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# Phosphorylation of Focal Adhesion Kinase at Tyrosine 861 Is Crucial for Ras Transformation of Fibroblasts*

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**Although elevated expression and increased tyrosine phosphorylation of focal adhesion kinase (FAK) are crucial for tumor progression, the mechanism by which FAK promotes oncogenic transformation is unclear. We have therefore determined the role of FAK phosphorylation at tyrosine 861 in the oncogenic transformation of NIH3T3 fibroblasts. FAK phosphorylation at tyrosine 861 was increased in both constitutively H-*Ras*-transformed and H-*Ras*-inducible NIH3T3 cells, in parallel with cell transformation. However, H-*Ras*-inducible cells transfected with the nonphosphorylatable mutant FAK Y861F showed decreased migration/invasion, focus forming activity and anchorage-independent growth, compared with either wild-type or kinase-defective FAK. In contrast to unaltered FAK/Src activity, the association of FAK and p130$^{CAS}$ was decreased in FAK Y861F-transfected cells, and FAK phosphorylation at tyrosine 861 enhanced this association *in vitro*. Consistently, FAK Y861F-transfected cells were defective in activation of c-Jun $NH_2$-terminal kinase and in expression of matrix metalloproteinase-9 during transformation. Taken together, these results strongly suggest that FAK phosphorylation at tyrosine 861 is crucial for H-*Ras*-induced transformation through regulation of the association of FAK with p130$^{CAS}$.**

Focal adhesion kinase (FAK)[1] is a non-receptor cytoplasmic tyrosine kinase that modulates multiple cell functions, including migration, proliferation, and survival (1, 2). Elevated expression and increased tyrosine phosphorylation of FAK have been reported in several types of malignant tumors, suggesting that FAK may play a role in tumor progression (3, 4), especially because the deregulation of processes normally regulated by FAK, namely adhesion-dependent cell growth, survival, and motility, are critical aspects of tumor progression (5, 6).

The ability of FAK to transduce downstream signals depends on its phosphorylation at tyrosine residues and its ability to interact with several intracellular signaling molecules, including the Src family kinases (7), p130$^{CAS}$ (8), Grb2 (9), and phosphatidylinositol 3-kinase (10, 11). FAK phosphorylation at tyrosine 397 results in its direct interaction with Src, which contributes to the transformation of fibroblasts (12, 13). Subsequent recruitment to the complex of proteins containing Src homology 2 (SH2) domains, including Grb2 and c-Crk, is likely to trigger adhesion-induced cellular responses, including changes to the actin cytoskeleton and activation of the Ras-mitogen-activated protein kinase pathway (14, 15).

p130$^{CAS}$ docking protein was initially identified as a major phosphotyrosine-containing protein in cells transformed by the v-*src* and v-*crk* oncogenes (16). In addition, p130$^{CAS}$ may be a substrate of v-Src kinase and a binding target for the SH2 domain of v-Crk (17) during retroviral transformation. Indeed, transfection of antisense p130$^{CAS}$ mRNA into *ras*- and v-*src*-transformed cells led to their reversion (18), and mouse embryonic fibroblasts lacking p130$^{CAS}$ were resistant to Src-induced transformation (19). p130$^{CAS}$-deficient mouse embryonic fibroblasts also showed impaired actin bundling and cell migration, and these properties were restored after re-expression of p130$^{CAS}$ (19), further indicating that this protein is essential in signal transduction during cell migration and transformation.

Phosphorylation of FAK at tyrosine 861 (FAK Tyr$^{861}$ phosphorylation) is especially interesting, because it is known to regulate migration of prostate carcinoma cells with increasing metastatic potential (20), as well as the migration and survival of vascular endothelial cells (21). In addition, FAK Tyr$^{861}$ phosphorylation is increased in metastatic breast cancer cells (22) and *ras*-transformed fibroblasts (23) and decreased in detransformed cells by trichostatin A (23). We therefore hypothesized that FAK Tyr$^{861}$ phosphorylation may regulate transforming activity in transformed/cancer cells. Here we report that FAK Tyr$^{861}$ phosphorylation is crucial for H-*Ras*-induced transformation by regulating the association between FAK and p130$^{CAS}$.

## EXPERIMENTAL PROCEDURES

*Reagents and Antibodies*—Doxycyclin and puromycin were purchased from Sigma, and fibronectin was purchased from Invitrogen. Monoclonal antibodies (mAbs) to phosphotyrosine (4G10) and p130$^{CAS}$ (8G4-E8) were purchased from UBI (Hauppauge, NY), mAb to HA (12CA5) was purchased from Roche Applied Science, mAbs to H-*Ras* (F235), GST (B-14), phospho-specific ERK (E-4), and ERK2 (K-23) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mAbs to JNK and phospho-specific JNK (pT183/pY185) were purchased from Cell Signaling Technology (Beverly, MA). Mouse mAb to FAK and rabbit polyclonal antibodies to phosphorylation site-specific FAK[PY$^{397}$] and FAK[PY$^{861}$] were purchased from BioSource Quality Controlled Biochemicals, Inc. (Morgan Hill, CA).

*Establishment of a Doxycyclin-regulated H-Ras NIH3T3 Cell Line*—Mouse wild-type H-*Ras* cDNA in pcDNA3.1 (Invitrogen) was a generous

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[1] The abbreviations used are: FAK, focal adhesion kinase; SH2, Src homology 2; mAb, monoclonal antibody; GST, glutathione *S*-transferase; JNK, c-Jun $NH_2$-terminal kinase; FBS, fetal bovine serum; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium.

gift from Dr. Zea Young Ryoo of the Catholic University of Korea. The full-length H-*Ras* (G12R) cDNA was enzymatically excised and subcloned, using the GeneTailer site-directed mutagenesis system (Invitrogen), into the BamHI site of the tetracycline-inducible vector, pTRE-IRES-EGFP, a generous gift from Dr Hong Jian Zhu of the Ludwig Institute for Cancer Research (Melbourne, Australia). To obtain NIH3T3 cells with doxycyclin-induced (a tetracycline derivative) H-*Ras* (G12R) expression, pTRE-H-*Ras*-IRES-EGFP and pEFpurop-Tet-on (the generous gift of Dr. Hong Jian Zhu) were cotransfected into NIH3T3 cells with FuGENE6 reagent (Roche Applied Science), and the cells were selected with puromycin. Positive clones were those that expressed H-*Ras* in the presence of doxycyclin, as shown by Western analysis using anti H-*Ras* antibody (F235 clone; Santa Cruz). These H-*Ras* NIH3T3 cells were grown in a 5% $CO_2$ atmosphere at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen), 60 mg/ml penicillin, and 100 mg/ml streptomycin. Puromycin (2 $\mu$g/ml) was added to the medium for H-*Ras* expression clones.

*Construction of Mutant FAK Mammalian Expression Vectors*—Site-directed mutagenesis of full-length cDNA encoding FAK in the pRC/CMV vector was performed using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA). The synthetic oligonucleotide, CCA ACA CAT CTT TCA GCC TGT GGG G ($Tyr^{861} \rightarrow$ Phe), was used to change tyrosine to phenylalanine. cDNAs encoding FAK and its mutant Y861F were inserted into pRC/CMV at the NotI/XbaI cloning sites, which generated in-frame fusions of a sequence encoding three HA epitopes (YPYDVPDYA) at the 3′ end of the FAK coding sequences. Expression vectors encoding epitope-tagged WT FAK (pKH3-FAK) and kinase-defective FAK (pKH3-kdFAK) were kindly provided by Dr. Jun-Lin Guan of Cornell University (Ithaca, NY).

*Transfections*—Transient transfections were carried out using LipofectAMINE reagent (Invitrogen), as described by the manufacturer. In brief, NIH3T3 and H-*Ras*-inducible NIH3T3 cells were plated in 100-mm dishes and grown to ~80% confluency. To each culture was added 5 ml of a mixture of 15 $\mu$l of LipofectAMINE and 4 $\mu$g of plasmid DNA, and the cells were incubated for 6 h at 37 °C in a 5% $CO_2$, 95% air incubator. To each was added 5 ml of DMEM containing 20% FBS, and the cells were further incubated for 24 h. The medium was then aspirated and replaced with 5 ml of DMEM containing 10% FBS.

*Immunoprecipitation and Immunoblotting*—The cultures were washed twice with phosphate-buffered saline, and the cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 2 mM $Na_3VO_4$) containing a protease inhibitor mixture (1 $\mu$g/ml aprotinin, 1 $\mu$g/ml antipain, 5 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin A, and 20 $\mu$g/ml phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 10,000 × *g* for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS-PAGE. For immunoprecipitations, each sample, containing 200–1,000 $\mu$g of total protein, was incubated with the relevant antibody for 2 h at 4 °C, followed by incubation with protein G-Sepharose beads for 1 h. Immune complexes were collected by centrifugation. The proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences), which were incubated with the appropriate primary antibodies, followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). The signals were detected by ECL (Amersham Biosciences).

*Focus Forming and Soft Agar Growth Assays*—In the focus forming assay, $5 \times 10^4$ H-*Ras*-transformed NIH3T3 cells were plated and incubated for 10–14 days, fixed in 99% methanol, and stained with Wright-Giemsa stain. For analysis of colony formation in soft agar, $1 \times 10^5$ cells in 2 ml of DMEM containing 10% FBS, 2 $\mu$g/ml puromycin, and 0.3% agarose were seeded in 6-mm plates containing a 0.6% agarose underlay. The cultures were fed every 4 days, and the formation of colonies was scored after 3 weeks.

*Tumor Cell Migration and Invasion Assay*—Fibronectin (10 $\mu$g/$\mu$l in phosphate-buffered saline) was added to each well of a Transwell plate (Costar; 8-$\mu$m pore size), and the membranes were allowed to dry for 1 h at 25 °C. The Transwell plates were assembled in a 24-well plate, and the lower chambers were filled with DMEM containing 10% FBS and 0.1% bovine serum albumin. Cells ($5 \times 10^4$) were added to each upper chamber, and the plate was incubated at 37 °C in 5% $CO_2$ for 3 h. The cells that had migrated to the lower surface of the filters were stained with 0.6% hematoxylin and 0.5% eosin and counted. For invasion assays, 24-well Transwell plates (Costar; 8-$\mu$m pore size) were coated with fibronectin (10 $\mu$g/$\mu$l) on the lower side of the membrane and with Matrigel (30 $\mu$g/$\mu$l) on the upper side.

*In Vitro FAK/Src Kinase Assays*—FAK immunoprecipitates were washed twice with 1× RIPA buffer and once with 10 mM Tris buffer. The pellets were dissolved in 20 $\mu$l of kinase buffer (10 mM Tris, pH 7.4, 10 mM $MnCl_2$, 2 mM $MgCl_2$, 0.02% Triton X-100), and the reactions were started by adding 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP, 1 $\mu$M cold ATP, and GST-paxillin and incubated at 25 °C for 5 min. For the Src kinase assay, Src immunoprecipitates were dissolved in 20 $\mu$l of kinase buffer (10 mM Tris, pH 7.4, 5 mM $MnCl_2$) and preincubated for 5 min at 25 °C. To each sample was added 2 $\mu$g of acid-denatured enolase as exogenous substrate, and the samples were incubated at 25 °C for 5 min.

*Cell Adhesion and Spreading Assays*—Cell adhesion and spreading assays were performed on fibronectin coated tissue plates essentially as described (24). Briefly, fibronectin was diluted in serum-free medium, added to tissue culture plates (2 $\mu$g/cm$^2$) and incubated at 25 °C for at least 1 h to allow its adsorption. After washing with phosphate-buffered saline, the plates were blocked by incubating them with 0.2% heat-inactivated bovine serum albumin for 1 h and then washed with serum-free medium (2 × 10 min). The cells were detached with 0.05% trypsin, 0.53 mM EDTA, suspended in serum-free medium containing 0.25 mg/ml of soybean trypsin inhibitor, harvested, resuspended in serum-free medium, plated onto fibronectin-coated plates, and incubated for various periods of time at 37 °C.

*GST Pull-down Assays*—Three cDNA constructs encoding proline-rich domains of FAK, PR1PR2 (amino acids 711–877), PR1PR2F (amino acids 711–877, $Tyr^{861} \rightarrow$ Phe), and FAK PR2 (amino acids 811–877), were generated by PCR amplification. The PCR products were cloned into the BamHI/EcoRI site of the pGEX-4T-1 expression vector (Amersham Biosciences). The recombinant proteins were purified on glutathione-Sepharose 4B columns, phosphorylated *in vitro* with purified Src (23, 25), and mixed with H-*Ras*-inducible NIH3T3 cell lysates. After incubation at 4 °C on a rotator for 2 h, the precipitated complex was eluted with SDS-PAGE sample buffer and resolved by SDS-PAGE.

*RNA Extraction and Reverse Transcription Polymerase Chain Reaction*—Total RNA was extracted from cultured cells and used as templates for reverse transcriptase. Aliquots of cDNA were amplified using primers for MMP-2 (5′-AATACCTGAA-3′ (forward) and 5′-AAGGGGAACTTGCAGT-3′ (reverse)); MMP-9 (5′-GCTTTGCTGCCCC-3′ (forward) and 5′-GGAAAGGCGTGTGCCAG-3′ (reverse)); and rat $\beta$-actin (5′-TGGAATCCTGTGGCATCCATGAAAC-3′ (forward) and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (reverse)). The amplification protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C (MMP-9 and $\beta$-actin) or 55 °C (MMP-2) for 30 s, and extension at 72 °C for 60 s. The PCR products (545 bp for MMP-2, 544 bp for MMP-9, and 349 bp for $\beta$-actin) were cloned and sequenced to confirm their identity.

*Gelatinase Activity*—Conditioned culture media from $5 \times 10^6$ cells were subjected to nonreduced SDS-PAGE containing 1 mg/ml of gelatin. The gels were washed three times with 2.5% (v/v) Triton X-100 for 30 min each at 25 °C and three times with water for 10 min each, incubated at 37 °C overnight in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% $NaN_3$, 10 mM $CaCl_2$, stained with Coomassie Brilliant Blue, and destained. Protease activity was visualized as a clear zone against a blue background.

## RESULTS

*FAK Phosphorylation at Tyrosine 861 Is Increased in H-Ras-transformed Cells*—To investigate the potential role of FAK phosphorylation in *Ras*-induced transformation, we assayed FAK $Tyr^{861}$ phosphorylation in two different *ras*-transformed cells. We found that, in contrast to FAK $Tyr^{397}$ phosphorylation, FAK $Tyr^{861}$ phosphorylation was dramatically higher in H-*Ras* transformed NIH3T3 and K-*ras* transformed rat2 cells than in their respective untransformed cells (Fig. 1*A*). To further examine the correlation of FAK $Tyr^{861}$ phosphorylation with transformation, NIH3T3 cells were transfected with a tetracycline-inducible H-*Ras* expression vector. In response to 2 $\mu$g/ml doxycyclin, these cells gradually expressed H-*Ras* (Fig. 1*C*) and exhibited a spindle-like morphology with small round cell bodies (Fig. 1*B*). In addition, FAK $Tyr^{861}$ phosphorylation was increased in a time-dependent manner over 96 h (Fig. 1*C*). In contrast, when these transformed cells were removed from doxycyclin containing medium and cultured in the absence of doxycyclin, H-*Ras* expression was decreased, cell morphology reverted to a normal phenotype (Fig. 1, *B* and *D*), and FAK $Tyr^{861}$ phosphorylation was decreased (Fig. 1*D*). Taken together, these data strongly suggest that increased FAK $Tyr^{861}$

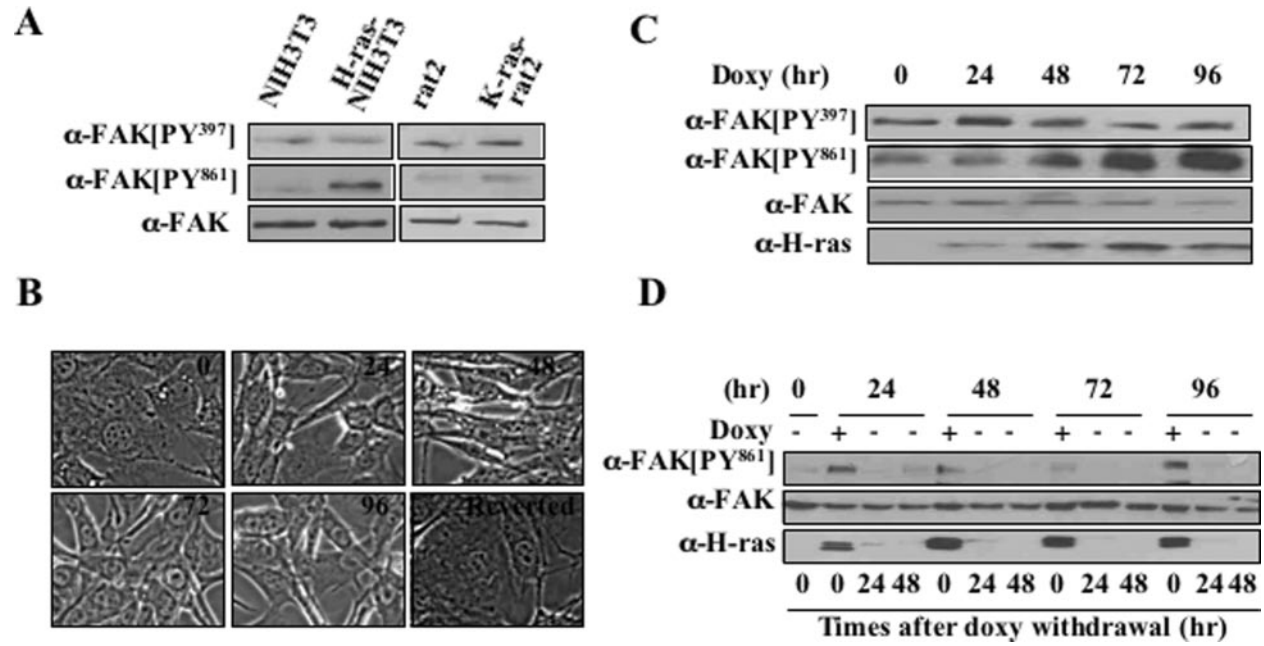


FIG. 1. **FAK phosphorylation at tyrosine 861 is increased in H-*Ras*-transformed cells.** *A*, cells were lysed, and site-specific FAK phosphorylation was analyzed by Western blotting with anti-FAK[PY$^{397}$] and anti-FAK[PY$^{861}$] antibodies. The amounts of proteins were monitored by stripping and reblotting the membranes with anti-FAK antibody. *B* and *C*, H-*Ras*-inducible NIH3T3 cells were treated with 2 $\mu$g/ml doxycyclin for the indicated periods of time. The photograph was taken under phase contrast optics with a digital camera. H-*Ras*-inducible NIH3T3 lysates extracted at the indicated times were immunoblotted with antibodies to FAK [PY$^{397}$], FAK[PY$^{861}$], FAK, and H-*Ras*. *D*, H-*Ras*-inducible NIH3T3 cells treated with (+) or without (−) 2 $\mu$g/ml doxycyclin for the indicated periods of time were detached and replated on culture plastic dishes in the absence of doxycyclin for the additional indicated periods of time. The photograph was taken under phase contrast optics with a digital camera (Reverted, shown in *B*). Total cell lysates were resolved by SDS-PAGE and subjected to immunoblotting with antibodies against FAK, FAK[PY$^{861}$], and H-*Ras*.

phosphorylation is correlated with the transformation of NIH3T3 cells.

*FAK Tyrosine Phosphorylation at 861 Is Required for H-Ras Transformation of NIH3T3 Cells*—To investigate the role of FAK Tyr$^{861}$ phosphorylation, we performed site-directed mutagenesis to replace tyrosine 861 with nonphosphorylatable phenylalanine residues (Y861F) and transfected HA-tagged Y861F into NIH3T3 cells (Fig. 2*A*). Compared with control cells, proliferations of wild-type FAK (wtFAK), kinase-defective FAK (kdFAK), and Y861F-transfected cells were not much altered, but cell migration was markedly increased in wtFAK-transfected cells and decreased in kdFAK- and Y861F-transfected cells (Fig. 2, *B* and *C*), implying that FAK Tyr$^{861}$ phosphorylation contributes to cell transformation through the regulation of migration rather than proliferation. Consistently, H-*Ras*-inducible NIH3T3 cells transfected with the Y861F mutant showed decreased migration, invasion, focus forming activity and anchorage-independent growth in soft agar (Fig. 3), indicating that FAK Tyr$^{861}$ phosphorylation is critical for H-*Ras* transformation of NIH3T3 cells.

*FAK Phosphorylation at Tyrosine 861 of FAK Regulates Its Interaction with p130$^{CAS}$*—Because FAK-mediated signaling involves interactions with the Src family kinases, FAK Tyr$^{861}$ phosphorylation may affect the activity of Src kinase. The *in vitro* kinase assay, however, showed that transfection of FAK Y861F mutant had no effect on the activity of Src (Fig. 4*A*). Similarly, FAK Tyr$^{861}$ phosphorylation had no effect on the activity of FAK (Fig. 4*B*), making it unlikely that FAK Tyr$^{861}$ phosphorylation regulates H-*Ras* transformation via the regulation of enzymatic activity. p130$^{CAS}$ has been shown to play a central role in transformation mediated by the v-*src* and H-*Ras* oncogenes (26), as well as being essential for FAK-mediated (27) cancer cell survival and migration (28). We found that overexpression of Y861F, but neither wtFAK nor kdFAK, caused a decreased interaction between p130$^{CAS}$ and FAK in

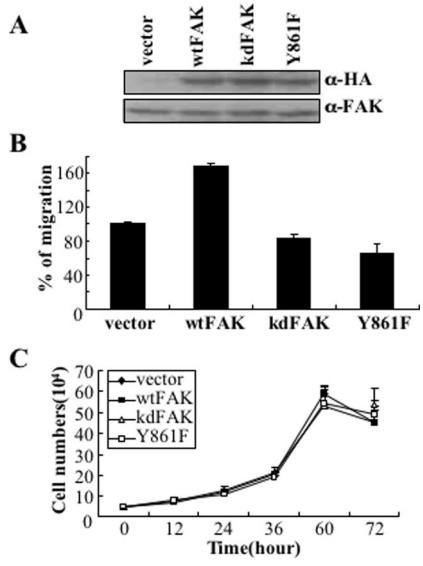


FIG. 2. **Overexpression of Y861F mutant leads to decreased migration of NIH3T3 cells.** NIH3T3 cells were transfected with empty vector (♦), wild-type FAK (wtFAK ■), kinase-defective FAK (kdFAK △), or Y861F (□) mutant FAK cDNA. *A*, total cell lysates were analyzed by SDS-PAGE followed by blotting with antibodies to HA and FAK. *B*, cells were split in 35-mm dishes ($5 \times 10^4$ cells/dish) and incubated for the indicated periods of time, and the numbers of cells were counted with a hemocytometer. *C*, cells were allowed to migrate on fibronectin-coated (10 $\mu$g/ml) Transwell plates for 2 h. After fixing and staining with 0.6% hematoxylin and 0.5% eosin, the number of migrated cells was counted. The results shown represent the averages of three independent experiments.

NIH3T3 cells (Fig. 5*A*). The interaction of p130$^{CAS}$ with FAK was increased in H-*Ras*-transformed cells but decreased in cells overexpressing Y861F (Fig. 5B), supporting the importance of the interaction of p130$^{CAS}$ with FAK in transforma-

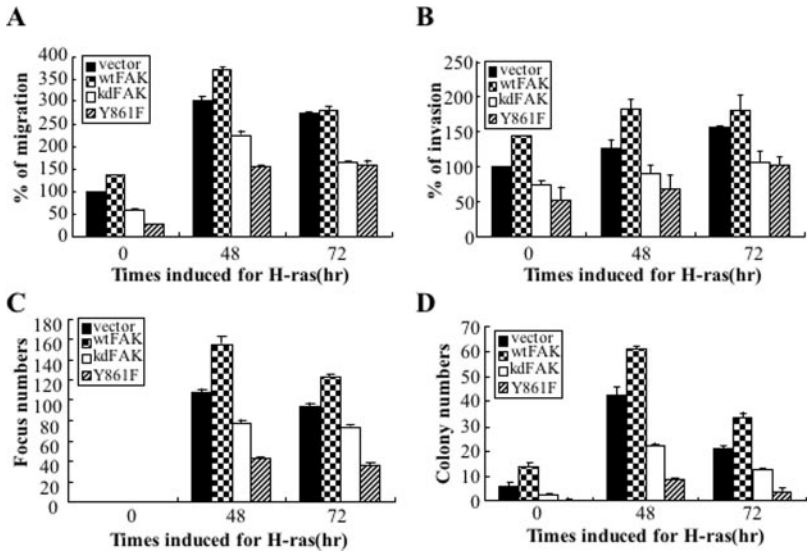


FIG. 3. **FAK phosphorylation at tyrosine 861 is essential for transforming activities in H-*Ras*-transformed NIH3T3 cells.** H-*Ras*-inducible NIH3T3 cells transfected with vector, wtFAK, kdFAK, or Y861F mutant of FAK cDNA were treated with 2 g/ml of doxycyclin for the indicated periods of time. *A*, cells ($5 \times 10^4$) were trypsinized and allowed to migrate on fibronectin-coated (10 $\mu$g/ml) Transwell plates for 2 h as described in the legend to Fig. 2. The percentages of cells migrating, relative to that of vector, are shown. *B*, cells ($5 \times 10^4$) were loaded onto the upper compartments of Matrigel-coated Transwell plates and incubated for 13 h, and the number of invasive cells was counted. The percentages of invasive cells, relative to that of vector, are shown. *C*, cells were replated and incubated for 14 days, and transformed foci were visualized by Wright-Giemsa staining and counted. This experiment was repeated three times with nearly identical results. *D*, cells ($1 \times 10^5$/dish) were seeded in soft agar and allowed to grow 21 days, and the number of viable colonies was counted. All of the results represent the averages of at least three independent experiments.

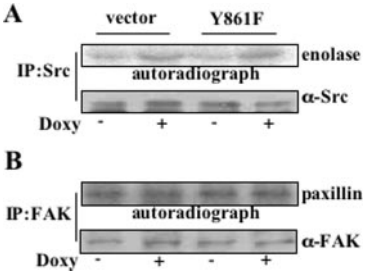


FIG. 4. **Overexpression of Y861F mutant does not affect the activity of FAK/Src kinases.** *In vitro* kinase assays were performed with Src (*A*) and FAK (*B*) immunocomplexes. Autoradiography of phosphorylated enolase and paxillin (*top panels*). The levels of protein in each immunoprecipitate (*IP*) were determined by immunoblotting with antibodies against Src and FAK (*bottom panels*).

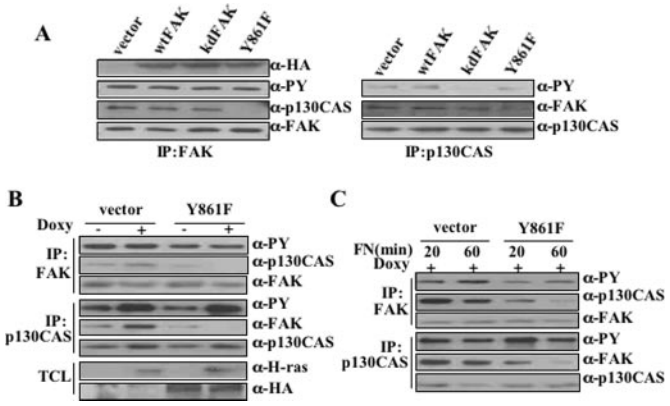


FIG. 5. **Mutation of tyrosine 861 to phenylalanine leads to reduced interaction with p130$^{CAS}$.** *A*, NIH3T3 cell extracts were immunoprecipitated with antibody to FAK (*left panel*) or p130$^{CAS}$ (*right panel*), and the amount of protein in each immunoprecipitate was determined by immunoblotting with antibodies to FAK, p130$^{CAS}$, HA, and phosphotyrosine (*PY*). *B*, H-*Ras*-inducible NIH3T3 cell lysates, FAK immunoprecipitates, and p130$^{CAS}$ immunoprecipitates were immunoblotted with the indicated antibodies. The levels of FAK and p130$^{CAS}$ protein in each were assayed by immunoblotting with specific antibodies. *C*, cells were plated on fibronectin-coated plates for the indicated periods of time. FAK and p130$^{CAS}$ immunoprecipitates were immunoblotted with the indicated antibodies. The level of protein in each immunoprecipitate was determined by immunoblotting with antibodies to FAK and p130$^{CAS}$.

tion. Interestingly, the total phosphorylation of FAK was decreased in Y861F-transfected cells, but there was no detectable change in p130$^{CAS}$ phosphorylation (Fig. 5*B*).

Because the integrin pathways are involved in the phosphorylation of FAK, the recruitment of adaptor proteins, and the subsequent activation of downstream effecter molecules, we assayed the interaction between p130$^{CAS}$ and FAK after plating the cells on fibronectin. Again, both the interaction of p130$^{CAS}$ with FAK and the phosphorylation of FAK were decreased in Y861F-transfected cells during spreading (Fig. 5*C*).

The proline-rich domain1 (PR1) of FAK is required for its interaction with the SH3 domain of p130$^{CAS}$ (8). Because FAK Tyr$^{861}$ is located between PR1 and PR2, FAK Tyr$^{861}$ phosphorylation may affect the interaction of this protein with p130$^{CAS}$. We therefore performed a GST pull-down assay using three GST-FAK recombinants (PR1PR2, PR1PR2F, and PR2) and H-*Ras*-inducible NIH3T3 whole cell lysate. We found that *in vitro* phosphorylation of GST-PR1PR2 (23, 25) caused a dramatic increase in the amount of p130$^{CAS}$ bound to GST-PR1PR2 (Fig. 6, compare the *second* and *third lanes* with the *fifth* and *sixth lanes*), whereas we were unable to detect p130$^{CAS}$ bound to *in vitro* phosphorylated GST-PR2. These results strongly suggest that FAK Tyr$^{861}$ phosphorylation regulates the interaction of FAK with p130$^{CAS}$.

*A Mutation in FAK Tyrosine 861 Results in Decreased JNK Activation and Matrix Metalloproteinase-9 Expression*—The FAK/p130$^{CAS}$ complex has been observed to regulate cell transformation through JNK-mediated transcriptional regulation (29). We found that the activation of JNK was consistently defective in H-*Ras*-inducible NIH3T3 transfected with the Y861F mutant (Fig. 7*A*). We also observed consistent decreases in the expression of MMP-9 mRNA (Fig. 7*B*), and gelatin zymography of cell conditioned media showed decreased expres-

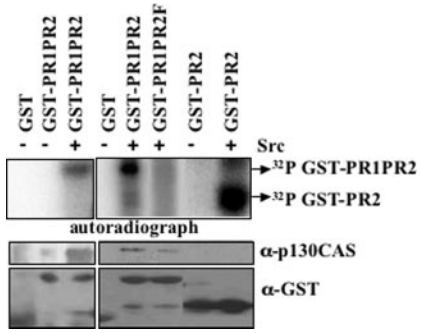


FIG. 6. **FAK phosphorylation at tyrosine 861 regulates interaction with p130$^{CAS}$ *in vitro*.** Purified recombinant GST, GST-FAK(PR1PR2), GST-FAK(PR1PR2F), and GST-FAK(PR2) fusion proteins were phosphorylated with active Src kinase. Autoradiography of phosphorylated GST-FAK(PR1PR2), GST-FAK(PR1PR2F), and GST-FAK(PR2) (*top panel*). The proteins bound to glutathione agarose beads were immunoblotted with anti-p130$^{CAS}$ and anti-GST antibodies (*bottom panel*).

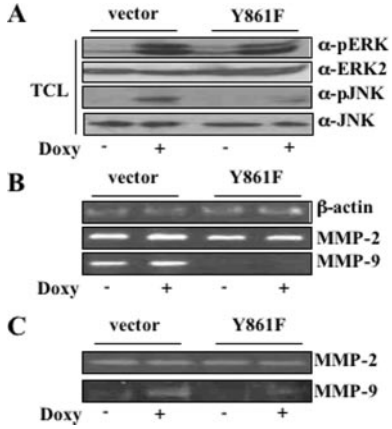


FIG. 7. **JNK activation and MMP-9 expression are decreased in Y861F-transfected cells.** *A*, cell lysates were immunoblotted with phospho-specific ERK and JNK antibodies. The level of protein in each lane was determined by immunoblotting with antibodies to ERK2 and JNK. *B*, total RNA was extracted, and expression of MMP-2 and MMP-9 mRNA was analyzed by reverse transcriptase-PCR, with β-actin mRNA used as a loading control. Representative results from three independent experiments are shown. *C*, gelatin zymography was performed with conditioned media. Migration of the active forms of MMP-2 and MMP-9 are shown.

sion of MMP-9 (Fig. 7*C*). These results confirmed that FAK Tyr$^{861}$ phosphorylation regulates the interaction of FAK with p130$^{CAS}$, resulting in the activation of JNK and the secretion of MMP-9.

## DISCUSSION

Although the relationship between FAK Tyr$^{861}$ phosphorylation and transformation and/or tumorigenesis has been noted (20–22, 30), the mechanism by which this phosphorylation affects cell phenotype has not been directly addressed. The results shown here indicate that FAK Tyr$^{861}$ phosphorylation regulates H-*Ras*-induced transformation of fibroblasts by regulating the interaction between FAK and p130$^{CAS}$. We observed a clear correlation between FAK Tyr$^{861}$ phosphorylation and the transformed morphology and activity in these cells.

The ability of FAK to integrate signals requires the integrity of tyrosine 397, a major autophosphorylation site that mediates the SH2-dependent binding of Src family kinases, which are crucial regulators of cell transformation. We therefore thought it likely that FAK Tyr$^{861}$ phosphorylation might regulate cell transformation by regulating FAK phosphorylation at tyrosine 397. We found, however, that FAK Tyr$^{861}$ phosphorylation did not affect FAK Tyr$^{397}$ phosphorylation or the phosphorylation of the endogenous FAK substrate, paxillin. Elevated Src activity has been shown to correlate with increased FAK phosphorylation in tumor cells, and a stable complex between FAK and activated Src has been described in Src-transformed cells (31), suggesting that increased Src activity/interaction could potentially contribute to transformation (4, 33). We found, however, that Src activity was not much different in cells transfected with FAK Y861F mutant compared with those transfected with vector and that the interaction of FAK with Src was not affected by this mutation (data not shown). Taken together, these findings indicate that it is unlikely that FAK Tyr$^{861}$ phosphorylation regulates cell transformation through FAK/Src complex activity.

p130$^{CAS}$ has been considered a putative substrate of the v-Src kinase and a binding target for the SH2 domain of v-Crk during retroviral transformation (17). Recently, p130$^{CAS}$ phosphorylation and the interaction of p130$^{CAS}$ with FAK have been reported as important in cell transformation (8, 35). Our data demonstrated that the association of FAK with p130$^{CAS}$ was decreased in FAK Y861F-transfected cells and that, *in vitro*, FAK Tyr$^{861}$ phosphorylation enhanced this association. In addition, the interaction of p130$^{CAS}$ with FAK was decreased during cell transformation in H-*Ras*-inducible NIH3T3 cells expressing the Y861F mutant. These results strongly suggest that FAK Tyr$^{861}$ phosphorylation regulates cell transformation through regulation of the interaction between FAK and p130$^{CAS}$.

Interestingly, although total FAK phosphorylation was decreased in Y861F-transfected cells, there was no detectable change of p130$^{CAS}$ phosphorylation, which is an important event leading to cell transformation and consistent with the unchanged Src and FAK activity observed in these cells. The signal downstream of the FAK/p130$^{CAS}$ complex may affect *ras* transformation of fibroblasts in several ways. The activation of p130$^{CAS}$ creates binding sites for adaptor proteins such as Crk, which is critical for cell transformation (32). Rac-mediated JNK activation downstream of the p130$^{CAS}$-Crk complex is essential for cell invasion (34) and acts to alter the transcriptional regulation of cell invasion-associated gene targets, including MMP-9 (29). In addition, FAK promotes cell motility and focal contact remodeling events, in part through the regulation of MMP-9 secretion (28). Our data demonstrated that both the activation of JNK and the secretion of MMP-9 were defective in the FAK Y861F mutant. These findings strongly support the notion that FAK Tyr$^{861}$ phosphorylation is crucial for FAK/p130$^{CAS}$-mediated cell transformation.

Because *in vitro* phosphorylated tyrosine 861 clearly enhanced the interaction of FAK with p130$^{CAS}$, it is likely that FAK Tyr$^{861}$ phosphorylation regulates this interaction. This result was somewhat unexpected, because this interaction is through the SH3 domain of p130$^{CAS}$ and the PR1 domain of FAK, in which tyrosine 861 is not present. It is possible, however, that FAK Tyr$^{861}$ phosphorylation serves to stabilize preexisting interactions by creating an additional SH2-binding site. We are currently attempting to identify another adaptor molecule that contains an SH2 domain and binds to FAK in a Tyr$^{861}$ phosphorylation-dependent manner.

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