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 formation 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 p130CAS 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 NH2-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 p130CAS.

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), p130CAS (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 RASmitogen-activated protein kinase pathway (14, 15).

p130CAS 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, p130CAS 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 p130CAS mRNA into ras- and v-src-transformed cells led to their reversal (18), and mouse embryonic fibroblasts lacking p130CAS were resistant to Src-induced transformation (19). p130CAS-deficient mouse embryonic fibroblasts also showed impaired actin bundling and cell migration, and these properties were restored after re-expression of p130CAS (19), further indicating that this protein is essential in signal transduction during cell migration and transformation. Phosphorylation of FAK at tyrosine 861 (FAK Tyr861 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 Tyr861 phosphorylation is increased in metastatic breast cancer cells (22) and ras-transformed fibroblasts (23) and decreased in de-transformed cells by trichostatin A (23). We therefore hypothesized that FAK Tyr861 phosphorylation may regulate transforming activity in transformed/cancer cells. Here we report that FAK Tyr861 phosphorylation is crucial for H-Ras-induced transformation by regulating the association between FAK and p130CAS.

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 p130CAS (84G-E8) were purchased from UBI (Hauppauge, NY), mAb to HA (12CA5) was purchased from Roche Applied Science, mAbs to H-Ras (F225), 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[Y861P] and FAK[Y861] 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
gift from Dr. Zeng Young Ryou of the Catholic University of Korea. The full-length H-Ras (G12R) cDNA was enzymatically excised and subcloned into the GeneTailer site-directed mutagenesis system (Invitrogen), into the BamHI site of the tetracycline-inducible vector, pTRE-IRE5-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-IRE5-EGFP and pEFpuro-Tet-off (the generous gift of Dr. Hong Jian Zhu) were cotransfected into NIH3T3 cells with FuGENE 6 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(G12R)-expressing cells were grown in 5% CO2–air–atmosphere of Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen), 100 mg/ml penicillin, and 100 mg/ml streptomycin. Puromycin (2 μg/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 (Tyr861→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% confluence. To each culture was added 5 ml of a mixture of 15 μl of LipofectAMINE and 4 μl of plasmid DNA, and the cells were incubated for 6 h at 37 °C in a 5% CO2 incubator. To each was added 5 ml of DMEM containing 20% FBS, and the cells were 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 Na3VO4) containing a protease inhibitor mixture (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 20 μg/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 μg of total protein, was incubated with the relevant antibody for 2 h at 4 °C, followed by incubation with Protein A-Sepharose (10 μl). These complexes were collected by centrifugation. The proteins were transferred onto polyvinylidene difluoride membranes (Amerham Biosciences), which were incubated with the appropriate primary antibodies, followed by species-specific horseradish peroxidase-conjugated secondary antibodies (Amerham Biosciences). The signals were detected by ECL (Amerham Biosciences).

Focus Forming and Soft Agar Growth Assays—In the focus forming assay, 5 × 104 H-Ras-transformed NIH3T3 cells were plated and incubated for 10–14 days, fixed in 9% methanol, and stained with Wright-Giemsa stain. For analysis of colony formation in soft agar, 1 × 104 cells in 2 ml of DMEM containing 10% FBS, 2 μg/ml puromycin, and 0.3% agarose in 6-mm plates were overlaid with a 0.6% agarose layer. 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 μg/ml) in phosphate-buffered saline) was added to each well of a Transwell plate (Costar; 8-μm pore size), and the membranes were allowed to dry for 1 h at 37 °C. The Transwell plates were assembled in a 24-well plate, the lower chambers were filled with DMEM containing 10% FBS and 0.1% bovine serum albumin. Cells (5 × 104) were added to each upper chamber, and the plate was incubated at 37 °C in 5% CO2 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, the Transwell plates (Costar; 8-μm pore size) were coated with fibronectin (10 μg/ml) on the lower side of the membrane with and with Matrigel (30 μg/ml) 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 μl of kinase buffer (10 mM Tris, pH 7.4, 10 mM MgCl2, 2 mM MgCl2, 0.02% Triton X-100), and the reactions were started by adding 10 μl of γ-32P-ATP, 1 μM cold ATP, and GST-paxillin and incubated at 25 °C for 5 min. For the Src kinase assay, Src immunoprecipitates were dissolved in 20 μl of kinase buffer (10 mM Tris, pH 7.4, 5 mM MnCl2) and preincubated for 5 min at 25 °C. To each sample was added 2 μg 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 culture plates essentially as described (24). Briefly, fibronectin was diluted in serum-free medium, added to tissue culture plates (2 μg/cm2) 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 μg/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, PRIPR2 (amino acids 711–887, PRIPR2F (amino acids 711–877, 877–919, Tyr861→Phe), were generated by PCR amplification. The PCR products were cloned into the BamHI/EcoRI site of the pGEX-4T-1 expression vector (Amerham 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′-AATACCTGGAATCCTTGAGATG-3′ (forward)) and 5′-AAGGCACCTG CCTGCCAG-3′ (reverse)), MMP-9 (5′-GCTTGGTCGCGCGC-3′ (forward)) and 5′-GGAAAGGGTCGTGCGCA-3′ (reverse)), and rat-α-actin (5′-TGGAACTTGGGAGGATGCGAC3′ (forward)) and 5′-AAAGCAGTCTCAGTGGT-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 β-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 β-actin) were cloned and sequenced to confirm their identity.

Gelatinase Activity—Conditioned culture media from 5 × 105 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% NaN3, 10 mM EDTA, 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 Tyr861 phosphorylation in two different ras-transformed cells. We found that, in contrast to FAK Tyr397 phosphorylation, FAK Tyr861 phosphorylation was dramatically higher in H-Ras-transformed NIH3T3 and K-ras transformed rat2 cells than in their respective untransformed cells (Fig. 1A). To further examine the correlation of FAK Tyr861 phosphorylation with transformation, NIH3T3 cells were transfected with a tetracycline-inducible H-Ras expression vector. In response to 2 μg/ml doxycyclin, these cells gradually expressed H-Ras (Fig. 1C) and exhibited a spindle-like morphology with small round cell bodies (Fig. 1B). In addition, FAK Tyr861 phosphorylation was increased in a time-dependent manner over 96 h (Fig. 1C). 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 Tyr861 phosphorylation was decreased (Fig. 1D). Taken together, these data strongly suggest that increased FAK Tyr861 phosphorylation
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 Tyr861 phosphorylation, we performed site-directed mutagenesis to replace tyrosine 861 with nonphosphorylatable phenylalanine residues (Y861F) and transfected HA-tagged Y861F into NIH3T3 cells (Fig. 2A). 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 Tyr861 phosphorylation is critical for H-Ras transformation of NIH3T3 cells.

FAK Phosphorylation at Tyrosine 861 of FAK Regulates Its Interaction with p130CAS—Because FAK-mediated signaling involves interactions with the Src family kinases, FAK Tyr861 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. 4A). Similarly, FAK Tyr861 phosphorylation had no effect on the activity of FAK (Fig. 4B), making it unlikely that FAK Tyr861 phosphorylation regulates H-Ras transformation via the regulation of enzymatic activity. p130CAS 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 p130CAS and FAK in NIH3T3 cells (Fig. 5A). The interaction of p130CAS with FAK was increased in H-Ras-transformed cells but decreased in cells overexpressing Y861F (Fig. 5B), supporting the importance of the interaction of p130CAS with FAK in transformation.

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[PY397] and anti-FAK[PY861] 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 μg/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[PY397], FAK[PY861], FAK, and H-Ras. D, H-Ras-inducible NIH3T3 cells treated with (+) or without (−) 2 μg/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[PY861], and H-Ras.

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 × 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 μg/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.

FIG. 3. FAK and Ras Transformation
tion. Interestingly, the total phosphorylation of FAK was decreased in Y861F-transfected cells, but there was no detectable change in p130CAS phosphorylation (Fig. 5B).

Because the integrin pathways are involved in the phosphorylation of FAK, the recruitment of adaptor proteins, and the subsequent activation of downstream effector molecules, we assayed the interaction between p130CAS and FAK after plating the cells on fibronectin. Again, both the interaction of p130CAS with FAK and the phosphorylation of FAK were decreased in Y861F-transfected cells during spreading (Fig. 5C).

The proline-rich domain1 (PR1) of FAK is required for its interaction with the SH3 domain of p130CAS (8). Because FAK Tyr861 is located between PR1 and PR2, FAK Tyr861 phosphorylation may affect the interaction of this protein with p130CAS. 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 p130CAS bound to GST-PR1PR2 (Fig. 6, compare the second and third lanes with the fifth and sixth lanes), whereas we were unable to detect p130CAS bound to in vitro phosphorylated GST-PR2. These results strongly suggest that FAK Tyr861 phosphorylation regulates the interaction of FAK with p130CAS.

A Mutation in FAK Tyrosine 861 Results in Decreased JNK Activation and Matrix Metalloproteinase-9 Expression—The FAK/p130CAS 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. 7A). We also observed consistent decreases in the expression of MMP-9 mRNA (Fig. 7B), and gelatin zymography of cell conditioned media showed decreased expres-
served a clear correlation between FAK Tyr861 phosphorylation and the interaction between FAK and p130 CAS. We observed as important in cell transformation (8, 35). Our data demonstrated that the association of FAK with p130CAS was decreased in FAK Y861F-transfected cells and that, in vitro, FAK Tyr861 phosphorylation enhanced this association. In addition, the interaction of p130CAS with FAK was decreased during cell transformation in H-Ras-inducible NIH3T3 cells expressing the Y861F mutant. These results strongly suggest that FAK Tyr861 phosphorylation regulates cell transformation through regulation of the interaction between FAK and p130CAS.

Interestingly, although total FAK phosphorylation was decreased in Y861F-transfected cells, there was no detectable change of p130CAS 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/p130CAS complex may affect ras transformation of fibroblasts in several ways. The activation of p130CAS creates binding sites for adaptor proteins such as Crk, which is critical for cell transformation (32). Rac-mediated JNK activation downstream of the p130CAS-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 Tyr861 phosphorylation is crucial for FAK/p130CAS-mediated cell transformation.

Because in vitro phosphorylated tyrosine 861 clearly enhanced the interaction of FAK with p130CAS, it is likely that FAK Tyr861 phosphorylation regulates this interaction. This result was somewhat unexpected, because this interaction is through the SH3 domain of p130CAS and the PR1 domain of FAK, in which tyrosine 861 is not present. It is possible, however, that FAK Tyr861 phosphorylation serves to stabilize pre-existing 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 Tyr861 phosphorylation-dependent manner.

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