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A truncated FAK lacking the FERM domain displays high catalytic activity but retains responsiveness to adhesion-mediated signals ☆

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Abstract

In order to determine the role of the FERM domain in the regulation of FAK phosphorylation at Tyr-397, the major autophosphorylation site, we generated a truncated FAK lacking a region of the N-terminus corresponding to amino acids 1–384 (FAKΔ384). FAKΔ384 showed a striking increase in phosphorylation, as compared with wild type FAK, in lysates of either HEK 293 or FAK−/− cells. Interestingly, the truncated form of FAK lacking the N-terminal domain retains responsiveness to integrin-mediated signals, as judged by its dephosphorylation by holding cells in suspension and by the recovery of the phosphorylation when replating the cells on fibronectin. We propose a model in which removal of FERM-mediated auto-inhibition is important to increase FAK catalytic activity but the translocation and clustering of this enzyme at the focal adhesions is required for maximal phosphorylation at Tyr-397.

Keywords: Integrin; Fibronectin; Auto-inhibition; Autophosphorylation; Focal adhesions; Focal adhesion kinase; FAK Tyr-397; Cell adhesion; Cell suspension; FAK−/− cells

The non-receptor tyrosine kinase p125 focal adhesion kinase (FAK) is a multidomain protein that localizes to regions of the cell that attach to the extracellular matrix, called focal adhesions [1]. FAK promotes the transmission of downstream signaling by binding and recruiting signaling and adapter proteins. Autophosphorylation of FAK at Tyr-397 [2,3], located N-terminal to the catalytic domain, creates a binding site for the tyrosine kinase Src and other downstream signaling effectors, including PI 3-kinase and phospholipase Cγ [1]. Subsequent Src-mediated phosphorylation of FAK at Tyr-576 and Tyr-577, located in the kinase activation loop, is important for the maximal activation of FAK and downstream signaling events [4,5]. A rapid increase in FAK phosphorylation is a prominent early event in cells stimulated by diverse signaling molecules that regulate cell proliferation, migration, and survival, including integrin-mediated cell adhesion, polypeptide growth factors and mitogenic agonists that act via heptahelical G protein-coupled receptors [1,6–10]. The biological importance of FAK-mediated signal transduction is underscored by the fact that this tyrosine kinase plays a fundamental role in embryonic development [11,12], and in the control of cell migration [4,13–15] and cell cycle progression [16]. Consequently, the mechanism(s) that regulates the catalytic activity of FAK is the subject of intense interest but remains incompletely understood.

FAK contains three major domains, an N-terminal domain, a central catalytic domain, and a C-terminal

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domain [1]. The N-terminal domain of FAK displays homology with FERM domains, which are conserved domains that mediate protein–protein interactions [17]. Recently, the FERM domain located in the N-terminal region of FAK has been proposed to repress the catalytic activity of the enzyme by intramolecular auto-inhibition [18–20], a mechanism of negative regulation in many protein kinases [21]. Upon integrin-mediated cell adhesion, FAK is known to localize to focal adhesions via its focal adhesion targeting (FAT) domain in the C-terminal region of the enzyme [22]. The cytoplasmic domains of integrins are thought to interact with the FERM domain of FAK, relieve FERM-mediated auto-inhibition, and activate the catalytic activity of the enzyme [18]. However, the precise role of the N-terminal region in the regulation of the autophosphorylation of FAK in response to cell-adhesion signals has been the subject of contrasting reports and remains to be clarified [18,22]. Here, we show that deletion of the N-terminal domain of FAK produces a form of the enzyme that displays enhanced catalytic activity but retains responsiveness to integrin-mediated signals.

Materials and methods

Cell culture. Stock cultures of HEK 293 cells and FAK−/− fibroblasts derived from FAK-null mouse embryos were maintained in 100 mm tissue culture plates by subculturing every 3–4 days in DMEM supplemented with 10% fetal bovine serum, at 37 °C in a humidified atmosphere containing 10% CO_2 and 90% air. For experimental purposes, cells were plated in 35 mm dishes at a density of 5×10^5 cells per dish 12–18 h prior to transfections.

Plasmid constructs and transfections. To generate the FLAG-tagged FAK construct (WT FAK), FAK coding sequence was amplified by PCR using Platinun *Taq*DNA polymerase high fidelity and Mouse FAK cDNA (ATCC) as template. The sense primer included a Kozak consensus sequence followed by nucleotides corresponding to positions 1–27 of Mouse FAK coding sequence and a *Bgl*II restriction site. The antisense primer contained a sequence complementary to nucleotides corresponding to positions 3135–3159 and a *Bam*HI restriction site. The *Bgl*II–*Bam*HI PCR fragment was cloned into pCMV-Tag 2B (Stratagene) digested with *Bam*HI. In order to obtain a FAK deletion mutant lacking amino acids 1–384 (FAKΔ384), the FAK PCR fragment was digested with *Sph*I, treated with the large fragment of polymerase I (Klenow) to fill-in the ends and cloned into pCMV-Tag 2B digested with *Eco*RV. Both constructs were verified by DNA sequence analysis and the products of expression analyzed by Western blot using murine monoclonal antibodies M2 and M5 (Sigma) against the FLAG epitope. HEK 293 cells and FAK−/− fibroblasts were transiently transfected with 1 μg DNA/35 mm dish, in Opti-MEM using Lipofectamine Plus according to the manufacturer's suggested conditions (Life Technologies, Gaithersburg, MD). Transfected cells were incubated for 24 h before analysis.

Cell suspension and stimulation with fibronectin. After 24 h of transfection, cells were harvested by limited trypsin/EDTA treatment (0.05% trypsin, 2 mM EDTA in PBS). Trypsin was inactivated by adding soybean trypsin inhibitor (0.5 mg/ml) with 0.25% bovine serum albumin in DMEM. Cells were collected by centrifugation, suspended in DMEM containing 0.1% bovine serum albumin, and held in suspension for the indicated times at 37 °C. Cell culture dishes (35 mm) were pre-coated with fibronectin (FN) purified from bovine plasma (10 μg/ml) in PBS overnight at 4 °C, rinsed with PBS, and warmed to 37 °C for 1 h prior to plating. Suspended cells were distributed onto ligand-coated dishes and incubated at 37 °C, and at indicated times following plating, protein extracts were made in 2× SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (200 mM Tris–HCl, pH 6.8, 1 mM EDTA, 6% SDS, 4% 2-mercaptoethanol, and 10% glycerol).

Immunoprecipitation. Cell lysis and immunoprecipitations were performed in ice-cold RIPA buffer as previously described before [23,24]. Briefly, cell lysates were clarified by centrifugation at 15,000 rpm for 10 min. Supernatants were transferred to fresh tubes and proteins were immunoprecipitated at 4 °C for 4 h with protein A–agarose linked to polyclonal anti-FAK (C-20) antibody. Immunoprecipitates were washed three times with lysis buffer and extracted in 2× SDS–PAGE sample buffer, by boiling for 10 min, and resolved by SDS–PAGE.

Western blotting. After SDS–PAGE, proteins were transferred to Immobilon membranes and subsequently blocked using 5% non-fat dried milk in PBS–0.1% Tween 20, pH 7.2, for 1 h at room temperature followed by incubation overnight at 4 °C with anti-FAK[pY397] (0.1 μg/ml) against the phospho-Tyr-397 of FAK or anti-FAK (C-20) (0.1 μg/ml) directed against the C-terminal region of FAK. The membranes were washed three times with PBS–0.1% Tween 20 and then incubated with secondary antibodies (horseradish peroxidase-conjugated donkey antibodies to rabbit NA 934V (1:5000) for 1 h at 22 °C. After washing three times with PBS–0.1% Tween 20, the immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection reagents. Autoradiograms were scanned using the GS-710 Calibrated Imaging Densitometer (Bio-Rad), and the labeled bands were quantified using the Quantity One software program (Bio-Rad).

In vitro kinase reactions. Anti-FAK immunoprecipitates prepared as described above were washed twice with protein kinase assay buffer (50 mM Hepes, pH 7.4, 0.1 mM EDTA, and 0.01% Brij 35) and resuspended in 20 μl of this buffer. Kinase reactions were started by adding 10 μl of ATP mix (2 μCi [γ-^{32}P]ATP, 1 mM Raytide, 30 mM $MgCl_2$, and 300 μM ATP). The reactions were carried out at 30 °C for 20 min and stopped on ice by adding 10 mM EDTA. Terminated reactions were combined with an equal volume of phosphoric acid, centrifuged at maximum rpm for 30 s and the supernatants were spotted onto P81 paper squares (Upstate Biotechnology, #20–134). After drying, the P81 paper squares were washed three times for 5 min each time with 0.75% phosphoric acid and once for 5 min with acetone. After the washing, the P81 squares were dried and radioactivity was read in a scintillation counter.

Materials. Bovine fibronectin was obtained from Sigma. Soybean trypsin inhibitor was from Gibco-BRL. Raytide was purchased from Calbiochem (Cambridge, MA). FAK polyclonal Ab C-20 was from Santa Cruz Biotechnology (Santa Cruz, CA). The phosphospecific polyclonal Ab anti-FAK[pY397] was obtained from BioSource International (Camarillo, CA). Horseradish peroxidase-conjugated donkey antibodies to rabbit (NA 934V) and ECL reagents were from Amersham-Pharmacia (Piscataway, NJ). All other reagents used were of the purest grade available.

Results and discussion

Deletion of the N-terminal of FAK enhances its catalytic activity

In order to determine the role of the FERM domain in the regulation of FAK phosphorylation at Tyr-397, the major autophosphorylation site, we generated a truncated FAK lacking a region of the N-terminus correspond-