

Targeting Membrane-localized Focal Adhesion Kinase to Focal Adhesions

ROLES OF TYROSINE PHOSPHORYLATION AND SRC FAMILY KINASES*

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In the present study, we examined regulation of activated focal adhesion kinase localization in focal adhesions. By using focal adhesion kinase fused to an inert transmembrane anchor, we found that the focal contact targeting region within focal adhesion kinase was preserved in the membrane-targeted fusion protein. However, upon tyrosine phosphorylation, full-length focal adhesion kinase became excluded from focal adhesions. This negative regulation of localization could be abolished by mutating key amino acid residues of focal adhesion kinase shown previously to be involved in adhesion-mediated signal transduction. Hyper-phosphorylation of endogenous focal adhesion kinase induced by pervanadate resulted in a similar reduction of localization at focal adhesions. We also show here that Src family kinases are essential for the phosphorylation-dependent exclusion of focal adhesion kinase from focal adhesions. We propose here a molecular model for the tyrosine phosphorylation-dependent regulation of focal adhesion kinase organization involving Src kinases and an inhibitory phosphorylation of the C-terminal (Tyr-925) tyrosine residue.

Adhesion-mediated signaling triggers tyrosine phosphorylation of a multitude of proteins, including paxillin, tensin, and focal adhesion kinase (FAK)¹ (1–4). FAK phosphorylation following adhesion depends on its association with Src family kinases, leading to the formation of multimolecular signaling complexes in which FAK serves as a scaffold (5–7). Following activation, residue Tyr-397 of FAK becomes auto-phosphorylated, resulting in the formation of an SH2-docking site (5). Src family kinases then associate with Tyr-397 and phosphorylate several other tyrosine residues in FAK, including Tyr-925 that resides within the focal adhesion targeting (FAT) sequence at

the FAK C terminus (5). The adaptor protein Grb2 then binds phosphorylated Tyr-925 and forms a signaling complex that includes the nucleotide exchange factor Sos and the small GTP-binding protein Ras (5). This sequence of events contributes to activation of the ERK1/2 response (5). Two additional pathways lead to FAK-mediated activation of ERK1/2: one involves binding of the adaptor protein p130^{Cas} to the proline-rich region of FAK via an SH3 domain interaction, and the second is the result of FAK association with the adaptor protein Shc (8–10).

The mechanisms that underlie FAK activation are not yet clear. Although FAK lacks any myristoylation or farnesylation signals, membrane translocation of FAK could be initiated by direct association with the β_1 integrin tail (11). Close indirect interaction between FAK and integrin cytoplasmic tails may also be mediated via talin or paxillin (12, 13). Indeed, several studies (11, 14) have demonstrated that a membrane-translocated form of FAK (following fusion to an inert transmembrane anchor) results in an adhesion-independent, constitutive phosphorylation of the molecule. This phosphorylated state of FAK depends on its association with Src and is accompanied by constitutive association with several signaling molecules including Cas and Grb2 (11, 14). Integrin/FAK association may be essential for adhesion-mediated signaling, which can protect cells against apoptosis (11). However, adhesion-mediated signaling responses are transient, pointing to the activity of negative regulatory mechanisms (15, 16). For example, the adhesion-induced ERK1/2 response is rapid, reaching a peak by 15 min, followed by a swift decay (15–17). Potential negative regulators of FAK activities include the phosphatases Shp-2, PTP-PEST, and the dual specificity tumor suppressor phosphatase PTEN (18–20).

In the present study, we examined whether membrane association of FAK affects its interaction with focal adhesions and whether such interaction is regulated by the phosphorylation state of FAK. We found that constitutively activated FAK, induced by membrane translocation, is excluded from focal adhesions of murine 3T3 and primary embryonic fibroblasts, as well as primary human fibroblasts. The exclusion of hyper-phosphorylated FAK from focal adhesions was prevented by mutating to arginine the Lys-454 residue (in the ATP-binding site, making this a kinase-crippled mutation after a Lys-to-Arg point mutation), Tyr-397 (the Src family kinase docking site), or Tyr-925 (the C-terminal Grb2 docking site). All of these residues are known to contribute to the FAK/Src family kinase signaling complex. Moreover, induction of hyper-phosphorylation by pervanadate resulted in a similar reduction of FAK at

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¹ The abbreviations used are: FAK, focal adhesion kinase; ERK, extracellular signal-regulated kinase; FAT, focal adhesion targeting; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; SH, Src homology; CMV, cytomegalovirus.

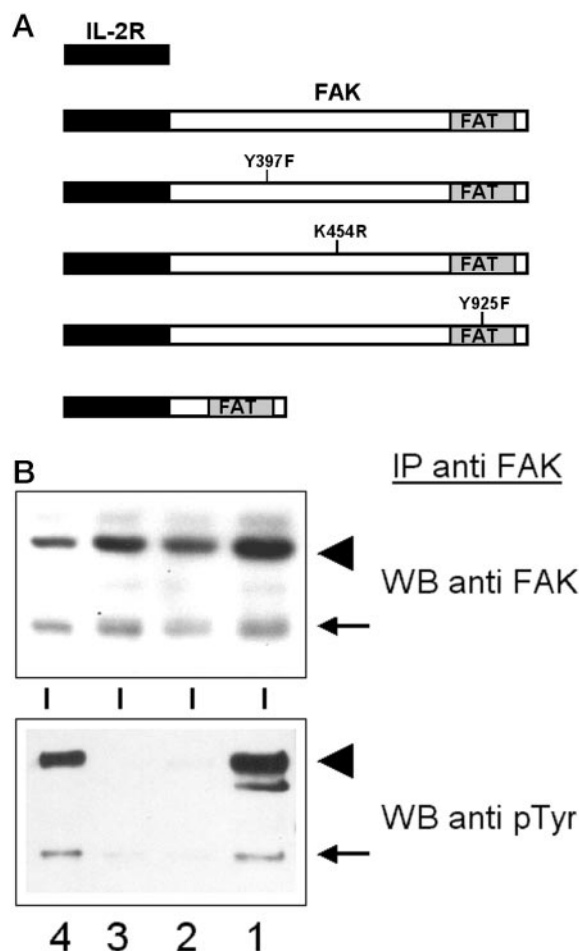


FIG. 1. Generation and expression of IL-2R/FAK chimeras. A, full-length wild type murine FAK and its mutant variants (Y397F, K454R, and Y925F) were fused with the non-signaling IL-2R α subunit. Non-fused IL-2R served as a negative control. IL-2R fused to the C terminus of FAK corresponding to FRNK was also utilized. B, murine 3T3 cells were transfected with IL-2R/FAK (lane 1), IL-2R/FAK^{Y397F} (lane 2), IL-2R/FAK^{K454R} (lane 3), or IL-2R/FAK^{Y925F} (lane 4). After 48 h the cells were incubated in suspension for 1 h and then lysed, and immunoprecipitation (IP) with anti-FAK monoclonal antibody was performed, followed by Western blotting (WB) with anti-FAK monoclonal antibody (top panel) or anti-phosphotyrosine (bottom panel). Arrowheads indicate the location of chimeric FAK molecules, and arrows indicate the location of endogenous FAK.

focal adhesions without affecting vinculin localization. We found that the localization of a wild-type FAK transmembrane chimera to focal adhesions improved when Src or a combination of Src, Yes, and Fyn were absent. We suggest that the hyperphosphorylation of juxtamembrane FAK inhibits its localization to focal adhesions. Furthermore, this hyper-phosphorylation and subsequent interference with FAK localization and function is regulated by Src family kinases. This study demonstrates a novel regulatory mode of FAK subcellular localization controlled by specific tyrosine phosphorylation.

MATERIALS AND METHODS

Construction of Chimeric Receptors—Chimeric transmembrane receptors were constructed using standard methods (21). The sequences of all constructs were confirmed by nucleotide sequencing, and protein expression from the constructs described in this study was confirmed following transfection by both immunofluorescence staining and Western immunoblotting. The general approach to generate a chimera with each type of cytoplasmic domain was to insert partial or full-length cDNA molecules into *HindIII-XhoI* or *HindIII-XbaI* restriction sites of the plasmid vector pCMV/IL-2R (22). This vector is driven by the CMV promoter, and it expresses the extracellular and transmembrane domains of the non-signaling α subunit of the interleukin-2 receptor

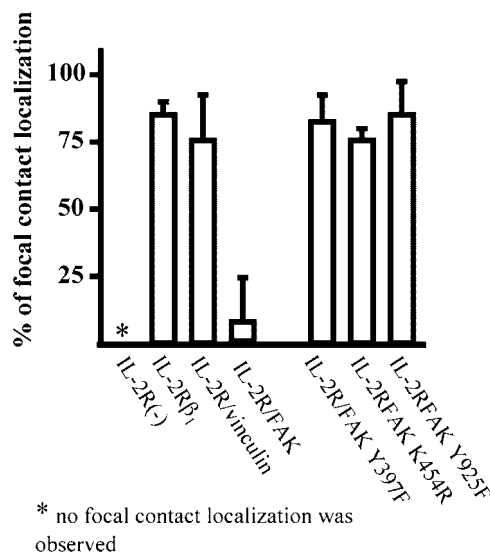


FIG. 2. Localization patterns of IL-2R chimeras in primary human fibroblasts. Human foreskin fibroblasts were transfected with the IL-2 receptor gp55 subunit in chimeric combination with full-length vinculin, FAK (wild type), or FAK with Y397F, K454R, or Y925F mutations. Non-fused IL2R served as a control. Forty eight hours following transfection, the cells were stained for IL-2 receptor and vinculin or phosphotyrosine to confirm focal contact localization. At least 50 transfected cells (stained for IL-2R) were counted for each experiment, and the percentage of cells with focal adhesion-localized chimera was calculated. Each chimera was examined in 3–5 independent transfections, and the average and S.E. of these experiments are presented.

(IL-2R) as a fusion protein with any molecule of interest as the cytoplasmic domain, as illustrated in Fig. 1A. Mouse FAK cDNA clone (pT7-7-FAK) was obtained from the American Type Culture Collection (Manassas, VA). Full-length mouse FAK cDNA was inserted into pCMV/IL-2R as follows. A PCR product encoding the first 500 bp of FAK cDNA was generated using pT7-7-FAK as template with a primer containing a *HindIII* site followed by 15 nucleotides encoding the first 5 amino acids of FAK (GenBank™ MUSFAK, residue 110): 5'-GGCTA-GAAGCTTATGGCAGCTGCTTAT-3', and the antisense primer 5'-GG-TGCTTCTAGAGGCTACTTGATCAGC-3' corresponding to nucleotides 586–602 followed by an *XbaI* site. The PCR product was ligated into CMV/IL-2R using its *HindIII* and *XbaI* sites. An *AccI-XbaI* fragment was then removed from the ligation product and replaced by an *AccI-XbaI* fragment from pT7-7-FAK, creating a full-length IL-2R/FAK chimera that included the FAK 3' non-translated region. The FAK^{K454R} mutant was generated by converting codon 454 encoding lysine to an arginine residue, and FAK^{Y397F} and FAK^{Y925F} were obtained by replacing the tyrosine residue corresponding to amino acid position 397 or 925 with a phenylalanine residue, using PCR-directed mutagenesis. Mutations were confirmed by DNA sequencing. IL-2R/FRNK chimera was kindly provided by T. Takino (CDBRB, NIDCR, National Institutes of Health, Bethesda).

Cells and Transfections—Human foreskin fibroblasts were kindly provided by Susan S. Yamada (NIDCR, National Institutes of Health, Bethesda). NIH 3T3 cells were kindly provided by J. Silvio Gutkind (NIDCR, National Institutes of Health, Bethesda). pp60^{c-Src} null cells (*src*^{-/-}), derived from mice homozygous for a disruption of the *Src* gene, as well as wild-type mouse embryo fibroblasts (wt), were kindly provided by P. Schwartzberg and H. Varmus (NHGRI, National Institutes of Health, Bethesda). Fibroblasts homozygous for disruption of the *Src*, *Fyn*, and *Yes* genes were generously provided by P. Soriano (Fred Hutchinson Cancer Center, Seattle). All cell lines were maintained in culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, penicillin, streptomycin (Biological Industries, Beit Ha'emek, Israel), and 1 mM sodium pyruvate (Sigma). Electroporation of these cells with 30 μ g of DNA for each chimeric IL-2R/cytoplasmic protein construct was performed as described previously at 170 V and 960 microfarads with a Bio-Rad Gene Pulser but without any thymidine block (22). Some transfections were performed using LipofectAMINE Plus (Invitrogen) utilizing standard protocols. Treatment of cells with vanadate was performed using activation to pervanadate as described (23). Briefly, vanadate was activated to pervanadate with hydrogen peroxide, and excess hydrogen peroxide

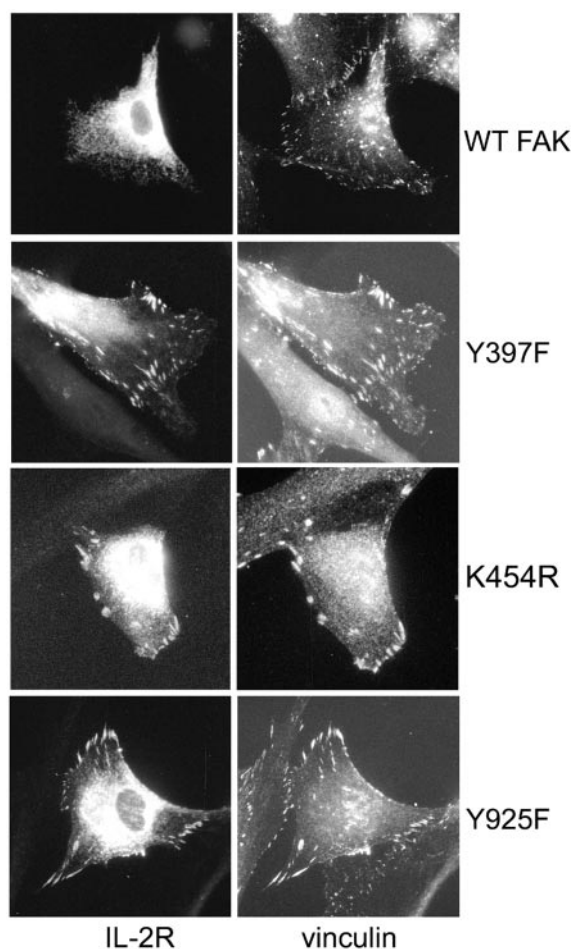


FIG. 3. Localization of IL-2R/FAK chimeras in primary human fibroblasts. Human foreskin fibroblasts were transfected with the IL-2 receptor in chimeric combination with full-length FAK (WT) or FAK with Y397F, K454R, or Y925F mutations. Forty-eight hours after transfection, the cells were prepared for immunofluorescence analysis of the localization of IL-2 receptor (left panels) and vinculin (right panels). Note that the wild-type (WT) FAK chimera failed to localize to focal adhesions. In contrast, the Y397F, K454R, and Y925F chimeras localized effectively to focal adhesions.

was removed with catalase as described (23). The solution was added to regular culture medium at a final concentration of 0.1 mM pervanadate, and cells were fixed for immunofluorescence after incubation for 5–120 min. Controls were untreated or treated with an equal quantity of control hydrogen peroxide/catalase solution.

Immunological Reagents—Mouse monoclonal antibodies 7G7/B6 against the human IL-2 receptor α subunit (Tac antigen) and anti-phosphotyrosine 4G10 were from Upstate Biotechnology, Inc. (Lake Placid, NY). FITC-conjugated rat anti-human IL-2 receptor antibody MCA350F was purchased from Serotec (Raleigh, NC). Mouse anti-human vinculin monoclonal antibody was kindly provided by V. Kotliansky (Biogen, Boston). Mouse monoclonal antibody against FAK was purchased from Transduction Laboratories (Lexington, KY).

Immunoprecipitation and Western Blotting—Murine 3T3 cells were transfected with the indicated plasmids, and 48 h later the cells were rinsed with PBS containing 1 mM sodium orthovanadate and lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0, 20 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Protein extracts were subjected to SDS-PAGE and Western blotting.

Indirect Immunofluorescence—Transfected cells were stained by indirect immunofluorescence as described previously (24). Cells were fixed with 4% formaldehyde in PBS for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. The cells were then incubated with the indicated first (45 min) and second (45 min) antibodies in PBS. Anti-FAK, anti-vinculin or anti-phosphotyrosine antibodies were used at 10 μ g/ml followed by staining with secondary Alexa- or Cy3-conju-

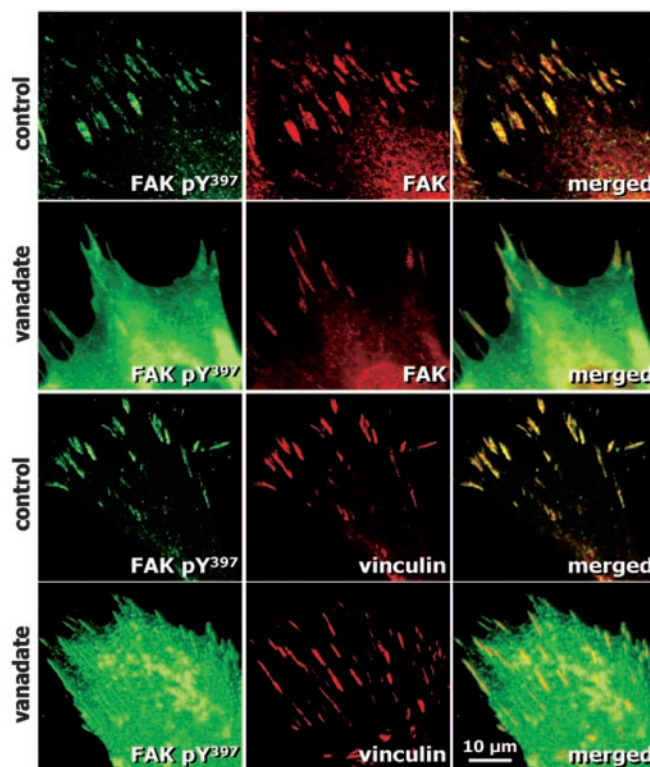


FIG. 4. Pervanadate treatment induces reduction of FAK at focal adhesions. Primary human fibroblasts were cultured overnight on glass coverslips and then treated with 0.1 mM pervanadate or the hydrogen peroxide/catalase control solution for 30 min as described under “Materials and Methods.” Double indirect immunofluorescence staining of phospho-FAK³⁹⁷ (FAK pY³⁹⁷ in green) and FAK or vinculin (in red) was performed. Note that in controls, both FAK and vinculin co-localize in focal adhesions with phospho-FAK³⁹⁷ (yellow in panels where red and green localizations are merged). In contrast, treatment with pervanadate not only disperses phospho-specific staining of FAK³⁹⁷ diffusely throughout the cell, but it also decreases total FAK in focal adhesions without affecting the focal adhesion localization of vinculin. Scale bar, 10 μ m.

gated goat anti-mouse antiserum diluted 1:200. In some experiments, FITC-conjugated rat anti-human IL-2 receptor antibody was utilized at a 1:50 dilution.

Digital Fluorescence Image Analysis of Subcellular Distribution—The system used for quantitative fluorescence microscopy and image analysis is described in detail elsewhere (25). Briefly, the specimens were examined with a DeltaVision digital microscope system (Applied Precision). Images of focal adhesions were segmented by the Water algorithm following high pass filtration (subtracting from each pixel the average over $4 \times 4 \mu$ m area around the particle). The parameters in Water were adjusted to the typical dimensions of focal adhesions and were kept constant for all the analyses. To analyze the relationships between fluorescent probes (IL-2R or phosphotyrosine), focal adhesions were segmented separately, and fluorescence intensities were measured for each probe in the segmented focal adhesions. Ratio images were calculated and presented in a spectral, log scale, color look-up table that ranged from blue for low label ratios (≤ 0.1) to red for high label ratios (≥ 10). To utilize this 2 order-of-magnitude range optimally, and to compensate for the differences in photon yields of different fluorescent labels, all the ratios were normalized linearly by a constant that shifted their average toward a ratio value of 1. In order to compare fluorescence intensity visually, spectrum scale presentations of filtered fluorescence data were presented using a blue-to-red linear spectrum scale.

RESULTS

Expression and Tyrosine Phosphorylation of Transmembrane FAK Chimeras—It has been established that FAK can be activated by membrane translocation to mimic its proximity to the plasma membrane in focal adhesions (11, 14). In order to examine whether the phosphorylation of specific tyrosine resi-

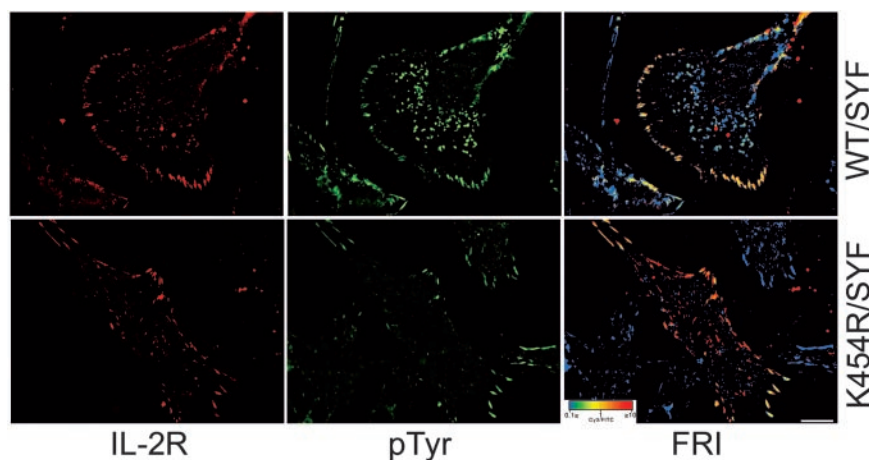


FIG. 5. **IL-2R/FAK chimeras localization in *src/yes/fyn*^{-/-} murine fibroblasts.** Murine embryo fibroblasts lacking expression of Src, Yes, and Fyn (*SYF*) were transfected with the IL-2 receptor in chimeric combination with full-length FAK (WT) or FAK with a K454R mutation in the kinase activation loop (K454R). One hour after plating on fibronectin, the cells were prepared for immunofluorescence analysis of the localization of IL-2 receptor (IL-2R and Cy3 labeling, *red images*) and phosphotyrosine (*pTyr*, FITC labeling, *green images*). Ratio images of IL-2 receptor to phosphotyrosine are shown for each type of transfectant in the panels on the *right*. As shown in the ratio scale bar, high ratios (IL-2R in excess) appear *red*, and low ratios (phosphotyrosine (*pTyr*) in excess) appear *blue*, and regions of relative identity between the intensity of the two labels appear *yellow*. Note that both the wild type and K454R FAK chimeras localized to focal adhesions. These adhesions appear *yellow* in the ratio images, indicating that relative equal amounts of the two fluorescence intensities (Cy3 and FITC) are found in these focal adhesions. *FRI*, fluorescence ratio imaging. *Scale bar*, 10 μ m.

dues can regulate the subcellular localization of highly phosphorylated FAK, we generated a membrane-targeted form of FAK by fusing it to the non-signaling α subunit of the IL-2R as an inert transmembrane anchor (Fig. 1A). The IL-2R tac antigen (α subunit) was utilized to follow the specific location of the transfected FAK molecules in cells.

In addition, we constructed several FAK transmembrane chimeras mutated at residues previously shown to be involved in functions of the wild-type molecule (Fig. 1A). First, we examined the levels of FAK tyrosine phosphorylation following expression of the chimeras in murine 3T3 cells. Previous studies indicated that membrane translocation was sufficient to induce FAK tyrosine phosphorylation even when the cells were maintained in suspension (11, 14). In agreement with these studies, we found that membrane-translocated FAK becomes hyper-phosphorylated (Fig. 1B). As reported previously, mutations of residues K454R (kinase-mutated) and Y397F (inactive Src family SH2 docking site) significantly reduced FAK hyper-phosphorylation (Fig. 1B) (11, 14). Mutating residue Tyr-925 had no apparent effect on the overall level of FAK phosphorylation (Fig. 1B). In concurrence with previous studies, we observed trans-phosphorylation of endogenous cytoplasmic FAK in cells expressing highly phosphorylated wild type FAK or Y925F chimeras (Fig. 1B) (14).

Subcellular Localization of Hyper-phosphorylated FAK and Its Mutants—Our previous studies indicated that transmembrane chimeras containing β integrin tails or cytoskeletal molecules (*e.g.* vinculin) as their cytoplasmic fusions can localize to focal adhesions, in a similar manner to their wild-type counterparts (Fig. 2) (22). We tested whether the FAK C-terminal segment designated the focal adhesion targeting (FAT) region is indeed functional when located close to the plane of the membrane. Therefore, we fused the FAK C terminus corresponding to the FRNK region to IL-2R (Fig. 1A). When expressed in human foreskin fibroblasts, this IL-2R/FRNK chimera localized to focal adhesions (not shown).

However, as shown in Figs. 2 and 3, IL-2R/FAK that contains the full-length FAK molecule did not localize to focal adhesions of primary human fibroblasts. Lack of IL-2R/FAK localization was also observed in murine 3T3 cells and primary murine fibroblasts (not shown). In a similar manner, the IL-2R molecule without any cytoplasmic tail that served as a negative

control was diffusely distributed on the cell membrane in agreement with previous studies (Fig. 2) (22). The inability of the FAK chimera to localize was not due to destruction of cell-matrix adhesion sites, as confirmed by double-labeling the transfected cells with anti-vinculin antibody, demonstrating that focal adhesions persist in the transfected cells (Fig. 3). The lack of hyper-phosphorylated FAK localization has been reported previously and may depend on the cell type being examined (11). One possibility was that FAK turnover within focal adhesions might decrease, thereby reducing the rate of incorporation of the chimeric form into already-established focal adhesions. To test this hypothesis, the transfected cells were detached and replated on fibronectin-coated coverslips. After 10, 30, 60, and 120 min, the cells were fixed, permeabilized, and double-stained for the IL-2R portion of the chimera and for vinculin. These double-localization analyses demonstrated that even though focal adhesions were formed in the transfected cells (as confirmed by vinculin staining), the chimera was not incorporated into these structures (not shown). This analysis indicates that the IL-2R/FAK chimera is specifically excluded from focal adhesions.

To test whether the localization of endogenous FAK is also sensitive to its tyrosine phosphorylation state, cells were treated with pervanadate for 5, 15, 30, 60, or 120 min; they were then fixed, permeabilized, and double-stained with anti-Tyr-397-phosphorylated FAK and anti-FAK or with anti-Tyr-397-phosphorylated FAK and anti-vinculin. At the earliest time point examined (5 min), a substantial increase in diffusely localized Tyr-397-phosphorylated FAK was observed concomitant with a loss of hyper-phosphorylated FAK from focal adhesions that became striking by 15–30 min (Fig. 4). Because phospho-FAK remaining in focal adhesions might theoretically have been masked by the large increases in general cytoplasmic phospho-FAK staining, we also compared the localization of total FAK protein after pervanadate treatment. Total FAK localized to focal adhesions was also substantially reduced, whereas double-staining with anti-vinculin confirmed that the focal adhesions remained intact (Fig. 4). These analyses of endogenous FAK confirm that the amount of FAK in focal adhesions is reduced upon its hyper-phosphorylation. In order to examine the regulation of FAK localization to focal adhesions by specific tyrosine phosphorylation, we transfected hu-

man foreskin fibroblasts with IL-2R chimeras containing FAK mutated at the K454R residue (a kinase active-site mutation), Y397F (the Src family kinase docking site), or Y925F (the Grb2 docking site). As shown in Figs. 2 and 3, the IL-2R/FAK Y397F, K454R, and Y925F mutant chimeras localized properly to focal adhesions of these cells. Identical focal adhesion localization patterns were detected with the FAK-mutated chimeras in murine 3T3 fibroblasts and murine embryonic fibroblasts (not shown).

Regulation of FAK Localization to Focal Adhesions by Src Family Kinases—Src family kinases are important for generating adhesion-mediated tyrosine phosphorylation of FAK (5, 7). We hypothesized that the presence of Src family kinases may be necessary for regulating FAK association with focal adhesions. Therefore, we transfected the wild-type IL-2R/FAK chimera into primary embryonic murine fibroblasts, which served as the control for null fibroblasts. As demonstrated for primary human fibroblasts and murine 3T3 cells, the chimera did not localize to the focal adhesions of these cells (not shown). However, when transfected into Src-deficient fibroblasts (Src $-/-$) the wild type IL-2R/FAK chimera localized to focal adhesions in ~100% of the transfected cells (not shown).

Since Src family kinases Fyn and Yes may also bind to and phosphorylate FAK, we transfected the wild type IL-2R/FAK chimera into fibroblasts obtained from triple Src family null embryos (SYF $-/-$), and we examined its subcellular localization. In this experiment, the wild-type IL-2R/FAK chimera (as well as FAK mutants chimeras) localized effectively to focal adhesions of ~89% of the transfected cells (Fig. 5). FAK mutant chimeras (e.g. FAK K454R) also demonstrated similar localization to focal adhesions of SYF $-/-$ cells (Fig. 5).

DISCUSSION

Appropriate adhesion-mediated signaling responses require tight regulatory mechanisms. Indeed, signaling responses mediated by focal adhesion kinase have been shown to be activated by protein tyrosine kinases (e.g. Src family kinases) or down-regulated by specific phosphatases (e.g. Shp-2 and PTEN) (5, 7, 18–20). However, adhesion-mediated signaling may be mediated by a variety of molecularly and structurally distinct cell-matrix adhesion sites that include focal adhesions, fibrillar adhesions, three-dimensional matrix adhesions, and podosomes (4, 27, 29). In fact, cell-matrix adhesion sites not only mediate physical association of cells with the extracellular matrix but may also function as specialized sites that initiate and regulate signaling responses (30–32). Although focal adhesion targeting regions have been identified in several cytoskeletal and signaling molecules, including FAK, the regulation of these regions still remains to a large extent unclear. One interesting exception is vinculin. It was shown that vinculin/talin and vinculin/actin associations are regulated by the intramolecular interactions of the vinculin N-terminal head domain with its C-terminal tail (33, 34). Phosphatidylinositol 4,5-bisphosphate may govern the vinculin head/tail association, thereby controlling its molecular associations within focal adhesions (33, 34). Mutations in the proline-rich region of vinculin, which links the head and neck domains, may affect the localization of the molecule (35).

In the present study, we evaluated for the first time the regulation of focal adhesion targeting of FAK by specific tyrosine phosphorylation. We also tested the contribution of Src family kinases to the regulation of FAK targeting to focal adhesions. Following association with the phosphorylated FAK Tyr-397 residue, Src family kinases phosphorylate additional tyrosine residues on FAK (5). Tyr-925 is one of several tyrosine residues reported to be phosphorylated by Src family kinases, generating a docking site for the adaptor protein Grb2 and a

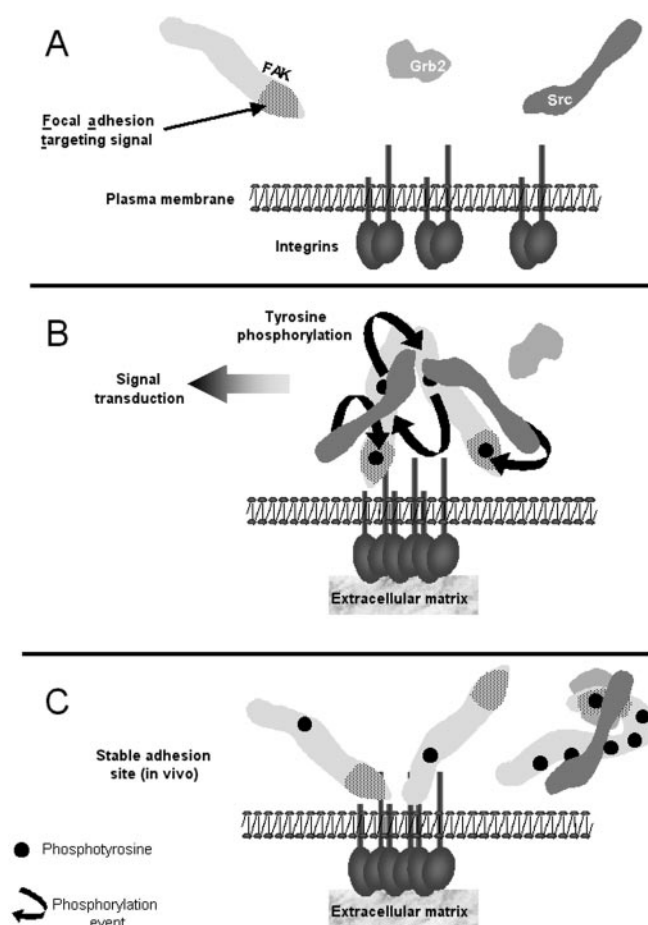


FIG. 6. Regulation of FAK localization by specific tyrosine phosphorylation. A, in non-adherent cells FAK is not phosphorylated. B, upon adhesion, FAK is recruited to the plasma membrane and clustered and subsequently phosphorylated by adjacent FAK molecules and Src family kinases. Specific phosphorylated tyrosine residues bind signaling molecules that generate signaling responses (e.g. mitogen-activated protein kinase). C, simultaneously to the progression of the biochemical signaling responses, FAK-signaling complexes inhibit the focal adhesion localization targeting sequences thereby reducing the association of FAK with cell-extracellular matrix adhesion sites.

signaling complex, resulting in activation of the ERK1/2 response (5). It is interesting to note that Tyr-925 resides within the region defined as the FAT sequence of FAK (Fig. 1) (36). Recent studies have indicated that in some cell types, FAK molecules that reside close to the plasma membrane are phosphorylated at residue Tyr-397. These molecules, however, are predominantly diffusely distributed, and only a minor fraction is localized in specific domains (37). In contrast, the predominant *in vivo* state of residue Tyr-925 is apparently unphosphorylated (26). Although focal adhesions of cells *in vitro* contain high levels of tyrosine-phosphorylated molecules, non-phosphorylated or partially phosphorylated FAK molecules appear to be the predominant form of this molecule associated with matrix adhesions *in vivo* (29). Tyr-397-phosphorylated FAK does not localize to “three-dimensional matrix adhesions” in fibroblasts attaching to three-dimensional matrices *in vitro*, although it can be found in small focal complexes. Similarly, α_5 integrin co-localizes with FAK in mouse embryonic tissue sections but not with Tyr-397-phosphorylated FAK (29).

We speculate that hyper-phosphorylated FAK is excluded from focal adhesions for a variety of reasons. Phosphorylation that includes Tyr-925 may generate a constitutive association with Grb2, as shown in previous studies (11). The constitutive signaling complex might sterically block the focal adhesion

targeting site, thereby inhibiting the association of activated FAK with focal adhesions (Fig. 6). Alternatively, the phosphorylation *per se* could have an inhibitory effect. The fact that the highly phosphorylated IL-2R/FAK Y925F mutant does localize to focal adhesions indicates that inhibition of FAK incorporation into focal adhesions does not stem merely from total tyrosine phosphorylation of the molecule but from the phosphorylation of specific residue(s). As depicted in Fig. 1A, Tyr-925 resides within the FAT segment of FAK. Therefore, the specific tyrosine phosphorylation of the Tyr-925 residue may be important for the regulation of FAK localization to focal adhesions. An additional mechanism that may explain the failure of highly phosphorylated FAK to localize to focal adhesions requires a higher affinity association of hyper-phosphorylated FAK with other molecular complexes, the FAK "scaffold." These complexes may be tied up elsewhere within the cytoplasm, and the off-rates of these interactions significantly altered such that availability of FAK for standard focal adhesion targeting (e.g. via paxillin and talin) is decreased. Alternatively, the constitutively hyper-phosphorylated FAK may have more restricted mobility in the plane of the membrane due to interactions (directly or indirectly via phosphatidylinositol phosphate) with membrane lipoproteins.

The common denominator of these putative mechanisms is the basic principle that tyrosine residues on FAK may function as molecular binary switches ("transistors"), cycling between states of reactivity according to their phosphorylation level. Tyr-397 is trans-phosphorylated by nearby FAK molecules and thus is regulated by the local density of the molecules localized at the plasma membrane (Fig. 6). Phosphorylation at this site determines whether FAK will bind Src family kinases and possibly other molecules, e.g. phospholipase C γ (28). Src family kinases expand the biochemical signal to additional tyrosine residues, including Tyr-925 (Fig. 6). Phosphorylation of Tyr-925 may require its molecular association with an Src family kinase domain. Such association may be transient, but at high molecular density of FAK and Src family molecules close to the inner plasma membrane plane it may become more continuous. This specific state is achieved by use of the chimeric FAK, which may represent the early stages of cell adhesion, where FAK is recruited to the plasma membrane via direct and/or indirect interactions with integrin tails. A previous study (11) indicated that anti-apoptotic signals, mediated by cell adhesion, may be inhibited by peptides that inhibit direct interactions between FAK and integrin tails. This inhibition was effective only during the early stages of cell adhesion (11).

Therefore, several tyrosine residues of FAK may have a dual function as follows: 1) regulation of biochemical signaling responses, by directly controlling FAK enzymatic activity and its associations with signaling molecules; and 2) regulation of FAK localization by modulation of intermolecular and/or intramolecular interactions. This concept may apply to other molecules as well. The two regulatory modes may be linked to an inte-

grated mode combining the subcellular localization of active signaling complexes. In the case of FAK, hyper-phosphorylated molecules on specific tyrosine residues may be selectively excluded from cell-matrix adhesion sites.

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