when it is artificially dimerized, suggesting a potential role for transphosphorylation between FAK molecules in its activation (10). In fact autophosphorylation of FAK at Tyr-397 is critical for most of the cellular functions of FAK including the phosphorylation of FAK at other residues (11–13). This is inferred from the observation that mutation of this residue to phenylalanine blocks the phosphorylation of FAK at residues within the kinase domain and in the carboxyl terminus (11). Because mutation of Tyr-397 to phenylalanine disrupts binding of Src to FAK, it is believed that Src plays an important role in phosphorylating FAK (14). Indeed, inhibition of the Src family kinases using pharmacological inhibitors like PP2, or genetically removing Src family kinases from cells inhibits integrin-dependent phosphorylation of FAK both at Tyr-397 and other residues, suggesting that Src plays a key role in regulating the activity of FAK (15, 16).

We and others have suggested that the amino-terminal domain of FAK, which contains a region sharing some sequence similarity to FERM domains, may play an important role in regulating the activity and phosphorylation state of FAK in cells (10, 17). Large truncations of the amino terminus of FAK are hyperphosphorylated, suggesting that the amino terminus exerts an inhibitory influence on the catalytic domain of FAK (10, 17, 18). We have also shown that the amino terminus of FAK is capable of interacting with the kinase domain, and have suggested that this interaction is responsible for the inhibitory
Role of FAK FERM-like Domain in Its Regulation

influence the amino terminus has on the activity of FAK within the full-length FAK molecule (17). We proposed a model in which the localization of FAK to forming the focal adhesion allows FAK to encounter a regulator that binds to the amino terminus and disrupts the interaction between the amino terminus and catalytic domain of FAK allowing the activation of FAK (17). Consistent with this model a group recently defined several mutations within the amino terminus of FAK that are underphosphorylated in growing cells, suggesting the possibility that these mutations are unable to interact with the upstream regulator and thus are unable to be fully activated (19).

Although it has clearly been established that the amino terminus of FAK has an influence over the activity and phosphorylation state of FAK, the exact nature of this role and how it influences the regulation of FAK remains controversial. In this study we identify two single point mutations within the first subdomain of the amino terminus of FAK that alter the phosphorylation state and regulation of FAK within the cell. We show that one of these point mutations leads to hyperphosphorylation of FAK and weakens the ability of the amino terminus to interact with the kinase domain. We also show that these mutations, which alter the phosphorylation of FAK, also affect FAK functions in the cell, promoting cell cycle progression and cell migration. Together, these data strongly suggest a role for the first FAK subdomain of the FERM domain in its normal regulation and function in the cell.

MATERIALS AND METHODS

Monoclonal anti-HA-conjugated agarose beads, mouse antibody against BrdU, protein A-Sepharose, human plasma fibronectin (FN), polyl-lysine, and bromo-deoxyuridine (BrdU) were purchased from Sigma. Monoclonal anti-HP-conjugated agarose beads, mouse antibody against BrdU, protein A-Sepharose, human plasma fibronectin (FN), polyl-lysine, and bromo-deoxyuridine (BrdU) were purchased from Sigma. The Src inhibitor PP2 and the control compound PP3 were purchased from Calbiochem. Lipofectamine and Plus reagent were purchased from Invitrogen. CHO cells were maintained in F-12 medium supplemented with 10% fetal bovine serum (Invitrogen). CHO cells were maintained in F-12 medium supplemented with 10% fetal bovine serum (Invitrogen). NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% calf serum. Cells were transfected with mammalian expression plasmids as indicated using Lipofectamine2000 following the manufacturer’s instructions. Experiments were conducted 24 h following transfection, which were serum starved for an additional 18 h. The cells were then briefly trypsinized, resuspended in Dulbecco’s modified Eagle’s medium containing a final concentration of 0.5 mg/ml soybean trypsin inhibitor (Sigma), washed twice in Dulbecco’s modified Eagle’s medium, and held in suspension for 60 min. Indicated cells were lysed. Cells replated on FN or polyl-lysine were added to plates coated with 10 μg/ml FN or 100 μg/ml polyl-lysine for 60 min after being held in suspension. For experiments where cells were replated on FN in the presence of either the Src inhibitor PP2 or the control compound PP3, cells were serum starved overnight. The following day the cells were placed into suspension for 60 min in the presence of either 10 μM of the Src inhibitor PP2 or 10 μM of the control compound PP3. The cells were then replated on FN-coated dishes for 30 min in the presence of the appropriate compound before being lysed.

For cell transplantation or cell migration experiments, cells were washed twice in phosphate-buffered saline and then lysed with a buffer containing 1% Triton X-100, 5% glycerol, 50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 20 μg/ml leupeptin. Lysates were cleared by centrifugation, and total protein concentration was determined using the Bio-Rad protein assay. Immunoprecipitations were carried out by incubating cell lysates with appropriate antibodies and protein A-Sepharose 4B beads (Sigma), or by incubating lysates with monoclonal HA antibody conjugated to agarose (Sigma). After washing, immune complexes were resolved using SDS-PAGE. Immunoblotting was carried out using horseradish peroxidase-conjugated IgG as a secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) or a sheep anti-mouse ECL antibody (Amersham, Arlington Heights, IL). ECL detection was then carried out using the Amersham Biosciences ECL system for detection.

BrdU Incorporation Assays—Indicated HA-tagged constructs were transiently expressed in NIH3T3 cells. Cells were recovered for 3 h in 10% calf serum following the transfection, and then serum starved for 18 h to arrest the cells in G0. BrdU incorporation assay was conducted as described in Ref. 20. Briefly, cells were released from BrdU by replating the cells in 10% calf serum and 150 μM BrdU. Following the indicated hours of growth, the cells were fixed, treated with DNase I, and processed for immunofluorescence assay. The percentage of nontransfected and transfected cells that had incorporated BrdU are represented as a mean of 4 experiments of 50–100 cells counted per condition. The error bars represent ± S.D.

Immunofluorescence Staining—Cells were processed for immunofluorescence staining as described previously (20). The primary antibodies used were: anti-HA polyclonal antibody Y-11 (1:200), anti-FAK pasilin monoclonal antibody (1:100), and Texas Red-conjugated phalloidin (1:50; Molecular Probes, Eugene, OR). The secondary antibodies used were: Texas Red-conjugated goat anti-mouse antibody (1:100; Jackson ImmunoResearch Laboratory, West Grove, PA) or fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (1:100; Jackson ImmunoResearch Laboratory, West Grove, PA).

Cell Migration Assay—CHO cells were transiently transfected with the indicated constructs, and pEGFP. Cells were serum starved overnight following the transfection. Prior to assay a fluorescence image of the cells was taken and used to approximate the transfection efficiency. Cells were detached from plates using 0.5 mg/ml soybean trypsin inhibitor (Sigma), and washed two times. Cells were resuspended in F-12 media supplemented with 0.2% fetal bovine serum. 1.25 × 104 cells
were placed into the upper well of a Boyden chamber apparatus (neuroprobe). The bottom well of the Boyden chamber contained either F-12 supplemented with 0.2% fetal bovine serum, or F-12 supplemented with 0.2% fetal bovine serum and 5 µg/ml FN as chemotacticant. Boyden chambers were placed in a 5% CO₂ incubator at 37 °C for 6 h. The remaining cells were lysed in 1% Triton X-100, 5% glycerol, 50 mM Tris, pH 8.0, and 200 mM NaCl, and 25 µg were run on an SDS-PAGE gel and immunoblotted as indicated. The polycarbonate membrane (neuroprobe) between the two chambers was removed and fixed in methanol for 10 min, after which it was removed, air dried, and stained with Giemsa stain. The cells on the upper side of the membrane were placed onto a coverslip and visualized and counted at ×100. Each data point represents the average of four experiments, each of which includes a minimum of 7 wells per condition for the 5 µg/ml FN condition. The error bars represent 1 S.D. The remaining cells were run on an SDS-PAGE gel and immunoblotted as indicated.

RESULTS

The amino-terminal domain of FAK contains a region that shares some sequence similarity with the FERM domains of several proteins (21). Crystal structures of several FERM domains are known, and they reveal a compact structure in which three distinct subdomains come together to form the overall clover-like structure (22–24). To narrow down which residues within the amino-terminal region of FAK are involved in influencing its catalytic activity, we compared the amino acid sequence of the FERM-like region of FAK to that of several other FERM domain containing proteins (25). We then compared this sequence alignment to the crystal structure of ezrin to define where the three subdomains might be within the FERM-like region of FAK (24). We created three fragments corresponding to the three subdomains comprising residues 1–127, 128–260, and 261–376, respectively, and co-expressed a myc-tagged version of each fragment, the myc-tagged entire amino terminus (residues 1–400) or vector alone with HA-tagged full-length WT-FAK in CHO cells (see Fig. 1a). As shown in Fig. 1b, expression of the whole amino terminus inhibited phosphorylation of FAK at tyrosine 397 consistent with our previously reported data showing that expression of the amino-terminal domain of FAK inhibited total phosphorylation of full-length FAK (17). Interestingly, two of the small fragments of the FERM domain of FAK, 1–127 and 128–260, were capable of inhibiting its phosphorylation at tyrosine 397 in trans, suggesting that more than one region of the FERM domain of FAK is involved in its regulation. A third fragment from residues 261 to 376 did not effect the phosphorylation of full-length FAK. Similar expression levels of the exogenously expressed full-length FAK were confirmed by blotting whole cell lysates with the C-20 antibody that recognizes the carboxyl terminus of FAK. The doublet recognized by this antibody is composed of a lower band corresponding to endogenous FAK, and an upper band corresponding to the HA-tagged exogenous FAK. To verify the identity of the upper band as HA-tagged FAK and to determine total phosphorylation, the lysates were immunoprecipitated by anti-HA followed by Western blotting analysis with antiphosphotyrosine antibody PY20. Fig. 1c shows that FAK fragments 1–127, 128–260, and 1–400, but not 261–375, inhibited phosphorylation of HA-tagged FAK. Together, these results suggest that regions of FAK corresponding to the first and second subdomain of other FERM domains are important for the regulation of FAK. They also suggest that the presence and phosphorylation of Tyr-397 in the larger 1–400 amino-terminal construct is not important to its ability to inhibit the phosphorylation of FAK.

Mutation of FERM Domain Alters Phosphorylation of FAK—We and other groups have shown that truncation mutants of FAK as short as 125 residues are hyperphosphorylated, suggesting that these residues are likely to be important in the autoregulation of FAK (17, 18). The ability of a fragment consisting of the first 127 amino acids to inhibit the phosphorylation of FAK in trans underscores the likely importance of these residues in the regulation of FAK. To further define what residues are involved in the influence of the amino terminus of FAK on its own catalytic activity, we compared the sequence alignment of the FERM-like region of FAK to that of other FERM domain containing proteins (25). We looked for amino acid positions within this sequence alignment where a charged residue was often present. Based on these comparisons, we chose four residues within the first subdomain of the FERM-like region of FAK to mutate to alanine, Lys-38, Lys-78, Asp-101, and Arg-125 (Fig. 2a). An HA-tagged version of each of the mutants, WT-FAK, or the Δ375 truncation mutant of FAK were then transiently transfected into NIH3T3 cells. Fig. 2b shows three of the mutations, K78A, D101A, and R125A, showed decreased Tyr-397 phosphorylation of the K38A mutant showed increased phosphorylation at this residue consistent with our previously reported data showing that expression of the amino-terminal domain of FAK to that of several other FERM domain containing proteins (25). We then compared this sequence alignment to the crystal structure of ezrin to define where the three subdomains might be within the FERM-like region of FAK (24). We created three fragments corresponding to the three subdomains comprising residues 1–127, 128–260, and 261–376, respectively, and co-expressed a myc-tagged version of each fragment, the myc-tagged entire amino terminus (residues 1–400) or vector alone with HA-tagged full-length WT-FAK in CHO cells (see Fig. 1a). As shown in Fig. 1b, expression of the whole amino terminus inhibited phosphorylation of FAK at tyrosine 397 consistent with our previously reported data showing that expression of the amino-terminal domain of FAK inhibited total phosphorylation of full-length FAK (17). Interestingly, two of the small fragments of the FERM domain of FAK, 1–127 and 128–260, were capable of inhibiting its phosphorylation at tyrosine 397 in trans, suggesting that more than one region of the FERM domain of FAK is involved in its regulation. A third fragment from residues 261 to 376 did not effect the phosphorylation of full-length FAK. Similar expression levels of the exogenously expressed full-length FAK were confirmed by blotting whole cell lysates with the C-20 antibody that recognizes the carboxyl terminus of FAK. The doublet recognized by this antibody is composed of a lower band corresponding to endogenous FAK, and an upper band corresponding to the HA-tagged exogenous FAK. To verify the identity of the upper band as HA-tagged FAK and to determine total phosphorylation, the lysates were immunoprecipitated by anti-HA followed by Western blotting analysis with antiphosphotyrosine antibody PY20. Fig. 1c shows that FAK fragments 1–127, 128–260, and 1–400, but not 261–375, inhibited phosphorylation of HA-tagged FAK. Together, these results suggest that regions of FAK corresponding to the first and second subdomain of other FERM domains are important for the regulation of FAK. They also suggest that the presence and phosphorylation of Tyr-397 in the larger 1–400 amino-terminal construct is not important to its ability to inhibit the phosphorylation of FAK.

Mutation of FERM Domain Alters Phosphorylation of FAK—We and other groups have shown that truncation mutants of FAK as short as 125 residues are hyperphosphorylation...
the exogenously expressed FAK constructs were expressed at similar levels, suggesting that differences in their phosphorylation were not attributable to differences in their expression level. Similar results were found in CHO cells (data not shown). To determine the total phosphorylation of the FAK mutants, we immunoprecipitated the exogenously expressed FAK from whole cell lysates using HA-conjugated agarose. Again antiphosphotyrosine immunoblots revealed that the D101A and R125A mutations had no effect on the general tyrosine phosphorylation of FAK (Fig. 2c). The K78A mutant showed decreased phosphorylation, and the K38A and Δ375 mutations were strongly hyperphosphorylated. Anti-HA immunoblots of the immunoprecipitates confirmed that equivalent amounts of the exogenously expressed FAK or FAK mutants were present. Similar results were found in CHO cells (data not shown). These results confirm that the K38A mutant of FAK is strongly hyperphosphorylated when expressed in mammalian cells.

To see whether the increased tyrosine phosphorylation of the K38A mutant can increase the phosphorylation of a FAK-dependent substrate in mammalian cells, we analyzed phosphorylation of paxillin by FAK and its mutants. As shown in Fig. 2d both WT-FAK and the K78A mutant of FAK were capable of inducing weak tyrosine phosphorylation of GFP-paxillin. Interestingly, the K38A mutant was capable of inducing strong
phosphorylation of paxillin, to an extent similar to the hyperactive \(\text{H9004} \_{375}\) mutant. These data suggest that the \(\text{K38A}\) mutant is a hyperactive point mutant that shows similarity to the \(\text{H9004} \_{375}\) amino-terminal domain truncation mutant. This is the first demonstration that a single point mutation within the FERM-like region of FAK can result in hyperphosphorylation and activity of FAK.

**Regulation of K38A through Integrins and Focal Adhesions**—The phosphorylation state of FAK is regulated by the adhesion state of the cell through the binding of integrins to their extracellular matrix ligands and the formation of focal adhesions (1, 2, 26). To see whether the regulation of the FAK mutants by the binding of the \(\beta1\) integrin to FN is intact we expressed HA-tagged WT-FAK, the K38A mutant, or the K78A mutant in NIH3T3 cells and replated the cells onto FN- or poly-L-lysine-coated dishes. 25 \(\mu\)g of whole cell lysates were immunoblotted with antibodies against PY397 (top panel) or HA (bottom panel). This suggests that the defect in phosphorylation of this mutant in growing cells is not because of an inability of this mutant to become activated, but because of a defect in maintaining its phosphorylation in growing cells, or because of a lack of phosphorylation on residues other than Tyr-397. These results show that the hyperphosphorylated K38A mutant of FAK is constitutively active.

FAK localizes to discrete punctate structures, termed focal adhesions, where the cell maintains close contact with the underlying extracellular matrix (27). Previous studies have shown that the carboxyl-terminal region of FAK is both necessary and sufficient for the localization of FAK into these structures (28). However, recently a group that made several FAK and pyk2 chimeras suggested that the amino terminus of FAK may have some influence over this localization (29). To confirm that neither of the mutations that affect the phosphorylation of FAK alters its cellular localization, we transiently transfected HA-tagged WT-FAK, the K38A mutant, or the K78A mutant into NIH3T3 cells. The cells were processed for indirect immunofluorescence against either HA (green) or paxillin (red) in the left panels, or HA (green) and phallolin (red) in the right panels.
Red-conjugated phallodin to visualize actin structures. As shown in Fig. 3b both of the mutants showed a punctate focal adhesion localization similar to WT-FAK. Neither mutation had an obvious effect on the localization of paxillin to focal adhesions or actin structures as shown or on the localization of vinculin or the presence of PY20 staining focal adhesions (data not shown). These results show that aberrant phosphorylation of either mutation is neither caused by nor effects the localization of FAK at focal adhesions.

Regulation of K38A Mutant through Src—The tyrosine kinase Src has been suggested to play an integral part in regulating the phosphorylation of FAK (1, 3–5). However, we observed previously that a truncation mutant lacking 400 amino acids from the amino terminus of FAK (Δ400) is still capable of being phosphorylated despite the lack of a binding site for Src (17). This suggested that truncation of the amino terminus may lead to Src-independent phosphorylation through alleviating the autoinhibition of the activity of FAK imposed by the presence of the amino terminus. To test this more stringently, we constructed Δ375 truncation mutants that either contained the Y397F mutation or the K454R mutation (KD), which renders FAK catalytically inactive. These mutants of FAK or the corresponding mutations within the full-length FAK background were transiently transfected into NIH3T3 cells. As shown in Fig. 4a, either mutation of Y397F to disrupt Src binding, or mutation of K454R to disrupt the catalytic activity of FAK ablated the phosphorylation of the full-length FAK constructs (right three lanes). Mutation of Y397F within the Δ375 truncation did not alter its phosphorylation. Inactivation of the truncations catalytic activity, however, did completely disrupt its phosphorylation, suggesting that the heightened phosphorylation of this construct is dependent on its own activity and is not being mediated through another tyrosine kinase (left three lanes). As expected both the Y397F mutation and the K454R (KD) mutation disrupted phosphorylation at Tyr-397 for both the full-length and deletion mutant. These data suggest that removal of the autoinhibitory influence of the amino terminus through a truncation mutation can allow FAK to phosphorylate itself independent of its ability to bind to Src.

To ascertain whether the K38A mutation is also independent of Src for its phosphorylation we placed this mutation into either the Y397F or K454R background. As shown in Fig. 4a (middle lanes) shows that mutation of K38A to render it catalytically inactive (K38A/KD) disrupted its phosphorylation as visualized by a phosphotyrosine immunoblot. Although this mutant is hyperphosphorylated to a similar extent as the truncation mutant, Δ375, we found that the K38A/Y397F double mutant was not strongly phosphorylated. As expected, both the K38A/Y397F and K38A/KD double mutants showed no detectable phosphorylation at Tyr-397 as judged by pY397 immunoblot. These mutants were expressed in similar amounts as revealed by immunoblots against the HA tag of the exogenously expressed mutants, suggesting the differences in phosphorylation are not related to differences in protein expression. Similar results were obtained in CHO cells (data not shown). This result suggests that unlike truncation of the amino terminus, overall phosphorylation of the K38A mutant is still dependent on phosphorylation of Tyr-397.

To further clarify the role of Src in phosphorylation of FAK and its mutants, we transiently transfected WT-FAK, the K38A mutant, or the Δ375 truncation mutant into CHO cells. Cells were replated onto FN in the presence of the Src inhibitor PP2 or the control compound PP3. As shown in Fig. 4b, both total phosphorylation and phosphorylation at Tyr-397 are strongly reduced when full-length wild type FAK is replated in the presence of the Src inhibitor PP2, but not the control compound PP3, reiterating the importance of Src activity to both phosphorylation of wild type FAK at Tyr-397 and its subsequent phosphorylation at other residues. The truncation mutant, Δ375, remains both phosphorylated at Tyr-397 and at other residues in the presence of Src inhibitor PP2, confirming that removal of the amino terminus through a truncation mutation renders FAK independent of the activity of Src. Interestingly, the effect of PP2 on the K38A mutant is between its effect on full-length wild type FAK and the truncation mutant. Although the presence of PP2 clearly reduces the overall phosphorylation of the K38A mutant, the mutant is still clearly phosphorylated. In contrast, the phosphorylation of the K38A mutant at Tyr-397 is only slightly reduced in the presence of Src inhibitor PP2. Similar results were found in NIH3T3 cells (data not shown). These data suggest that the mutation of K38A reduces the dependence of FAK on Src for its phosphorylation, but does not make it completely Src independent. It also suggests that the clear hyperphosphorylation of the K38A mutant, and by analogy the Δ375 mutant is not entirely attributable to being rendered Src-independent, but is likely because of some other structural alteration or mechanism.

K38A Weakens the Interaction between Amino Terminus and Kinase Domain—Previously we showed that the amino-termi-
NK38A fragments were expressed in comparable amounts tagged full-length FAK (panel). Both the NWT and do so. The expression of the wild type amino terminus, or was capable of reducing the phosphorylation of FAK at Tyr-397, we found that vector alone, pkH3-NWT, or pkH3-NK38A with pHAN WT-K38A point mutation and co-transfected CHO cells with pkH3 amino terminus from residues 1 to 400 that contained the myc-tagged kinase domain. 1 mg of whole cell lysates were immunoprecipitated from whole cell lysates with HA-conjugated agarose. As shown in Fig. 5, all three exogenously expressed proteins were present in similar amounts in the immunoprecipitates. WT-FAK and the K78A mutant of FAK were immunoprecipitated from whole cell lysates with antibodies against the myc tag (middle panel). 25 μg of whole cell lysates were blotted with antibodies against the myc tag (bottom panel). b, CHO cells were co-transfected with myc-tagged WT-FAK and pkH3 vector alone, pkH3 NWT, or pkH3 NK38A. Cells were serum starved overnight and then replated onto FN-coated dishes for 30 min prior to lysis. 25 μg of whole cell lysates were blotted with antibodies against PY397 (top panel), myc (middle panel), or HA (bottom panel).

FIG. 5. The K38A mutation disrupts the interaction between FAK and its kinase domain. a, 293T cells were co-transfected with HA-tagged WT-FAK or K38A, K78A, or Δ375 FAK mutants and the myc-tagged kinase domain. 1 mg of whole cell lysates were immunoprecipitated with HA-agarose. The top half of the immunoprecipitates was immunoblotted with antibodies against HA (top panel). The bottom half of the immunoprecipitates was immunoblotted with antibodies against the myc tag (middle panel). 25 μg of whole cell lysates were blotted with antibodies against the myc tag (bottom panel). b, CHO cells were co-transfected with myc-tagged WT-FAK and pkH3 vector alone, pkH3 NWT, or pkH3 NK38A. Cells were serum starved overnight and then replated onto FN-coated dishes for 30 min prior to lysis. 25 μg of whole cell lysates were blotted with antibodies against PY397 (top panel), myc (middle panel), or HA (bottom panel).

The K38A mutation was capable of interacting with its own kinase domain (17). To test whether the K38A mutation affects the interaction between the amino terminus and the kinase domain, we co-transfected 293T cells with pkH3-vector alone, WT-FAK, or FAK mutants K38A, or K78A with the myc-tagged kinase domain of FAK. The full-length FAK constructs were immunoprecipitated from whole cell lysates with HA-conjugated agarose. As shown in Fig. 5, all three exogenously expressed proteins were present in similar amounts in the immunoprecipitates. WT-FAK and the K78A mutant of FAK were capable of precipitating the myc-tagged kinase domain to similar degrees, however, the K38A mutant of FAK was only weakly capable of precipitating the kinase domain. The myc-tagged kinase domain was present in similar amounts of whole cell lysates under each condition. Similar results were obtained in CHO cells (data not shown). These data show that the K38A mutation weakens the interaction between the kinase domain and the amino terminus, and suggests that this disruption is sufficient to allow hyperphosphorylation of this mutant.

K38A Mutant Does Not Inhibit the Phosphorylation of FAK in Trans—Previously we showed that expression of fragments of the amino terminus of FAK were capable of inhibiting the phosphorylation of FAK in trans (17). To test whether the K38A mutant was capable of inhibiting the phosphorylation of FAK in trans, we constructed a HA-tagged fragment of the amino terminus from residues 1 to 400 that contained the K38A point mutation and co-transfected CHO cells with pkH3 vector alone, pkH3-NWT, or pkH3-NK38A with pHAN WT-FAK containing a myc tag. As shown in Fig. 5b, we found that although co-expression of the wild type amino terminus (NWT) was capable of reducing the phosphorylation of FAK at Tyr-397, the K38A mutant amino terminus (NK38A) was unable to do so. The expression of the wild type amino terminus, or mutant amino terminus did not effect expression of the myc-tagged full-length FAK (middle panel). Both the NWT and NK38A fragments were expressed in comparable amounts making it unlikely that differences in protein expression contribute to the different effects of the two constructs (lower panel). This result provides further support for our hypothesis that the hyperphosphorylation of the full-length K38A mutant is because of a weakening of the interaction between the amino terminus and kinase domain caused by the mutation, which in turn weakens the inhibitory effect the amino terminus has on the activity of FAK resulting in hyperphosphorylation.

Effect of Mutations on Cell Cycle Progression—To determine whether the K38A mutant was capable of influencing the ability of FAK to promote cell cycle entry, NIH3T3 cells were transiently transfected with WT-FAK, the K38A mutant of FAK, or the Δ375 truncation mutant of FAK, and BrdU incorporation assays were used to assess the effect of these mutants on cell cycle progression. As shown in Fig. 6a, the effect of the K38A mutant was similar to that of the Δ375 mutant, with both being able to increase the percentage of cells that had entered S phase over that of the WT-FAK. These results show that mutations that alter the phosphorylation of FAK can impact its ability to function in cells.

We next tested whether the fragment of the amino terminus bearing the K38A mutation was capable of inhibiting cell cycle progression. NIH3T3 cells were transfected with either pkH3 NWT or NK38A, and BrdU incorporation assays were performed. As shown in Fig. 6b the WT amino terminus was capable of decreasing the percentage of cells that had entered S phase at 18 and 24 h when compared with the percentage of nontransfected cells. However, the fragment of the amino terminus bearing the K38A mutation did not have a significant effect on the percentage of cells that had entered S phase. This suggests that the ability of the wild type amino terminus to inhibit cell cycle progression is because of its ability to inhibit the phosphorylation of FAK as the point mutation that weakens the interaction between the kinase domain and amino terminus, and is unable to inhibit the phosphorylation of FAK in trans and is unable to inhibit cell cycle progression.

Effect of FAK Mutants on Migration—To examine the effect of the K38A mutant on cell migration, we transiently transfected CHO cells with the pkH3 vector alone, WT-FAK, the K38A mutant of FAK, the K78A mutant of FAK, or the Δ375 truncation mutant of FAK, and pEGFP to approximate the number of cells transfected under each condition. The cells were then subjected to a Boyden chamber migration assay as described under “Materials and Methods.” As shown in Fig. 7, few cells migrated to the lower side of the chamber when no FN was present, and neither expression of WT-FAK nor any of the mutants affected the ability of the cells to migrate in the absence of a FN stimulus. The presence of FN in the lower chamber causes a robust increase in the number of cells that migrate through the membrane. Expression of WT-FAK slightly increases the number of migrated cells. Expression of the Δ375 mutant of FAK causes no significant change in the number of cells that migrated toward FN. The K78A mutant of FAK that is underphosphorylated in growing cells also does not promote cell migration. However, the K38A mutant causes a 2-fold increase in the ability of cells to migrate toward FN. A HA blot of whole cell lysates showed that each construct was expressed in similar amounts under these conditions. The ability of the K38A mutation of FAK to promote directed cell migration is consistent with the strong hyperphosphorylation of this mutant under a variety of conditions, and shows that increasing the activity and phosphorylation of FAK through mutagenesis of the amino terminus can affect the function of FAK within the cell. It also suggests that the inability of the Δ375 mutant of FAK to promote cell migration is not directly linked to its hyperphosphorylation, but is instead because of...
cell population that were BrdU positive. The data are presented as the percentage of cells in the untransfected HA and BrdU. The percentage of HA positive cells that were BrdU was scored, as was the percentage of cells in the untransfected population that was BrdU positive. The data are presented as the average from four experiments ± 1 S.D.

![Image](https://example.com/image)

**Fig. 6.** The K38A mutant increases cell cycle entry. a, NIH3T3 cells were transfected with HA-tagged WT-FAK, or K38A, K78A, or Δ375 mutants of FAK. Cells were serum starved for 18 h prior to replating onto glass coverslips in the presence of 10% calf serum and 150 μM BrdU. Cells were processed for indirect immunofluorescence against HA and BrdU. The percentage of HA positive cells that were BrdU positive was scored, as was the percentage of cells in the untransfected cell population that were BrdU positive. The data are presented as the average from four experiments ± 1 S.D.

b, NIH3T3 cells were transfected with pkH3 NWT or pkH3 NK38A, and serum starved for 18 h prior to replating the cells on glass coverslips in the presence of 10% calf serum and 150 μM BrdU. Cells were then processed for indirect immunofluorescence following 18 or 24 h of growth against the HA tag of the exogenously expressed protein, and BrdU. The percentage of HA positive cells that where BrdU positive was scored, as was the percentage of cells in the untransfected population that was BrdU positive. The data are presented as the average from four experiments ± 1 S.D.

the lack of a critical region within the amino terminus of FAK that is necessary for cell migration.

**DISCUSSION**

Several recent studies have highlighted the importance of the amino-terminal FERM-like domain of FAK in the regulation of its activity and phosphorylation state by integrins (10, 17–19). Previously we showed that truncation mutants of the amino-terminal domain of FAK were hyperphosphorylated when expressed in mammalian cells and showed increased activity in vitro, suggesting that the amino-terminal domain of FAK was capable of exerting an inhibitory influence over full-length FAK. We also characterized an interaction between the amino terminus of FAK (residues 1–400) and its kinase domain, and showed that expression of the amino terminus in trans could inhibit the phosphorylation of FAK (17). In this article we identify a novel point mutation within the amino-terminal domain of FAK, K38A, which both allows hyperphosphorylation of FAK and disrupts the ability of the amino terminus to suppress the catalytic activity of FAK (see Figs. 2 and 5b). This mutant appears to selectively disrupt the inhibitory function of the amino-terminal region of FAK as the mutation does not disrupt the ability of FAK to promote cell migration, as do larger deletions of the amino terminus (Δ375) (see Fig. 7). Other functions of FAK including its catalytic activity, localization to focal adhesions, ability to promote cell cycle progression, and ability to phosphorylate a FAK substrate, paxillin, are not negatively affected by the K38A mutation, suggesting that this mutation does not grossly disrupt the structure of the amino-terminal domain of FAK, or of FAK in general (see Figs. 2, 3, and 6). This is the first report of a single point mutation outside of the catalytic domain of FAK that is capable of hyperphosphorylation, and it corroborates earlier findings that used larger deletions within the amino terminus to implicate this region as critical to the normal cellular regulation of FAK.

The amino-terminal domain of FAK contains a region of 300 amino acids that shares low sequence similarity with FERM domain containing proteins such as ezrin, talin, and band 4.1 (21). Many structures of FERM domains both isolated and in the context of larger protein complexes are known (22–24). We used both the sequence similarity within the amino terminus of FAK and comparison to known FERM domain structures to investigate the role of the amino terminus in the inhibitory influence the amino terminus of FAK has on its own activity as a kinase and phosphorylation state. FERM domain structures consist of clover-like overall architecture made up of three subdomains (22). Our initial experiment using fragments of FAK corresponding to each of the three subdomains suggested that the first subdomain of FAK likely played an important role in FAK autoinhibition (Fig. 1). This was then corroborated by our mutagenesis results, which suggested that individual mutation of two charged residues within this region could dramatically alter the phosphorylation state and activity of FAK within the cell. We found that mutation of Lys-38 to alanine resulted in hyperphosphorylation of FAK, whereas mutation of Lys-78 to alanine led to underphosphorylation of FAK (Fig. 2). Comparison of the sequence alignment between the canonical FERM domains and FAK reveals that a positively charged residue is conserved at the position corresponding to Lys-38 in FAK. For the K38A mutation the presence of hydrophobic residues immediately surrounding this residue are also well conserved, suggesting that the structure of this region may be significant to FERM domain structure in general. It has been proposed that the first subdomain of FERM domains may contain potential binding sites (22). Our study is the first to show that a single point mutation in this region selectively disrupts a FERM domain function of FAK. Interestingly a study by Chen et al. (30), also implicated the first subdomain of FAK in the ability to bind to and activate the tyrosine kinase Etk and to promote directed cell migration. The ability of the K38A mutation to promote directed cell migration suggests that this residue is not critical for the migratory function of the amino-terminal domain of FAK, and implies that other residues within the FERM-like region of FAK are involved in promoting cell migration.

In many FERM domain containing proteins, the FERM domain functions to control the cellular activity of the protein. This is generally achieved by an inhibitory self-interaction, as is the case for ezrin where the interaction of the FERM domain of ezrin with its carboxyl terminus masks the ability of the
carboxyl terminus to bind to actin (31). In all known structural studies of FERM domain self-interactions it is residues within the second and third subdomains that create the binding surface for both the intramolecular self-interactions and for interactions with other proteins, which serve to activate the protein (22, 32). However, our results suggest that the inhibitory self-interaction of FAK is not directly analogous to the self-interactions of other FERM domain containing proteins. Our results strongly implicate the first subdomain of the FERM domain of FAK as critical for its ability to inhibit the activity and function of the whole molecule. Previously we showed that truncation mutants from the amino terminus of FAK as short as 125 amino acids were hyperphosphorylated. This was supported by the ability of a small fragment of the amino terminus, consisting of residues 1–127, to inhibit phosphorylation of full-length FAK at Tyr-397 (see Fig. 1). Our mutagenesis results also produced a single point mutation in this region, K38A, which lead to hyperphosphorylation of FAK and increased the ability of FAK to phosphorylate a FAK-dependent substrate, paxillin, in cells (see Fig. 2), suggesting the importance of the first subdomain of FAK in its autoregulation.

Interestingly, a second mutagenesis study considering residues within the second and third subdomains of the FERM-like region of FAK recently identified a mutation that was capable of interfering with the ability of FAK to become activated (19). This mutant, which contains three lysines, Lys-216, Lys-218, and Lys-221 mutated to alanine, did not disrupt the interaction between FAK and its kinase domain, or the catalytic activity of the mutant in vitro, but the mutant fails to become highly phosphorylated when cells were stimulated with FN (19). This mutation is in contrast to the K78A mutation reported in this paper, which appears underphosphorylated in growing cells (at steady-state), but is capable of being phosphorylated when stimulated with FN (see Figs. 2 and 3). This suggests that the K78A mutation is not defective for activation of FAK downstream of integrin pathways, but is defective in maintaining its phosphorylation in adherent cells.

The presence of mutations in the second subdomain at residues Lys-216, Lys-218, and Lys-221, which do not become phosphorylated when cells are stimulated with FN, suggests that the second subdomain may contain a binding site for a FAK activating protein. The presence of these three mutations has been suggested to disrupt the interaction between FAK and a potential activating protein, and it is believed to be underphosphorylated because its ability to be activated downstream of integrin-dependent pathways is disrupted (19). Our data suggests that the first subdomain of the FERM-like region of FAK contributes to an interaction between the FERM domain and the kinase domain that serves to keep FAK in an inactive state until it is activated. However, our data does not rule out possible involvement of the second subdomain in the interaction with the kinase domain.

Strikingly, the residue (Arg-8) corresponding to Lys-38 of FAK in the ezrin structure (24), and the residues (Thr-164,
Asp-166, and Glu-169 in ezrin) corresponding to the Lys-216, Lys-218, and Lys-221 mutant of FAK lie on opposite ends of the same face of the FERM domain of ezrin (see Fig. 8). By analogy to ezrin or other FERM domain containing structures, Lys-38 is likely positioned in the beginning of the first β strand of the first subdomain with the positively charged side chain facing away from the interior of the structure where it could participate in an interaction with another protein. The mutations within the second domain identified by Dunty et al. (29) are at the end of an α-helix with the side chains facing out from the helix. Such an arrangement of residues would lend itself to the mode of regulation we are proposing, as the region in the second subdomain where an activating protein is likely to bind may be accessible in the inactive FAK molecule, whereas the other end of the domain is participating in the inhibitory interaction. Binding of a potential activator to the residues in the second FERM subdomain of FAK, however, may be capable of sterically blocking the first interaction of the subdomain with the kinase domain, as the two sets of residues may be still lying on the same face of the molecule. It is also possible that residues along this face of the molecule from both the first and second subdomains participate in both the interaction with the kinase domain and interaction with an activating protein. The presence of a FAK mutant that is underphosphorylated and appears to be unable to become activated suggests that these two interactions are distinguishable. Although structural comparison to other FERM domains suggests a mode of regulation that is in keeping with the current mutagenesis data, any structural interpretation must be viewed cautiously because FAK has very low sequence similarity with other FERM domains containing proteins (27%) and because of the presence of a 35-amino acid region amino-terminal to the FERM domain, which is large enough to have its own structural contribution to the amino terminus of FAK (25). However, lacking more direct structural information such comparisons are useful for proposing hypothetical models and interpreting the existing data.

Although the K38A point mutation leads to clear hyperphosphorylation of FAK and appears to disrupt the inhibitory influence this region has on the activity of FAK, we found several differences between this point mutant and the larger deletion mutant of FAK, Δ375, in terms of their ability to be regulated through phosphorylation of Tyr-397 and Src. First, the Δ375 mutant of FAK maintains high levels of phosphorylation when Tyr-397 is mutated to phenylalanine, whereas the K38A mutant, like full-length WT-FAK, is not strongly phosphorylated in the same background (see Fig. 3a). However, phosphorylation of both mutants is blocked by mutation of K454R, which renders FAK catalytically inactive suggesting that phosphorylation of the Δ375/F397 mutant is mediated through its own activity and is not dependent of the activity of another tyrosine kinase (see Fig. 3o). Second, phosphorylation of the Δ375 mutant is not strongly affected by the presence of the Src inhibitor PP2, whereas general phosphorylation of the K38A mutant (as judged by PY20 blot) is strongly decreased in the presence of the inhibitor, and phosphorylation at Tyr-397 (as detected by PY397 blot) is more moderately affected (see Fig. 3b). The K38A mutant appears between the stark inhibition of phosphorylation in the presence of PP2 seen for the full-length molecule, and the lack of inhibition in the presence of PP2 seen for the deletion mutant.

These results suggest that the K38A point mutation differentiates between two regulatory events important for the activation of FAK. One event is an activating event, which would remove the amino terminus away from the kinase domain of FAK and allow phosphorylation at Tyr-397. The K38A mutant bypasses this step because its ability to interact with the kinase domain of FAK is compromised (see Fig. 5). The Δ375 truncation also bypasses this step, because it lacks the FERM-like region of FAK. Thus both of these mutants exist in an already activated state and do not need an interaction with a second protein to disrupt the interaction between the amino terminus and the kinase domain, and allow phosphorylation of Tyr-397. Such a step presumably is necessary for activation of WT-FAK.

The second step key to full FAK activation is the recruitment of Src to phosphorylated Tyr-397 and subsequent phosphorylation of FAK at other residues. This step is key to many FAK signaling functions as evidenced by the consequences of the Y397F mutation. The K38A mutant although able to bypass the first activating event, still requires Src activity and phosphorylation of Tyr-397 to become phosphorylated at other residues. The Δ375 mutant, however, appears to bypass this regulatory step as well and remains phosphorylated both in the absence of Tyr-397 phosphorylation (as in the Δ375/Y397F mutant), and in the absence of Src activity. Binding of Src to phosphorylated Tyr-397 may stabilize the linker region between the amino terminus and the kinase domain and thus may promote further phosphorylation at other residues by Src or by FAK itself. The ability of the Δ375 mutant of FAK to become phosphorylated in the absence of Tyr-397 phosphorylation could be attributed to an increased accessibility of Src to its catalytic domain because of the removal of the bulky amino terminus. However, the strong phosphorylation of this mutant in the presence of the Src inhibitor PP2, and its lack of phosphorylation in the catalytically dead background (Δ375/KD) suggest that the Δ375 mutant is capable of phosphorylating itself at other residues. Perhaps loss of the amino terminus in the truncation mutant leads to an ability to transphosphorylate itself through intermolecular phosphorylation that is structurally impossible in the context of the full-length protein, even once the full-length protein is activated. This interpretation is supported by the study of Toutant et al. (10), which used an antibody system to show that this mutant was capable of intermolecular transphosphorylation. However, it remains possible that other residues within the amino terminus play a more direct role in directing the phosphorylation of FAK through Src. Our results also imply that the Src event is secondary to the “activating” event because the mutation that bypassed the primary activating event can still be effected by interfering with the activity of Src.

Previous studies have provided strong support for involvement of the amino-terminal domain of FAK in regulation of its central catalytic domain (10, 17–19). In this paper we have corroborated previous studies, which showed that large truncation mutants of FAK are hyperphosphorylated and have increased catalytic activity in vitro, by identifying a single point
mutation within the first subdomain of the FERM-like region of FAK that is hyperphosphorylated. The large distance of this mutation from the kinase domain, and relative conservative nature of this mutation suggest that the hyperphosphorylation seen in the larger truncations is not a simple artifact but can be duplicated with a simple point mutation. This paper also directly links the first subdomain of the FERM-like region of FAK to its regulation, and we suggest that it is the first subdomain that is likely to interact directly with the kinase domain of FAK and is responsible for its inhibitory influence on the activity of FAK.

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Residues within the First Subdomain of the FERM-like Domain in Focal Adhesion Kinase Are Important in Its Regulation
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