Specific tyrosine phosphorylation of Focal Adhesion Kinase mediated by Fer tyrosine kinase in suspended hepatocytes

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Running head: Fer-mediated phosphorylation of FAK in suspended cells.

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SUMMARY

Cell adhesion to the extracellular matrix (ECM) can activate signaling via focal adhesion kinase (FAK) leading to dynamic regulation of cellular morphology. Mechanistic basis for the lack of effective intracellular signaling by non-attached epithelial cells is poorly understood. To examine whether signaling in suspended cells is regulated by Fer cytoplasmic tyrosine kinase, we investigated the effect of ectopic Fer expression on signaling in suspended or adherent hepatocytes. We found that ectopic Fer expression in Huh7 hepatocytes in suspension or on non-permissive poly-lysine caused significant phosphorylation of FAK Tyr577, Tyr861, or Tyr925, but not Tyr397 or Tyr576. Fer-mediated FAK phosphorylation in suspended cells was independent of c-Src activity or growth factor stimulation, but dependent of cortactin expression. Consistent with these results, complex formation between FAK, Fer, and cortactin was observed in suspended cells. The Fer-mediated effect correlated with multiple membrane protrusions, even on poly-lysine. Together, these observations suggest that Fer may allow a bypass of anchorage-dependency for intracellular signal transduction in hepatocytes.

INTRODUCTION

Whereas normal and most transformed cells cannot trigger intracellular signal activation in suspension, they efficiently stimulate diverse intracellular signaling pathways upon integrin-mediated cell adhesion to ECM proteins, resulting in an anchorage-dependency [1, 2]. Adhesion-mediated intracellular signal cascades regulate the activity and localization of numerous signaling molecules that subsequently modulate morphological changes via actin reorganization [3-5]. Such morphological adaptation to extracellular cues can be a prerequisite of diverse cellular functions including cell division and migration [6]. Cell adhesion-mediated actin rearrangement and morphological changes can involve activation of focal adhesion kinase (FAK), c-Src family kinase, and Rho GTPase family [7]; however, it is unclear how transduction of these intracellular signals is restricted when cells are detached from substrates.

The non-receptor tyrosine kinase FAK contains an NH_2-terminal domain that binds the cytoplasmic domain of β1 integrin [8], a central kinase domain, and a COOH-terminal domain containing proline-rich sequence motifs and a region required for focal adhesion targeting (FAT) [9, 10]. In response to integrin engagement with the ECM, FAK is autophosphorylated predominantly on Tyr^{397}, which is the consensus binding site for the SH2 domain (c-Src homology 2) of c-Src [11]. Interaction of c-Src with FAK leads to phosphorylation of FAK on other tyrosine residues including Tyr407, Tyr576, Tyr577, Tyr861, and Tyr925 [12]. FAK, Src, and paxillin form a signaling complex at cellular focal adhesions (FAs); assembly of this complex is normally initiated by autophosphorylation of FAK [13]. Therefore, FAK phosphorylation and activation is dependent on cell adhesion and is considered as an index of the cell adhesion process. Since FAK is involved in diverse cellular functions including adhesion, spreading, survival, and migration, it can be considered an important target molecule for inhibition of tumor

progression [14].

Fer [15] and Fps/Fes kinase form a unique two-member subfamily of cytoplasmic tyrosine kinases. Fer contains F-BAR domain, a SH2 domain, and a COOH-terminal tyrosine kinase domain [16, 17]. The structural characteristics of the F-BAR domain distinguish Fer from other cytoplasmic tyrosine kinases, such as c-Src or FAK. Fer is implicated in the regulation of cell-matrix and cell-cell adhesions that are mediated by focal adhesions and adherence junctions [18, 19]. The roles of Fer in cell adhesions probably involve its effects on cortactin phosphorylation and/or actin rearrangement [20]. Fer was also shown to cause detachment of Rat-2 fibroblasts from the substratum, most of which could be reattached with viability when they had been detached from the substratum for less than 24 h [19], indicating that the Fer-mediated detachment was not apoptotic. Therefore, we hypothesized that Fer may transduce signal pathways even in cells in suspension, since Fer might allow Rat-2 fibroblasts to be anchorage-independent to a certain degree.

In this study, we tested this hypothesis by examining the effects of expression of wildtype (WT) or mutant Fer in suspended or adherent hepatocytes on the phosphorylation of FAK, which is inert in suspended cells. We used Huh7 cells, which express endogenous Fer at a barely detectable level, since Huh7 cells would have Fer-related signaling components. Interestingly, we found that expression of WT Fer caused phosphorylation of FAK on Tyr861 or 925 residues even in suspension or on non-permissive poly-lysine in a c-Src-independent but cortactin-dependent manner, whereas residues Tyr397 or 576 were phosphorylated only in adherent hepatocytes.

MATERIALS AND METHODS

Cell culture: Huh7, HepG2 (ATCC) or SNU368, SNU398, SNU423, SNU449, SNU886 (Korean Cell Bank, Seoul) human hepatocytes were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, WelGENE) and 50 μg/ml gentamycin (Calbiochem) at 37℃ and 5% CO_2.

Cell lysate preparation and Western blot: Cells were transiently transfected with the following constructs: pcDNA3.1-GFP, pEGFP-human Fer WT, pEGFP-human Fer KD (kinase dead, D743N), or pEGFP-human Fer R483Q (nonfunctional SH2 domain containing mutant) [[21], kind gifts from Dr. Naoki Mochizuki, National Cardiovascular Center Research Institute, Osaka, Japan]; pcDNA3.1-GFP-cortactin WT, pcDNA3.1-GFP-cortactin NH_2-terminal (aa 1-334), or pcDNA3.1-GFP-cortactin COOH-terminal (aa 336-542). shRNA against Fer was made by cloning unique 21 nucleotide (^{84}aaa gaa att tat ggc cct gag^{104}) of the human fer mRNA (accession no. J03358) into pSUPER vector [15]. Hepatocytes were transiently transfected for 48 h using Welfect-EX reagent (WelGENE) according to the manufacturer's protocols and then kept in suspension or replated on ECM-coated dishes precoated with 10 μg/ml fibronectin (Fn), 10 μg/ml collagen type 1 (Cl; Chemicon), or 10 μg/ml poly-L-lysine (PL; Sigma), as described previously [22]. In certain cases, cells were pretreated with PP2 (10 μM, A.G. Scientific Inc.), 30 min prior to the replating. For experiments involving growth factor treatment, recombinant EGF (100 ng/ml; Invitrogen) or PDGFαβ (30 ng/ml; Prospec-Tany TechnoGene, LTD., Rehovot, Israel) was added directly to the replating media for the last 5 min of the 15 min replating incubation period. Whole cell lysates were prepared as described previously [22]. Following normalization of protein concentration, lysates were analyzed by standard Western blots using phospho-Y^{397}FAK, phospho-Y^{925}FAK, phospho-Y^{416}Src, c-Src, Fer (Santa Cruz Biotech.);

phospho-Y^{118}Paxillin, Paxillin, phospho-Erk1/2, Erk1/2, cortactin (Cell Signaling Tech.); α-tubulin (Sigma); GFP, GST (ABGENT); phospho-Y^{407}FAK, phospho-Y^{576}FAK, phospho-Y^{577}FAK, phospho-Y^{861}FAK, FAK, phospho-tyrosine (BD Transduction Lab.); or phospho-S^{722}FAK (Chemicon).

Immunofluorescence microscopy: Cells were transiently transfected with pEGFP-Fer WT or pEGFP-Fer KD for 48 h as above then replated on glass coverslips precoated with 10 μg/ml fibronectin and incubated for 30 min at 37℃. Cells were fixed with 3.7% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS at room temperature (RT) for 10 min, and washed three times with PBS. The cells were then incubated with primary antibody against phospho-Y^{397}FAK, phospho-Y^{861}FAK, or phospho-Y^{925}FAK for 1 h at RT and washed with PBS three times for 10 min. Cells were then incubated with anti-rabbit IgG-conjugated TRITC (Chemicon) in a dark and humidified chamber for 1 h at RT. For actin staining, cells were incubated with phalloidin-conjugated rhodamine (Molecular Probes, Eugene, OR) for 1 h at RT. The cells on coverslips were washed three times with PBS, mounted with a mounting solution (ProLong® Gold antifade reagent; Invitrogen), and visualized by fluorescent microscopy (BX51TR, Olympus, Japan).

***Deletion Mutant of Fer*:** Fer deletion mutants were prepared by PCR methods using pEGFP-human Fer WT (total 2469 bp) as the template. F-BAR domain of Fer (bp 1-1377, 'F' construct), SH_2 domain (bp 1378-1650, 'S' construct), kinase domain (bp 1687-2469, 'K' construct), F-BAR plus SH_2 domain (bp 1-1650, 'FS' construct), SH_2 plus kinase domain (bp 1378-2469, 'SK' construct) were generated and their sequences were directly confirmed. Transfection of SH_2 domain alone caused a significant cytotoxicity not enough for any biochemical analysis.

Immunoprecipitation: Cells were transiently transfected with either mock or Fer WT plasmid for

48 h, and then kept in suspension or replated on fibronectin or poly-L-lysine as above. After 15 min, cells were washed with cold PBS and immediately lysed in immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 0.1% sodium deoxycholate, 1% NP-40, 1% Triton-X100, and protease inhibitors) on ice. In case of immunoprecipitation of Fer WT and deletion mutants using anti-GFP or HA antibody, the lysates were prepared with a 1% Brij58-containing buffer (HEPES 20 mM, pH 7.4., NaCl 150 mM, $MgCl_2$ 2 mM, $CaCl_2$ 2 mM, 1% Brij58, and protease inhibitors). The lysates were cleared by centrifugation at 13000 rpm for 20 min at 4℃. Antibodies against Fer, cortactin, GFP, or HA were added directly to the cell extracts with an equal amount of protein and incubated overnight at 4°C with rotation (60 rpm). After incubation, 30 μl of 50% slurry protein A/G sepharose beads (Upstate, Waltham, MA) was added to each sample, and incubation for an additional 2 h at 4°C with rotation (60 rpm) was done. Immunoprecipitates were collected by centrifugation (7000 rpm for 3 min at 4°C) and washed twice with ice-cold lysis buffer and three times with cold PBS before elution by boiling for 5 min in 2X sample buffer. The eluted proteins were then separated by SDS-PAGE and probed by standard Western blot analysis, in parallel with whole cell lysates.

In vitro pull-down assay: The following GST fusion proteins were prepared: Recombinant GST alone (Cont), GST-FAK_{PRIPR2} (aa 711-877), GST-$FAK_{PRIPR2F}$ (aa 711-877 with Y861F), $FAK_{Y397/407}$ (aa 385-411), or GST-FAK_{CD} (aa 677-1052). Expression of GST fusion proteins was induced for 3 or 6 h in the presence of 1.0 mM IPTG. Bacteria were pelleted and resuspended in a lysis buffer (50 mM Tris-HCl, pH 8.2, 2 mM $MgCl_2$, 0.2 mM $Na_2S_2O_5$, 10% glycerol, 20% sucrose, 2 mM DTT, and protease inhibitors) and then sonicated 12-15 times for 10 sec at 4°C. Lysates were incubated with glutathione-sepharose 4 fast flow (Amersham

Biosciences) overnight at 4°C with rolling-over (60 rpm) and then washed twice with lysis buffer and three times with PBS at 4°C. Recombinant GST proteins (5.0 μg/reaction) bound to the beads were incubated overnight at 4°C with the Huh7 cellular extracts (100.0 μg/reaction) in a modified RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate, 0.1% sodium deoxycholate, 1% NP-40, and protease inhibitors) washed twice with modified RIPA buffer, and then three times with ice-cold PBS. The precipitated complex was eluted with SDS-PAGE sample buffer and resolved by SDS-PAGE.

In vitro Fer kinase assay: Cells were transiently microporated (Digital Bio) with either pEGFP-human Fer WT or KD plasmids for 48 h. The whole cell lysates were prepared and an equal amount of proteins were immunoprecipitated with anti-GFP antibody as above. The PBS-washed immunoprecipitates were mixed with recombinant GST-FAK_{CD} (aa 677-1052) (5 μg/reaction) for reaction, as explained previously [23].

RESULTS

Fer-mediated phosphorylation of specific tyrosine residues of FAK in suspended hepatocytes

The focus of this study was to understand how suspended cells become inert to the transduction of intracellular signaling. We hypothesized that normal and most malignant epithelial cells do not allow intracellular signal transduction when in suspension, due to both the lack of cell adhesion-mediated integrin activation and the presence of regulatory mechanism(s) that restrict activation of intracellular signaling molecules. We were especially interested in Fer non-receptor tyrosine kinase, since Fer expression induces detachment of Rat-2 fibroblasts from substrates but these cells remain viable even after being detached for 24 h [19]. We therefore examined whether expression of Fer causes signaling activation in suspended Huh7 hepatocytes. Since Huh7 cells minimally express Fer (see below) and thus they can have the Fer-related signaling components unlike Fer-null cells, most experiments were performed with Huh7 cells. We found that Fer overexpression into Huh7 cells resulted in phosphorylation of specific tyrosine residues of FAK even in suspended conditions; Specifically, Tyr407, 577, 861, and 925 were significantly phosphorylated in suspended Huh7 hepatocytes, comparable to cells adherent on fibronectin or collagen type 1 for 1 h, whereas Tyr397, 576, and Ser722 residues were phosphorylated only when cells were adherent (Figure 1A, upper). Phosphorylation of these residues in suspended cells was abolished by transfection of kinase-dead Fer (KD, D743N mutant) (Figure 1A). Phosphorylation of FAK Tyr407 (pY^{407}FAK) was quite different from the other residues, since it was independent of cell adhesion status and extracellular matrix type (Figure 1A). Fer-mediated phosphorylation of specific FAK tyrosine residues was not correlated with Tyr416 phosphorylation of c-Src family kinase (pY^{416}c-Src) (Figure 1A). Furthermore, the Fer WT-mediated effects were evident even in cells suspended for only 15 min

following 1 h rolling-over to null-out basal signaling activity (Figure 1B); this effect was also independent of pY^{416}c-Src. In addition, phosphorylation of paxillin Tyr118 (pY^{118}paxillin), an *in vitro* FAK substrate [24], was also clearly observed in suspended cells with overexpression of Fer WT, but not Fer KD, although in adherent cells pY^{118}paxillin was comparable between cells expressing WT or KD Fer expression (Figure 1B). Poly-lysine precoated dishes were generally used to mimic the suspended condition, since cells attach to poly-lysine through electric charges only rather than through integrin/ECM interaction [25]. When the Fer effects were tested in cells replated on non-permissive poly-lysine, pY^{577}FAK, pY^{861}FAK, and pY^{925}FAK were significantly observed at levels comparable to those in suspension or on fibronectin, whereas pY^{397}FAK and pY^{576} were observed only in cells adherent on fibronectin (Figure 1C). Interestingly, pY^{118}paxillin was detectable to certain degrees in cells that were suspended or replated on poly-lysine (Figure 1C, lower panel). The Fer-mediated phosphorylation of specific tyrosine residues of FAK was abolished by cotransfection of shRNA against Fer (i.e., shFer), but not by control shRNA (Figure 1D), indicating that the phosphorylation of Tyr577, 861, or 925 of FAK in suspended cells was indeed caused by Fer. Fer was barely detected in Huh7 cells and SNU368 hepatocytes as well (Figure 1E). The Fer-mediated FAK phosphorylation in suspended conditions was confirmed in another hepatocyte cell line, SNU368 (Figure 1F).

Fer-mediated FAK phosphorylation is dependent on cortactin

Fer phosphorylates cortactin [26], which is involved in the organization of cortical actin [27]. Therefore, we examined whether the Fer-mediated effects observed in suspended hepatocytes involved cortactin. Fer-mediated induction of pY^{861}FAK and pY^{925}FAK in suspended conditions was further enhanced by transfection with WT cortactin, whereas pY^{397}FAK and pY^{576}FAK were not observed in suspended conditions, and were unaffected by additional cortactin expression (Figure 2A). However, the effects of additional cortactin expression on

pY^{577}FAK, pY^{861}FAK, and pY^{925}FAK were less significant when Fer-expressing cells were adherent, compared with those in suspended cells (Figure 2A). The Fer-mediated increase in pY^{861}FAK and pY^{925}FAK in suspended or adherent cells was abolished or significantly reduced respectively, by cotransfection of mutants of the cortactin NH_2-terminal region (with Arp2/3- and F-actin-binding domain) alone or COOH-terminal region (with SH_3 domain) alone (Figure 2B, lanes 7 to 12). In contrast, pY^{577}FAK in both suspended and adherent cells and cell adhesion-dependent pY^{397}FAK, pY^{576}FAK, and pS^{722}FAK did not depend on cortactin expression (Figure 2B, lanes 7 to 12). Therefore, in suspended cells, Fer-mediated pY^{861}FAK and pY^{925}FAK required cortactin, while pY^{577}FAK did not. Moreover, cell adhesion-dependent pY^{397}FAK, pY^{576}FAK, and pS^{722}FAK correlated with pY^{416}c-Src, whereas Fer-mediated pY^{861}FAK and pY^{925}FAK in suspended conditions did not (data not shown). Interestingly, the Fer-mediated effect depended on its kinase activity; the kinase-dead (KD) D743N mutant of Fer abolished the specific tyrosine phosphorylation of FAK in suspended cells (Figure 2C). In contrast, expression of the Fer R483Q mutant with a nonfunctional SH2 domain did not decrease Fer-dependent and Fer-independent FAK phosphorylations, compared with WT Fer (Figure 2C). In adherent cells, pY^{397}FAK was not changed by Fer mutants, whereas pY^{576}FAK was slightly reduced by Fer mutants, compared with Fer WT (Figure 2C). These observations indicate that Fer overexpression allows phosphorylation of specific FAK tyrosine residues in cells in suspended condition in a Fer activity- and cortactin-dependent manner.

Fer-mediated phosphorylation of FAK tyrosine 577, 861, and 925 does not require c-Src family kinase activity.

As shown above, the Fer-mediated effect on FAK phosphorylation in suspended cells did not appear to be correlated with pY^{416}c-Src levels (Figure 1). To confirm this, we next tested whether inhibition of c-Src family kinase by a specific inhibitor, PP2, abolished the Fer-mediated

effect. As expected, PP2 treatment did not decrease pY^{397}FAK (cell adhesion-dependent autophosphorylation) but abolished pY^{576}FAK in adherent cells (Figure 3), which is known to be targeted by c-Src [28], thus indicating that PP2 was functional. Levels of pY^{576}FAK, pY^{577}FAK, pY^{861}FAK, and pY^{925}FAK in adherent cells transfected with the Fer KD mutant were decreased by PP2 treatment, whereas levels of pY^{577}FAK, pY^{861}FAK, and pY^{925}FAK in suspended or adherent cells transfected with Fer WT were not changed by PP2 treatment (Figure 3). These observations confirm that Fer-mediated phosphorylation of specific FAK tyrosine residues in suspended cells is independent of c-Src family kinase activity.

Fer-mediated phosphorylation of specific FAK tyrosine residues was independent of growth factor stimulation.

Since Fer can be activated by growth factors [26, 29], we next examined whether the Fer-mediated phosphorylation of specific FAK tyrosine residues in suspension could be regulated further by growth factor-mediated signaling. Fer WT or KD mutant-transfected cells in suspended or adherent conditions were treated with EGF (100 ng/ml) or PDGFαβ (30 ng/ml) for the last 5 min of the 15 min replating period before harvesting lysates. In adherent Huh7 cells, EGF further enhanced signal activities (except for adhesion-dependent pY^{397}FAK autophosphorylation), whereas PDGFαβ had no effect (Figure 4), presumably because the treatment concentration was not high enough. However, in suspended conditions Fer-mediated specific FAK tyrosine phosphorylation was not changed by EGF treatment, indicating that the Fer effect was independent of growth factor stimulation (Figure 4). EGF-independent (in suspended conditions) or EGF-dependent (in adherent conditions) pY^{577}FAK, pY^{861}FAK, and pY^{925}FAK were abolished or greatly decreased, respectively, by expression of Fer KD (Figure 4). In suspended cells, Fer expression resulted in an obvious phosphorylation level of Erk1/2 (pErk1/2)

only when cells were treated with EGF, and the pErk1/2 level in adherent cells were much more enhanced by EGF treatment. In both suspended and adherent cells, EGF-enhanced levels of pErk1/2 were not changed by expression of Fer KD, compared with Fer WT (Figure 4), indicating that pErk1/2 is independent of Fer kinase activity.

Complex formation between FAK, Fer, and cortactin

We observed above that ectopic Fer expression resulted in enhanced phosphorylation of specific tyrosine residues of FAK in suspended cells, by a mechanism dependent upon Fer kinase activity and WT cortactin. Therefore, it is likely that these proteins associate with one another. It was previously shown that Fer associates with, and phosphorylates, cortactin through its SH2 domain [26]. Therefore, we examined whether Fer associated with FAK in suspended Huh7 cells. First, coimmunoprecipitation was performed using lysates from suspended or adherent cells transfected with mock construct or Fer WT. pY^{925}FAK was significantly co-precipitated by either anti-Fer (Figure 5A, middle panel) or anti-cortactin (Figure 5A, bottom panel) antibody in suspended and adherent cells expressing Fer WT. In suspended cells transfected with Fer WT, pY^{861}FAK was weakly coimmunoprecipitated with Fer, but not with cortactin (Figure 5A). pY^{576}FAK was not coimmunoprecipitated with Fer in suspended cells (Figure 5A). Together, pY^{925}FAK appeared to associate with Fer and cortactin but pY^{861}FAK seemed to bind with Fer in suspended hepatocytes, but pY^{567}FAK could bind to Fer only in adherent condition. We then examined which region of FAK might associate with Fer, through an *in vitro* pull-down assay using recombinant GST-FAK fusion proteins. Recombinant GST-FAK_{CD} fusion protein (containing aa 677-1052 of FAK) associated with Fer from both suspended and adherent cells, whereas the GST alone control did not (Figure 5B). However, the proline-rich domains of FAK (PR1PR2, aa 711-877) without or with Y861F mutation (i.e., PR1PR2F) did not appear to associate with Fer (Figure 5C). This observation also suggests that the SH3 domain of cortactin

in the Huh7 extracts did not mediate the association between Fer and recombinant GST-FAK_{PR1PR2}. This observation further indicates that the association between Fer and FAK in suspended conditions might not involve PR1PR2 with Tyr861, but may require other COOH-terminal regions of FAK. Furthermore, GST-$FAK_{397/407}$ (containing aa 385-411 of FAK) did not bind Fer, whereas GST-FAK_{CD} did (Figure 5D).

We examined next which region of Fer might bind to FAK. First, GFP-tagged Fer showed a binding to FAK, but GFP alone did not (Figure 6A). To dissect the FAK binding region in Fer, we generated HA-tagged deletion mutants of Fer; HA-F-BAR domain (Fer-F), HA-SH_2 domain (Fer-S), HA-kinase domain (Fer-K), HA-F-BAR/SH_2 domain (Fer-FS), and HA-SH_2/kinase (Fer-SK). Although HA-SH_2 was not tested due to SH_2 domain-mediated dominant cytotoxicity, the *in vitro* pull-down analysis showed that the F-BAR domain or F-BAR/SH_2 domain construct of Fer interacted with FAK (Figure 6B). Interestingly, the expressions of the deletion constructs were hardly equalized even with diverse transfection methods, presumably due to each domain-mediated effect on cell viability (data not shown). Fer-FS bound less to FAK, and Fer-FS expression level was also much lower, compared to Fer-F. Therefore, the binding of Fer-FS to FAK might not be much lower, compared to Fer-F, indicating that Fer-S might not additionally be involved in the binding to FAK (Figure 6B). FAK-binding Fer-FS was expressed less than Fer-SK that did not bind to FAK, indicating that Fer-S and/or Fer-K might not bind to FAK (Figure 6B). Together, these data suggest that FAK (presumably COOH-terminal aa 677-1052) associates with the F-BAR domain of Fer. Then we explored if Fer could *in vitro* phosphorylate GST-FAK_{CD}, using anti-GFP immunoprecipitates for GFP-tagged Fer wildtype or KD mutant as enzymes and recombinant GST-FAK_{CD} as a substrate. We found that Fer wildtype could phosphorylate tyrosines of GST-FAK_{CD} corresponding to Tyr861 and Tyr925 of FAK, whereas Fer KD mutant could not (Figure 6C).

Fer-mediated FAK phosphorylation allows sprouting even on non-permissive poly-lysine

The Fer-mediated phosphorylation of specific FAK tyrosine residues including Tyr925 in suspended cells (without EGF treatment) did not lead to Erk1/2 activity (Figure 4), although pY^{925}FAK is previously well-known to lead to Erk1/2 activity [30]. We previously showed that pY^{925}FAK results in actin rearrangement [31], and therefore wondered whether Fer-mediated phosphorylation at specific FAK tyrosine residues might regulate cell functions such as actin polymerization-mediated protrusion/sprouting, even on a non-permissive substrate such as poly-lysine. Cells transfected with either Fer WT or Fer KD were replated on poly-lysine-precoated dishes for 30 min prior to immunostaining for pY^{397}FAK, pY^{861}FAK, or pY^{925}FAK, or staining for actin. Cells transfected with Fer WT (tagged with GFP) showed dynamic protrusion/sprouting even on poly-lysine, where integrin-mediated signaling for actin organization would not be available, whereas cells transfected with Fer KD did not (Figure 7). Interestingly, among Fer WT-transfected cells, multiple protrusions showed positive staining for pY^{861}FAK, pY^{925}FAK, or actin, but not for pY^{397}FAK (Figure 7, left). Theses observations suggest that Fer-mediated phosphorylation of specific FAK tyrosine residues may lead to membrane protrusions even in non-adherent conditions.

DISCUSSION

In this study we observed that ectopic overexpression of Fer cytoplasmic tyrosine kinase in hepatocytes caused phosphorylation of specific FAK tyrosine residues even when the cells were in suspension. Specifically, Tyr577, 861, or 925 of FAK were significantly phosphorylated in suspended cells, to levels comparable to those of cells adherent on fibronectin or collagen type 1 for 1 h, whereas phosphorylation of Tyr397, 576, or Ser722 residues were observed only in adherent cells. Although a FAK-c-Src complex (formed via interaction between phosphorylated-Try397 and the c-Src SH2 domain, respectively) is known to phosphorylate other tyrosine residues [12, 32], c-Src family kinase appeared not to be involved in the Fer-mediated phosphorylation of FAK in suspended cells. Furthermore, we observed that Fer-mediated specific FAK tyrosine phosphorylation in suspended cells was independent of signaling emanating from growth factor receptors, although FAK is known to bind EGFR and PDGFR [33] and Fer can be activated by growth factor stimulation [29].

Instead, this study showed that cortactin was required for the Fer-mediated effect. Fer was previously shown to bind cortactin [26]. In this study, we observed formation of a triple complex between Fer, FAK, and cortactin in suspended hepatocytes: in particular, the COOH-terminal region (aa 677-1052) of FAK was involved in the association with the F-BAR domain of Fer. The F-BAR domain of Fer is known to interact with p120catenin [18], and Fer overexpression in fibroblasts increased phosphorylations of p120catenin and β-catenin with leading to loss of cell-cell and cell/ECM adhesions [19]. The SH2 domain of Fer tyrosine kinases was shown to mediate phosphotyrosine-dependent protein-protein interactions during their oncogenic kinase functions through regulatory intramolecular and intermolecular interactions including cortactin, EGFR, and PDGFR [26, 29]. Therefore, Fer may interact with

cortactin through its SH_2 domain and with FAK through its F-BAR domain, leading to a triple complex.

Interestingly, both Tyr861 and Tyr925 were specifically phosphorylated in suspended cells transfected with Fer and cortactin. However, GST-FAK_{PR1PR2} including Tyr861 did not bind to Fer, indicating that more than just PR1PR2 is needed to bind Fer. Coimmunoprecipitation between Fer and pY^{861}FAK was less significant than that between Fer and pY^{925}FAK in suspended cells transfected with Fer. Furthermore, expression of the Fer RQ (R483Q) mutant with a nonfunctional SH2 domain could still cause specific FAK phosphorylations in suspension, comparable to those by Fer WT, indicating that Fer may target more than just phosphorylated-tyrosine 861 in FAK. On the other hand, the F-BAR domain of Fer is importantly involved in the interaction with FAK_{CD} (aa 677-1052 for a COOH-terminal domain). SH_2 domain alone of Fer could not be tested for the binding to FAK, since its expression caused significant cytotoxicity not enough for any biochemical assay. We observed that proline-rich domains (PR1PR2 of aa 711-877) of FAK without or with the Y861F mutation, or NH_2-terminal $FAK_{397/407}$ (aa 385-411) fused to GST did not associate with Fer during an *in vitro* pull-down assay. Tests using GST-FAK_{kinase} (aa 416-676) were also failed to show binding to Fer, although we had technical problems in the preparation of GST-FAK_{kinase}-bound beads (data not shown). Together, these observations indicate that the FAK COOH-terminal domain (aa 677-1052) outside of the PR1PR2 domain is required for association presumably directly with the F-BAR domain of Fer and indirectly with SH_2 domain of Fer through cortactin, when hepatocytes are detached. Since we did not observe binding between recombinant GST-FAK_{PR1PR2} and Fer in Huh7 cell extracts, the SH3 domain of cortactin that would also be present in the extracts might not mediate the interaction between Fer and FAK. In stead, it appears likely that the NH_2- and COOH-terminal regions of cortactin are required for Fer-mediated phosphorylation of specific

FAK tyrosine residues.

What roles might cortactin play in Fer-mediated FAK phosphorylation in suspended cells? Cortactin is an actin binding protein, which appears to be importantly involved in polymerization of actin filaments proximal to plasma membranes (i.e., cortical actin), during endocytosis, lammellipodia formation, axon guidance, and tumor metastasis via podosome/invadopodia formation [27]. Fer is also known to traffic between focal adhesions and adherence junctions to regulate cell-ECM and cell-cell adhesions, and to play a role in actin rearrangement [16]. The Fps/Fes/Fer tyrosine kinases are implicated in actin cytoskeletal rearrangements [17, 34]. Fer can be activated by growth factors including platelet-derived growth factor (PDGF) [35] and granulocyte-macrophage-colony stimulating factor (GM-CSF) [36]; growth factor-activated Fer associates with and promotes tyrosine phosphorylation of cortactin [26]. Our results indicate that overexpression of Fer in hepatocytes caused pY^{861}FAK and pY^{925}FAK in a cortactin-dependent manner, since the Fer effect was abolished by a NH_2-terminal mutant of cortactin (aa 1-334 without SH3 domain for binding with WASP, dynamin 2, and WIP) or a COOH-terminal mutant of cortactin (aa 336-542 without the Arp3-binding NTA domain and F-actin binding repeat regions). Therefore, the role of cortactin in the regulation of actin organization appears to be important for Fer-mediated FAK phosphorylation in suspended cells. We thus speculate that Fer may cause FAK phosphorylation through formation of a complex between Fer and cortactin that is physically involved in cortical actin filament branches abundant in suspended cells. In other words, during Fer-mediated phosphorylation of specific tyrosines in FAK in suspended hepatocytes, cortactin or cortactin-mediated cortical actin filaments beneath plasma membrane of suspended cells may play presumably an important role by allowing a platform for the biochemical process. Furthermore, Fer overexpression resulted in multiple membrane protrusions that were enriched with actin filaments and immunostained for pY^{861}FAK or

pY^{925}FAK, but not for pY^{397}FAK, even on a non-permissive substrate of poly-lysine.

This study suggests that Fer may allow a bypass of anchorage-dependency for FAK phosphorylation and activation. Fer overexpression has been reported to cause detachment of Rat-2 fibroblasts without commitment to apoptosis [19]. Although our findings suggest that restricted Fer activity may play a role in the regulation of signal transduction involving FAK in suspended cells, it is unclear at this time how Fer itself is regulated in suspended hepatocytes. A recent report indicates that Rac-mediated Akt activation is observed in suspended, but not adherent cells, and is tightly regulated by SH_2-containing inositol 5'-phosphatase (SHIP) [37]. Although it was suggested that regulation of Rac1/Akt signaling by SHIP in suspended conditions plays a role in the plasticity of migratory white blood cells and in dissemination of tumor cells that undergo repeated attachment and detachment processes, these results may also suggest that SHIP mediates negative regulation of signaling in an adhesion status-dependent manner. In this study, Fer expression caused FAK phosphorylation in suspended hepatocytes, although its overexpression did not cause any further increase in FAK phosphorylation in adherent Rat-2 fibroblasts [19] and hepatocytes (this study). In addition, Fer was also shown to bind to protein phosphatase 1 (PP1) and Fer overexpression decreases the enzyme activity of PP1 [15]. It has been suggested that the Fer-PP1 complex may control the balance between 'signaling' and 'anti-signaling' cascade when PP1 is involved [15], thus we speculate that Fer may cause phosphorylation of specific FAK tyrosine residues even in suspension when Fer is released from any bound phosphatase, such as PP1. Further studies are needed to elucidate the roles of Fer, cortactin, and phosphatase(s) in the regulation of signaling activity in suspended cells, and specifically how Fer can be suppressed to inhibit signal transduction in suspended cells.

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FIGURE LEGENDS

Figure 1. Phosphorylation of specific FAK tyrosine residues in suspended Huh7 cells by overexpression of Fer tyrosine kinase. Huh7 cells (A to D) or SNU368 (F) hepatocytes were transiently transfected for 2 days with mock, Fer WT, or Fer kinase dead (KD) cDNA plasmid (A, C and F), or with Fer WT or Fer KD cDNA (B), or with Fer WT or Fer KD cDNA plus control shRNA (Cont) or shRNA against Fer (shFer) (D). The cells were trypsinized, suspended in DMEM-H with 1% BSA, washed twice, and then rolled over (60 rpm) for 1 h at 37^{o}C. Cells were then kept in suspension (S), or replated onto dishes precoated with fibronectin (Fn), collagen type I (Cl in A and F), or poly-lysine (PL) for 60 min unless otherwise indicated. Whole cell lysates were prepared, normalized, and subjected to immunoblotting for the indicated proteins. (E) Whole cell lysates from subconfluent cells were used for immunoblotting for Fer and α-tubulin. Data shown represent at least three independent experiments.

Figure 2. Fer-mediated phosphorylation of specific FAK tyrosine residues is dependent on cortactin. Huh7 cells were transiently transfected for 48 h with pEGFP (Mock), pEGFP-Fer WT, pEGFP-Fer KD, pEGFP-Fer RQ mutant, WT cortactin, NH_2-terminal (aa 1-334) cortactin mutant, or COOH-terminal (336-542) cortactin mutant expression vectors. Cells were then kept in suspension (S), or replated onto fibronectin (Fn)-precoated dishes for 60 min unless otherwise indicated. Cell lysates were prepared and processed for immunoblots using antibodies against the indicated proteins. Cortactin mutant of NH_2-terminal (N-ter) or COOH-terminal (C-ter) depicts aa 1-334 or aa 336-542 of cortactin, respectively. Data shown represent three different experiments.

Figure 3. Fer-mediated phosphorylation of specific FAK tyrosine residues does not require c-Src family kinase activity. Huh7 cells were transiently transfected with Fer WT or KD expressing plasmid for 48 h. Cells were trypsinized, suspended in DMEM-H with 1% BSA, washed twice, and then rolled over (60 rpm) for 1 h at 37^{o}C. After 30 min, a subset of cells were treated with 10 μM PP2. After rolling over for an additional 30 min (sample 0), cells were kept in suspension (Sus) or replated onto fibronectin (Fn)-precoated dishes for 15 min, prior to harvest. Cell lysates were processed for immunoblot analysis of the indicated proteins. Data shown are representative of three independent experiments.

Figure 4. Fer-mediated FAK phosphorylation in suspended cells is independent of growth factor stimulation. Huh7 cells were transiently transfected with Fer WT or KD plasmid, and manipulated for suspended (Sus) or Fn-adherent conditions, as in Figure 1. Cells were treated with EGF (100 ng/ml, E) or PDGFαβ (30 ng/ml, P) for the last 5 min of the replating incubation of 15 min. Cell lysates were prepared for immunoblotting using antibodies against the indicated proteins. Data shown represent three different experiments.

Figure 5. Complex formation between FAK, Fer, and cortactin in suspended cells. (A) Huh7 cells were transiently transfected with mock or Fer WT for 2 days. Cells were then kept in suspension (Sus) or replated on fibronectin (Fn)-precoated dishes for 15 min, before harvesting lysates. An equal amount of protein was immunoprecipitated with anti-Fer or -cortactin antibody. Immunoprecipitates and lysates were immunoblotted in parallel for the indicated molecules. (B - D) Huh7 cells were transiently transfected with mock construct or Fer WT for 48 h. The cells were suspended (Sus) or replated onto fibronectin (Fn)-precoated dishes for 15

min prior to preparation of cell lysates. Recombinant GST alone (Cont) or GST-FAK_{CD}, GST-FAK_{PR1PR2}, GST-$FAK_{PR1PR2F}$, or GST-$FAK_{Y397/407}$ fusion proteins were prepared and incubated with the Huh7 extracts as described in Materials and Methods. Pulled-down proteins and lysates were eluted by boiling in SDS-PAGE sample buffer and used in immunoblots for anti-GST or anti-Fer antibody in parallel with lysates. WCL depicts whole cell lysates. * indicates a proteolysis product. Data shown represent three different experiments.

Figure 6. The F-BAR domain of Fer interacts with and phosphorylates a COOH-terminal region of FAK. (A and B) Huh7 cells were transiently transfected with pEGFP (Cont) or pEGFP-Fer wildtype (WT) (A) or with HA-tagged deletion mutants of Fer (B). Two days later, whole cell lysates were prepared and an equal amount of proteins was immunoprecipitated with either anti-GFP (A) or anti-HA (B) antibody. The immunoprecipitates and whole cell lysates were in parallel immunoblotted for FAK or HA. Notice that the expressions of the deletion constructs were hardly equalized, presumably due to each domain-mediated effect on cell viability. (C) Whole cell lysates were prepared from the cells transiently transfected with either pEGFP-Fer wildtype or kinase dead for 48 h. The immunoprecipitates of the lysates using anti-GFP antibody were prepared and used in *in vitro* Fer kinase assay. As a substrate, recombinant GST-FAK_{CD} was prepared and used, as explained in Materials and Methods. Data shown represent three independent experiments.

Figure 7. Fer overexpression facilitates actin-enriched membrane protrusions on non-permissive poly-lysine. (A) Huh7 cells were transiently transfected with pEGFP-Fer WT or KD mutant for 48 h. The cells were then replated on poly-lysine-precoated coverslips for 30 min. After incubation, cells were fixed, permeabilized, and stained for pY^{397}FAK, pY^{861}FAK,

or pY^{925}FAK, or with phalloidin-conjugated with rhodamine, before analysis by fluorescent microscopy. Scale bar represents 20 μm. Representative images from three independent experiments are shown.

Min-A Oh, 2008
Fig. 1

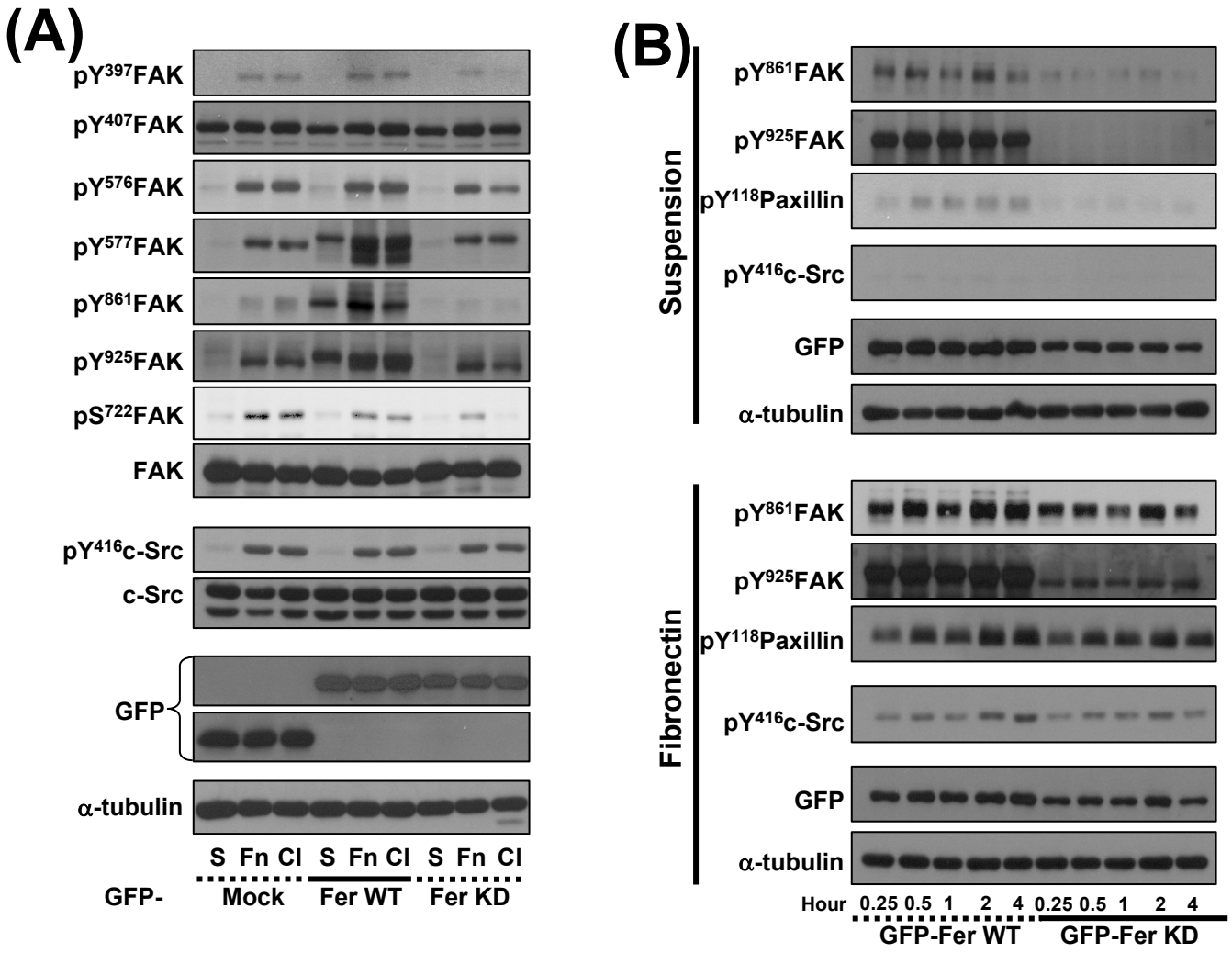

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Fig. 1

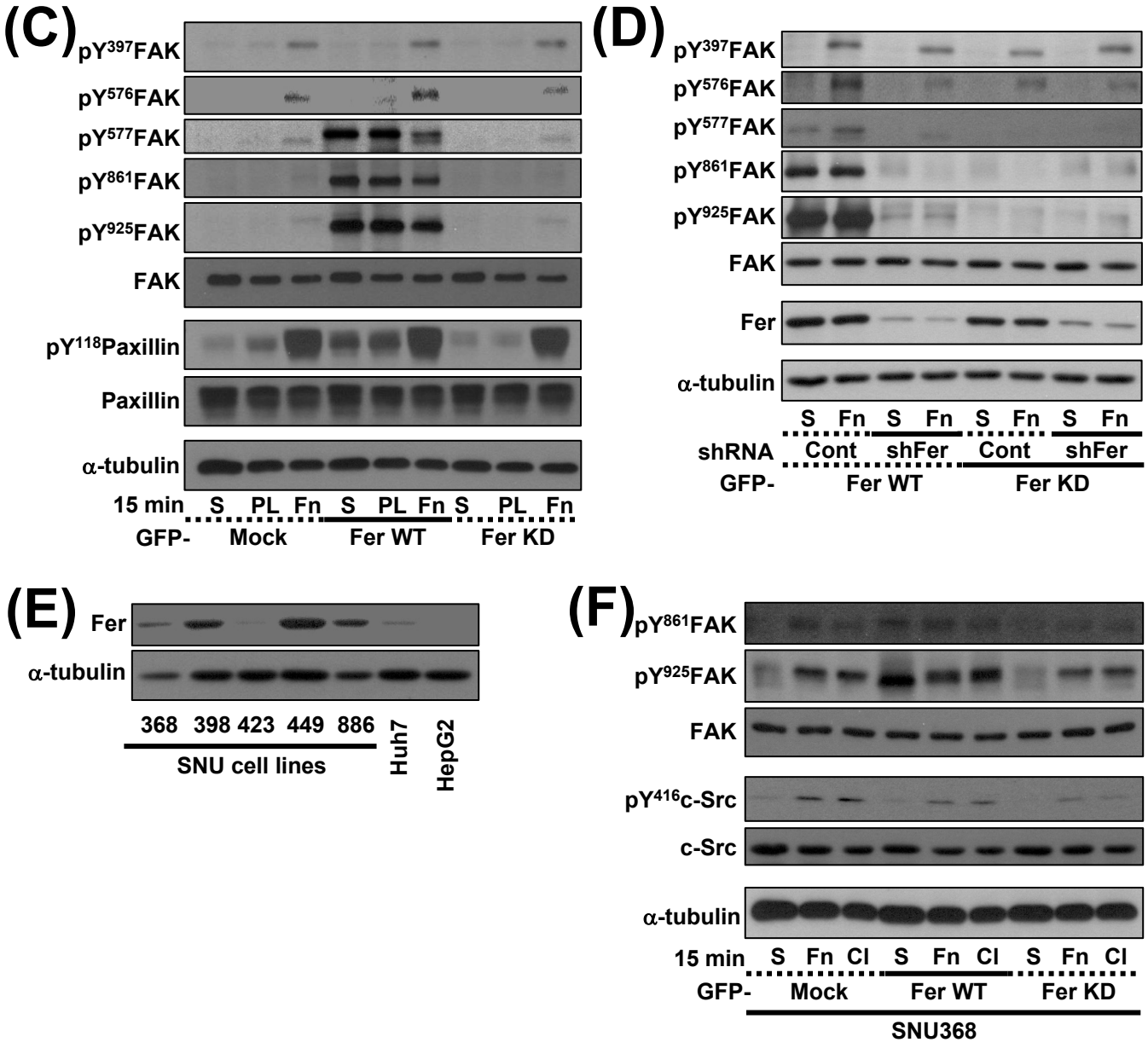

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Fig. 2

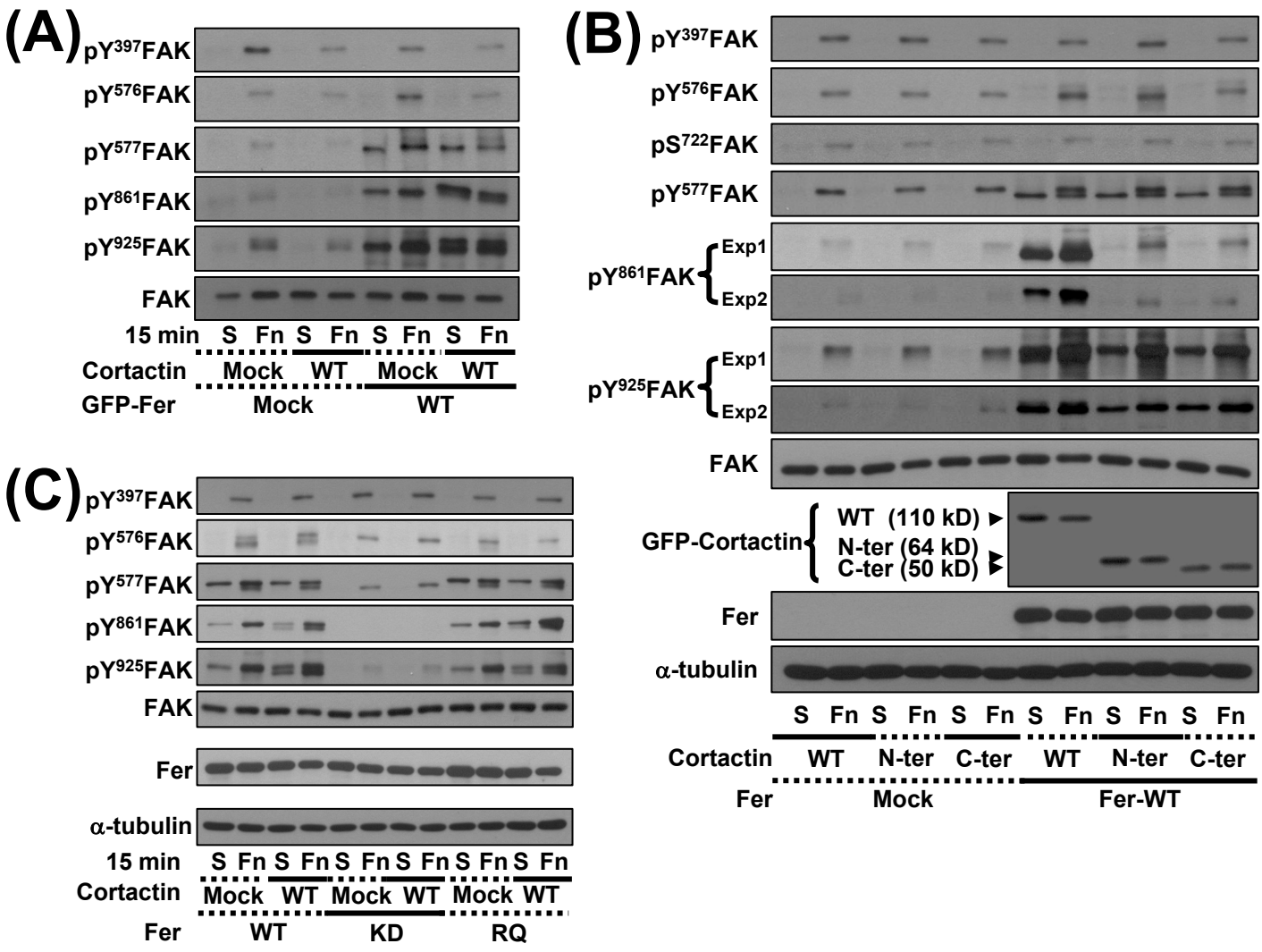

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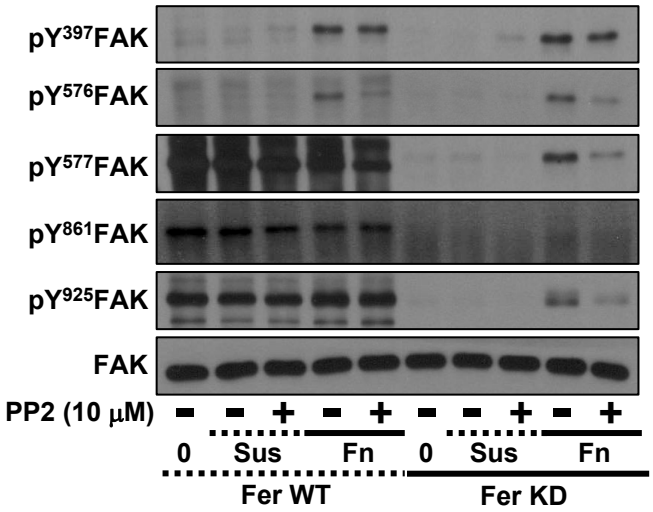
Fig. 3



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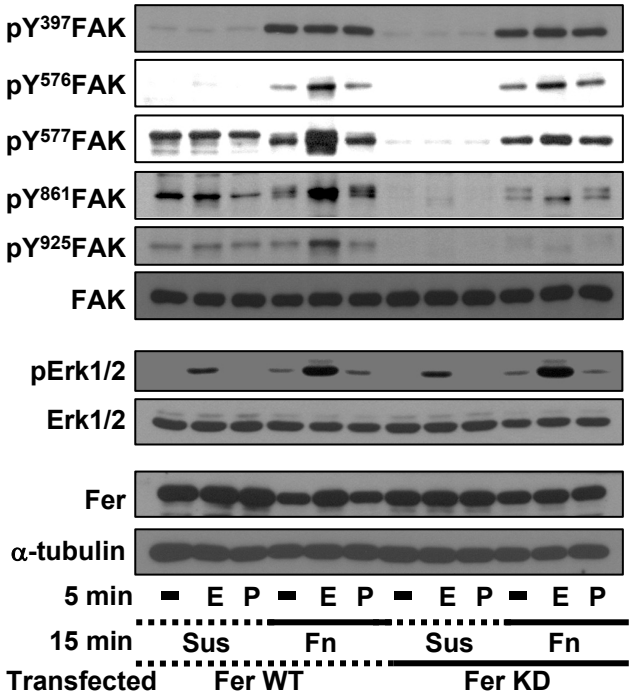

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Fig. 5

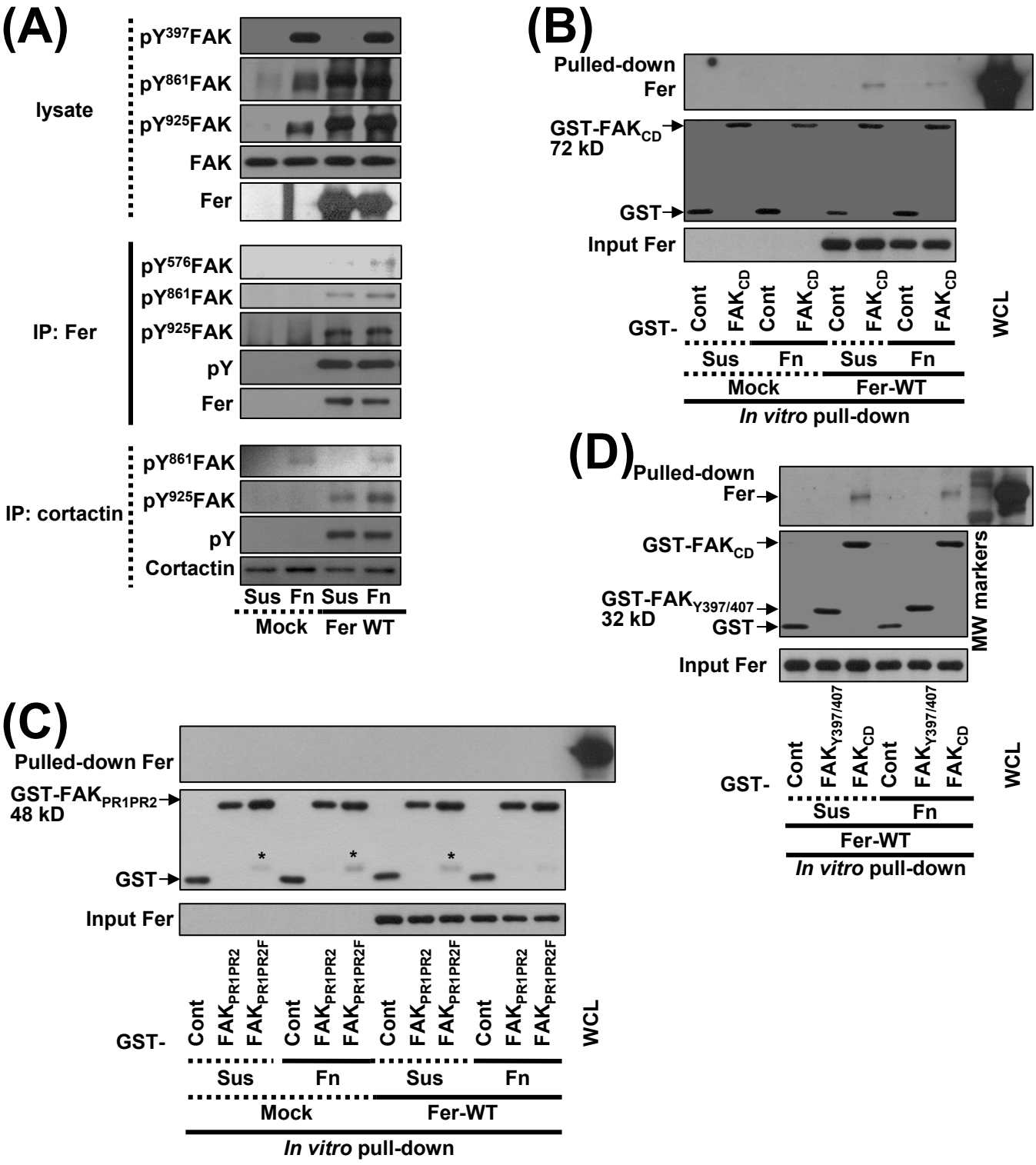

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Fig. 6

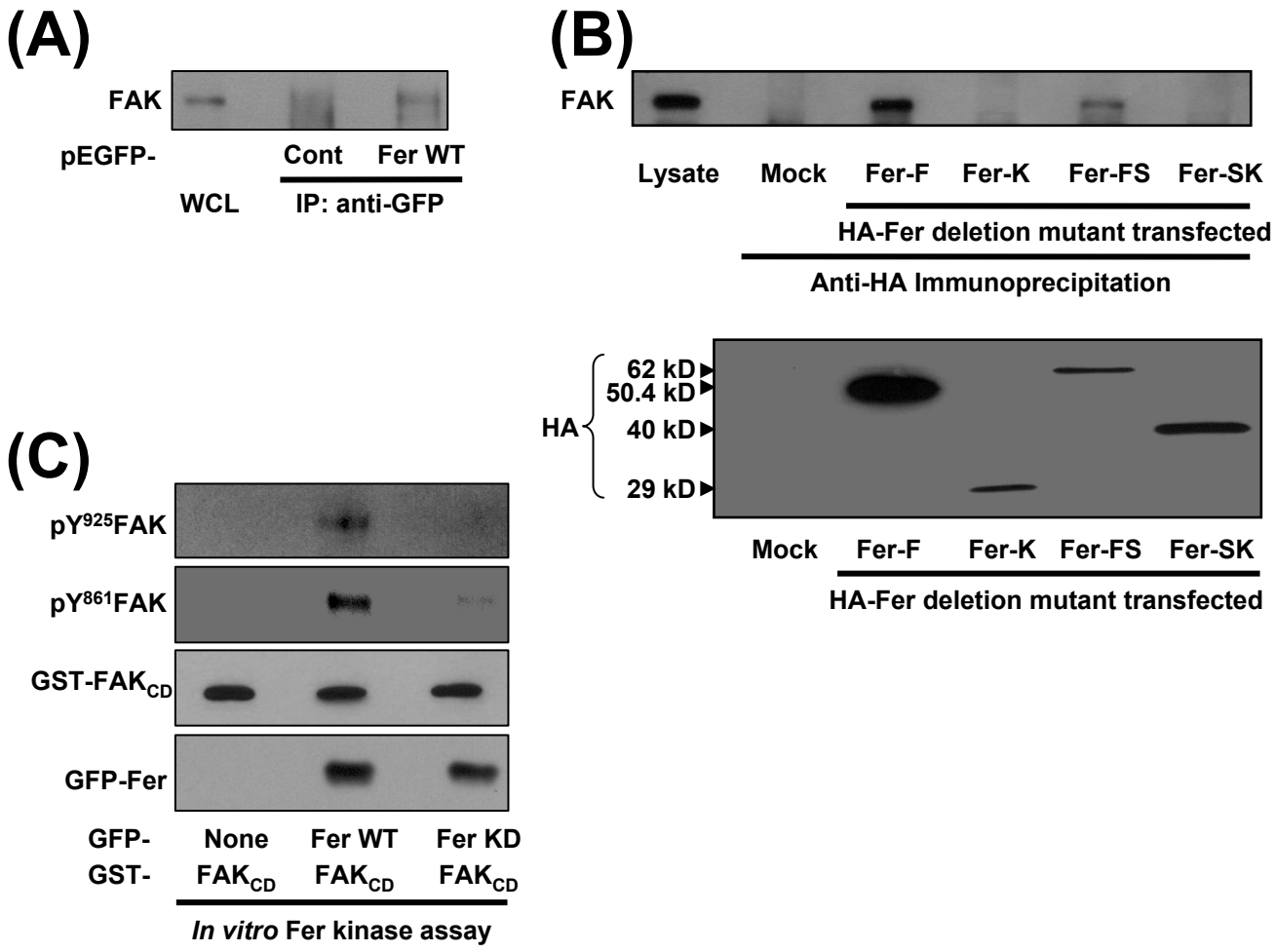

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Fig. 7