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Vanadate-Dependent FAK Activation Is Accomplished by the Sustained FAK Tyr-576/577 Phosphorylation

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Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase implicated in cell–matrix interaction and integrin signaling. It is well established that Tyr-397 is the FAK autophosphorylation site and Tyr-407, -576/577, -861, and -925 are the sites on murine FAK that are mediated by Src family kinases. To study how FAK is regulated by tyrosine phosphatase(s), cells overexpressing chicken FAK are treated with sodium vanadate. Both the phosphotyrosine content and the enzymatic activity of FAK are increased in response to vanadate. Interestingly, sustained FAK Tyr-576/577 and -863 phosphorylations are detected in vanadate-treated FAK overexpressors and are dependent on FAK autophosphorylation. Further analysis of sodium vanadate-treated FAK overexpressors reveals that the enhanced FAK kinase activity parallels its elevated Tyr-576/577 phosphorylation. Thus, we conclude that Src-mediated FAK phosphorylation is regulated by a tyrosine phosphatase(s) and may be of physioligical significance.

Key Words: **Src; FAK; tyrosyl phosphorylation; sodium vanadate.**

Focal adhesion kinase (FAK), a unique cytoplasmic protein tyrosine kinase, is localized at focal contacts (1) which contains a highly conserved catalytic domain flanked by large amino- and carboxyl-terminal domains devoid of any SH2 (Src homology domain 2) and SH3 (Src homology domain 3) motifs (1, 2). Tyrosyl phosphorylation of FAK is modulated by cell adhesion (2, 3), v-Src transformation (4,5) as well as the activation of several cell surface receptors (see review in Ref. 6). FAK is an important player in integrin signal transduction and consistent with this notion is the similar embryonic defects observed in FAK and fibronectin knock out mice (7, 8). The recent discovery of FAK-like tyrosine kinase, named PYK2 (9), CAKβ (10), RAFTK (11), or CADTK (12) implicates the existence of different FAK family members within a tissue regulating various cellular processes (13, 14, 15).

Tyr-397 is the FAK autophosphorylation site whose phosphorylation confers the binding site for Src family tyrosine kinases (16). The interaction between these two tyrosine kinases accelerates Src-mediated FAK phosphorylation. To date, Tyr-407, -576/577, -861, and -925 are identified as the sites on murine FAK that are phosphorylated by Src (17-20). Phosphorylation of Tyr-576/577 has been suggested to elevate the kinase activity of FAK (17), while Tyr-925 phosphorylation confers the site for Grb2 binding which may lead to the activation of Ras and triggers the MAPK kinase cascade (19, 20).

Previous studies of the human endothelial and Chinese hamster ovary cells demonstrated that sodium vanadate can strongly up-regulate the tyrosyl phosphorylation of FAK in a dose-dependent manner. And interestingly, FAK tyrosyl phosphorylation can in turn affect the assembly of focal adhesions and actin stress fibers (21). These results implicate that tyrosyl phosphorylation of FAK is regulated by a tyrosine phosphatase(s) and may have physiological significance. Since there are multiple tyrosine residues on FAK that can be phosphorylated by itself and/or Src family kinases, we are interested to know phosphorylation on which tyrosine residues may be tightly regulated in vivo. To address this point, chicken embryo (CE) cells are transfected with various *fak* cDNA constructs to generate FAK overexpressors, and the effects of sodium vanadate on these cells are determined. In this paper, we are going to report that (1) elevated phosphorylation on FAK Tyr-576/577 and Tyr-863 is detected in vanadate-treated FAK overexpressors; (2) the elevated Tyr-576/577 phosphorylation parallels with the increased FAK kinase activity detected in these cells; and (3) when Tyr-397 or Tyr-576/577 is mutated to phenylalanine, this vanadate-enhanced FAK kinase activity is abrogated. These results suggest that Src-mediated FAK phosphorylation is under delicate regulation in normal

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cells. By contrast, the constitutive phosphorylation of FAK Tyr-576/577 and Tyr-863 in cells expressing oncogenic Src may contribute to cellular transformation.

EXPERIMENTAL PROCEDURES

Cell, viruses, and cell lysis. Primary chicken embryo (CE) cells were prepared from 10-day-old chicken embryos (Spafas, Norwich, CT) as previously described (22). cDNA encoding $pp125^{FAK}$ and its mutants were cloned into retroviral expression vector RCAS A (BH), while cDNA encoding active $pp60^{c\text{-}Src}$ mutant (518amb) was cloned into RCAS B (BH) vector as described previously (23, 24). These constructs were individually transfected into CE cells by calcium phosphate precipitation method for generating FAK or Src overexpressing cells. To generate cells co-overexpressing both FAK and Src, Src virus-stock was collected from the cultured medium of *src(518amb)*-transfected cells and added into the FAK overexpressors growing dish. After 10 days, CE cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH7.4; 150 mM NaCl; 1% NP40; and 0.25% sodium deoxycholate), supplemented with 2 mM EGTA, 1 mM sodium vanadate, 12.5 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml α_2 macroglobulin (Boehringer-Mannheim), and 1 mM phenyl methylsulfonyl fluoride as described before (22). Protein amount in each lysate was determined by Bio-Rad protein assay kit (Bio-Rad).

Mutagenesis of FAK and generation of its expression constructs. The construction of 397F-FAK was described previously (16). For constructing 576F/577F- or 863F-FAK mutants, the *fak* cDNA was first subcloned into the plasmid $pALTER^{-1}$ mutagenesis vector (Promega). Then, the selected tyrosine residue was changed to phenylalanine by oligonucleotide-directed mutagenesis according to the provider's manual. The mutated sequence in each *fak* cDNA construct was confirmed by Sanger-dideoxy DNA-sequencing method before it was further subcloned into RCAS A(BH) vector.

Antibodies, immunoprecipitation, and immunoblotting. Rabbit antibody against FAK (BC3) was generated and described previously (1). Immunoprecipitation and immunoblotting of FAK with BC3 was performed as previously described (22). For detection of phosphotyrosine content, Western immunoblotting was performed with either polyclonal anti-phosphotyrosine antibody followed by [^{125}I]protein A blotting and X-ray autoradiography or horse radish peroxidase-conjugated mouse monoclonal antibody PY20 (Santa Cruz Biotechnology, Santa Cruz, Calilifornia) and detected by Enhanced Chemiluminescence method (Amersham, Arlington Heights, III, USA).

In vitro kinase reaction. After washing at least twice with RIPA buffer and twice with Tris-saline solution (50 mM Tris-Cl, 150 mM NaCl, pH7.2), the FAK immunocomplexes were prepared. Unless additional indication, the kinase reaction was performed in a 20-μl kinase mixture containing 20 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) (pH7.2), 5 mM $MnCl_2$, 5 mM $MgCl_2$, and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, NEN) for 10 min at 25°C. The kinase reactions were terminated by the addition of equal volume of 2X-concentrated Lamli sample buffer (25) and boiling for 5 min. The ^{32}P-labeled products were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), dried down, and visualized by autoradiography. For further phosphotryptic map analysis, the labeled FAK and its variants were excised and extracted from the gel as described by Boyle et al. (26).

To study the effect of sodium vanadate on the kinase activity of various FAK mutants, FAK overexpressors were treated with or without 500 μM sodium vanadate (buffered with 500 μM Hepes, pH7.4) for 30 min. Then, the FAK kinase reactions were carried out by incubating the FAK immunocomplexes with 10 μg poly(Glu-Tyr) in a 20-μl kinase mixture containing 20 mM PIPES (pH 7.4), 10 mM $MnCl_2$, 5 μM cold ATP, and 10 μCi of [γ-^{32}P]ATP for various time as indicated in the figure legends. The ^{32}P-labeled products were then either resolved in an SDS/PAGE and detected by autoradiography or precipitated by 20% TCA (trichloroacetic acid) at 4°C and filtrated through a Whatman glass microfiber filter (GF/C) followed by washes with ice-cold phosphate-buffered saline (150 mM NaCl, 15 mM sodium phosphate, pH 7.4) containing 5% TCA and 20 mM sodium pyrophosphate. The ^{32}P-labeled poly (Glu-Tyr) left on the membrane or excised from the gel were measured by Cherenkov counting.

Metabolic labeling of cells. FAK overexpressors and FAK/Src double overexpressors were incubated overnight with 0.5 mCi/ml carrier-free ^{32}P-orthophosphate (ICN, Costa Mesa, CA) in 90% phosphate-free DMEM plus 10% conditional medium. To study the effect of sodium vanadate on FAK phosphorylation, various FAK overexpressors were incubated with 500 μM sodium vanadate along with ^{32}P-containing medium for 60 min. Cellular lysates of ^{32}P-labeled cells were prepared as described above and immunoprecipitated with BC3 antibodies. FAK was then resolved and purified from an SDS/PAGE and subjected to tryptic phosphopeptide analysis as described below.

Tryptic peptide mapping by two-dimensional thin layer chromatography (TLC). In vivo ^{32}P-labeled FAK prepared from FAK overexpressors in the presence or absence of sodium vanadate or from cells expressing both Src and FAK were oxidized and digested with TPCK-treated trypsin as described by Boyle et al (26). Unless specifically indicated, phosphotryptic peptides were separated by electrophoresis (15 min at 1,000 V in pH 1.9 buffer [26]) followed by ascending chromatography (in phospho chromatography buffer [26]) on thin layer plates (Merck).

RESULTS

Enhanced FAK tyrosyl phosphorylation and enzymatic activity in response to sodium vanadate treatment. Previous results have demonstrated the enhancement of FAK tyrosyl phosphorylation in sodium vanadate-treated human endothelial cells and Chinese hamster ovary cells (21). To identify the tyrosine phosphatase-sensitive site(s) on FAK, CE cells overexpressing chicken FAK are generated and treated with sodium vanadate. As shown in Figure 1A, though similar levels of FAK are detected in both control and treated groups, there is at least 2-fold increase of FAK tyrosyl phosphorylation in response to vanadate. Consistent with previous observation (5, 27), the FAK activity in vanadate-treated cells exhibits a 2.5-fold increase (Fig. 1B).

Enhanced FAK Tyr-576/577 and -863 phosphorylation in sodium vanadate-treated FAK overexpressors. To identify the tyrosine phosphatase-labile sites on FAK, cells expressing FAK alone were metabolically ^{32}P-labeled in the presence or absence of sodium vanadate. The in vivo ^{32}P-labeled FAK under these two conditions were recovered, trypsinized and analyzed by 2D-TLC phosphotryptic mapping (Figs. 2A and 2B). Since the map of Src-mediated FAK was demonstrated in murine FAK (17-20), the 2D-TLC phosphotryptic map of the in vivo ^{32}P-labeled FAK prepared from cells expressing both Src and chicken FAK was also analyzed (Fig. 2C). The identities of Y1 and Y7 on Figure 2C were confirmed by Edman-degradation and their disappearance in 2D-TLC map of FAK when Tyr-397 and Tyr-863 were mutated to phenylalanines respectively (Fig. 2E and data not shown). In order to easily interpretate the results, the schematic drawing of

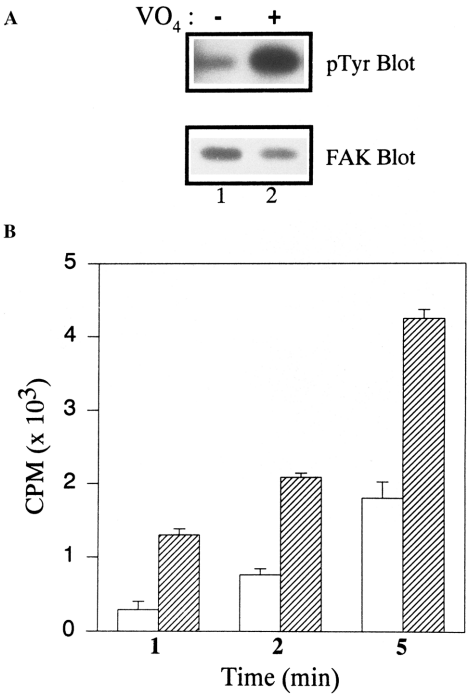

FIG. 1. Enhanced tyrosyl phosphorylation and enzymatic activity of FAK in response to sodium vanadate in FAK overexpressors. FAK was immunoprecipitated from lysates of FAK overexpressors pretreated with (lane 2 in panel A and hatched boxes in panel B) or without (lane 1 in panel A and open box in panel B) 500 μM sodium vanadate for 30 min. (A) One-quarter of the immunocomplexes was resolved with SDS–PAGE and Western blotted with polyclonal antibody against FAK (FAK Blot) or phosphotyrosine (pTyr Blot). (B) Three-quarters of the FAK immunocomplexes were assayed for *in vitro* kinase activity and the ^{32}P-labeled poly(Glu-Tyr) collected on Whatman glass microfiber filter (GF/C) was counted as described under Experimental Procedures. The results are shown in means ± S.D. for two independent experiments performed in triplicate.

FAK's 2D-TLC phosphotryptic map summarized from previous publications (17-20) and our unpublished data is exhibited in Figure 2D as a reference. The appearance of Y5/Y2 (i.e. Tyr-576 and/or Tyr-577), Y4 (i.e. Tyr-407), and Y7 (i.e. Tyr-863) in Figure 2B indicates that phosphorylation of these four Src-mediated sites on FAK are present in vanadate-treated cells. By contrast, these phosphotryptic peptides are not detected in control FAK overexpressors. Thus, these results implicate that Src-mediated FAK phosphorylation is sensitive to phosphatase and therefore should be well regulated within cells. Since FAK Tyr-576/577 phosphorylation has been demonstrated to positively regulate its kinase activity, thereby, consistent with vanadate-induced FAK activation (Fig. 1B) is the detection of sustained FAK Tyr-576/577 phosphorylation in vanadate-treated FAK overexpressors (Fig. 2B).

Disappearance of FAK Tyr-576/577 and -863 phosphorylation in cells expressing both 397F-FAK and Src. Tyr-397 is the autophosphorylation site of FAK and it is well established that FAK Tyr-397 phosphorylation mediates its association with Src family kinases. To substantiate our hypothesis that Tyr-576/577 and -863 are Src-mediated sites, cells expressing both Src and 397F-FAK were metabolically ^{32}P-labeled. The in vivo ^{32}P-labeled 397F-FAK was recovered, trypsinized and analyzed by 2D-TLC phosphotryptic mapping. As shown in Figure 3, no Y5 (i.e. Tyr-576/577) and Y7 (i.e. Tyr-863) are detected. These data indicate that indeed FAK Tyr-576/577 and -863 phosphorylation is mediated by Src.

Mutation of FAK at Tyr-397 or Tyr-576/577 will abrogate the sodium vanadate-induced FAK activation. To further prove that sodium vanadate-induced FAK activation is originated from FAK Tyr-576/577 phosphorylation, cells expressing wt-FAK and various FAK mutants are treated with sodium vanadate and the resulting pTyr content and the kinase activity of FAK are then analyzed. As shown in Figure 4A and 4C, sodium vanadate treatment can lead to the increase of both pTyr content and the kinase activity of wt- and 863F-FAK. By contrast, though the pTyr content of 576F/577F-FAK is increased in response to sodium vanadate, no enhancement of its kinase activity is detected. Interestingly, both pTyr content and kinase activity of 397F-FAK are not affected by vanadate treatment. This result indicates that phosphorylation of Tyr-397 is prerequisite for Tyrosine-576, -577 and -863 phosphorylation and Tyr-576/577 phosphorylation is critical for sodium vanadate-induced FAK activation. To further prove this point, cells overexpressing 397F-FAK were metabolically ^{32}P-labeled in the presence of sodium vanadate. The in vivo ^{32}P-labeled 397F-FAK was recovered, trypsinized and analyzed by 2D-TLC phosphotryptic mapping. As shown in Figure 2E, except a peptide run at the similar position of Y2, the phosphotryptic peptides such as Y1, Y4, Y5, Y7 are all disappeared. This result further indicates that FAK Tyr-397 phosphorylation is required for Src or its family members to mediate FAK phosphorylation.

DISCUSSION

Tyrosyl phosphorylation is an important post-translational modification involved in various cellular functions. The reversible tyrosyl phosphorylation is accomplished by the action of tyrosine kinases and phosphatases. After the cloning and characterization of FAK, it is now well established that FAK is an important player participated in integrin signal transduction. The elevated tyrosyl phosphorylation of FAK detected in Src transformed cells implicated that FAK was a direct substrate of Src family kinases. Indeed, Tyr-407, -576/577, -861, and -925 have been identified on murine FAK whose phosphorylation was mediated by Src and integrin activation. Interestingly, phoshorylation of Src-mediated sites was induced in

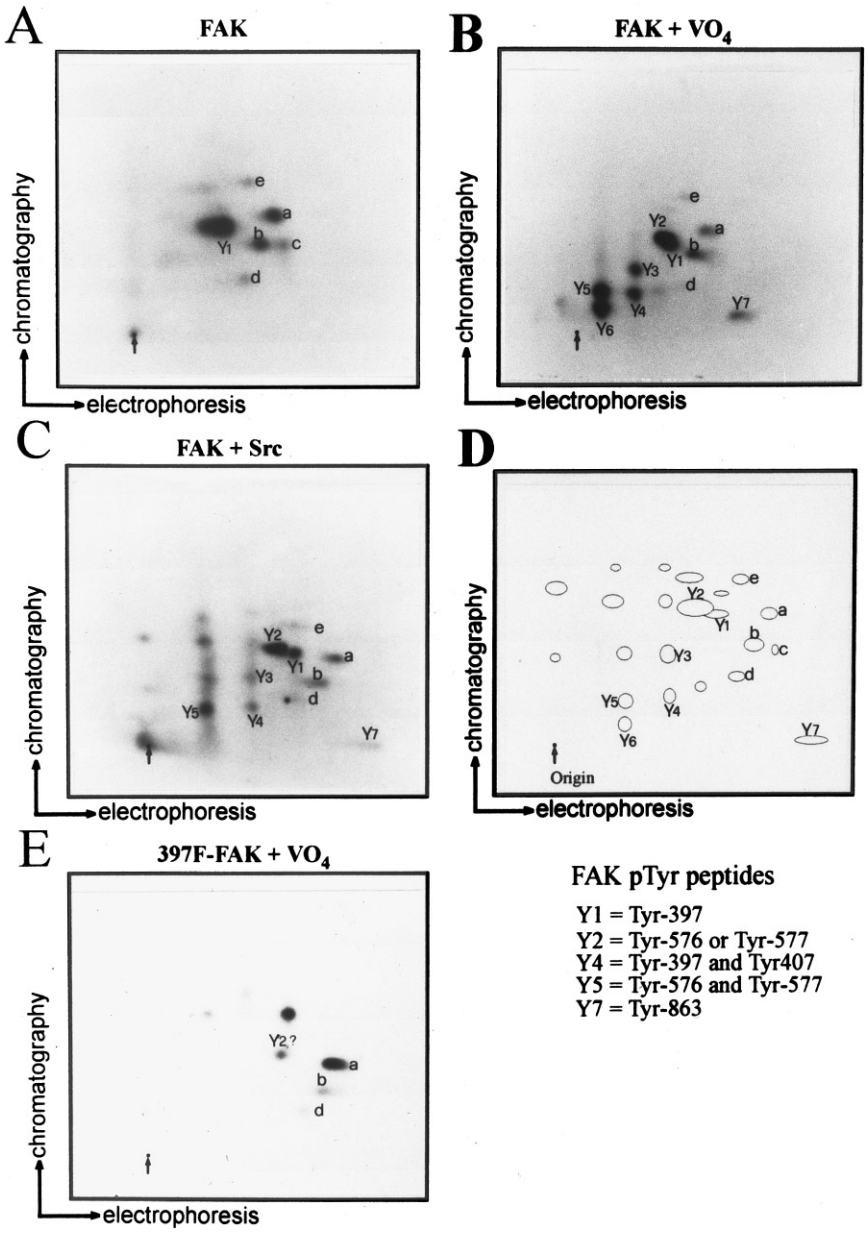

FIG. 2. Src-mediated FAK phosphorylation is also detected in sodium vanadate-treated FAK overexpressors. The *in vivo* ^{32}P-labeled FAK was immunoprecipitated from cellular lysates of FAK overexpressors (A, overnight labeling), FAK overexpressors treated with 500 μM sodium vandate (B, 1 h labeling), FAK and Src double overexpressors (C, overnight labeling), or 397F-FAK overexpressors treated with 500 μM sodium vanadate (E, 1 h labeling). The ^{32}P-labeled FAK purified from an SDS–PAGE was trypsinized and resolved with 2D-TLC as described under Experimental Procedures. In addition to Y1, peptides such as Y2, Y3, Y4, Y5, Y6, and Y7 appeared on the map of vanadate-treated FAK (panel B) and represented the ones containing FAK phosphatase-sensitive tyrosine residues. Except Y6, they were also present on the map of Src phosphorylated FAK (panel C). The relative positions of the phosphotyrosine-containing tryptic peptides derived from FAK were demonstrated in panel D as the reference.

vanadate-treated CE cells. Thus, Src-mediated FAK phoshorylation is crucial in cell adhesion and should be delicately regulated within cells. The failure of detecting Tyr-925 phosphorylation in chicken FAK could be due to its low stoichiometry in our system.

In this paper, we show that the enhancement of FAK tyrosyl phosphorylation in response to sodium vanadate is due to the increased phosphorylation of Tyr-576/577, -863, and possibly -397/407. Thus, Tyr-576/577 and -863 are the phosphatase-sensitive sites on chicken FAK which correspond to Tyr-576/577 and -861 on murine FAK. To pinpoint which phosphorylated tyrosine residue(s) on FAK is responsible for FAK activation induced by vanadate, the FAK kinase activity is determined in vanadate-treated CE cells overexpressing wt- and various FAK-mutants. Our data in-

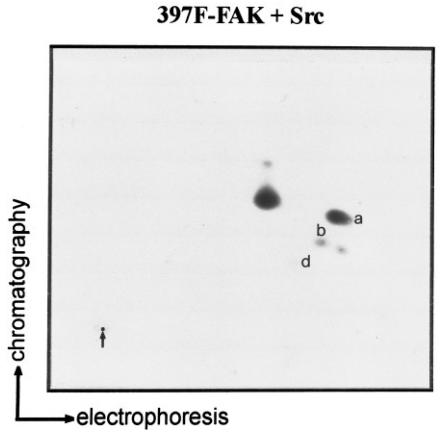

FIG. 3. Disappearance of FAK Tyr-576/577 and -863 phosphorylation in cells expressing both 397F-FAK and Src. *In vivo* ^{32}P-labeled FAK was immunoprecipitated from cellular lysates of 397F-FAK and Src double overexpressors (overnight labeling). The ^{32}P-labeled FAK was purified from SDS–PAGE, trypsinized, and resolved with 2D-TLC as described under Experimental Procedures. Compared to Fig. 2C, both Y5 and Y7 are absent in this map.

dicate that sustained Tyr-576/577 phosphorylation is responsible for the vanadate-induced FAK activation. Consistently, previous results have indicated that Tyr-576/577 phosphorylation elevated FAK kinase activity (17). Interestingly, phenylalanine mutated Tyr-397 abrogates the elevation of both FAK tyrosyl phosphorylation and kinase activity induced by vanadate treatment. Thus, this indicates that phosphorylated Tyr-397 is prerequisite for Tyr-576/577 phosphorylation. Indeed, analysis of the 2D-tryptic map of vanadate-treated 397F-FAK, little or none phosphorylated Tyr-576/577- and Tyr-863-containing peptides are detected (Figure 2E).

Focal adhesions (or focal contacts) are the sites where cytoskeleton actin filaments link to extracellular matrix through the transmembrane integrins. Since proteins that become tyrosyl phosphorylated after the engagement of fibronectin to integrin are mainly localized at the focal contacts and tyrosine kinase inhibitors can block the formation of focal adhesion after fibronectin stimulation (3, 28), it is speculated that tyrosyl phosphorylation is an important event required for the formation of focal contact. Among various cytoplasmic tyrosine kinases, Src family kinases and FAK have been demonstrated to be present in focal contacts where they can not only regulate the formation of this adhesive structure but also propagate signals from extracellular matrix down to the cytoplasm. Meanwhile, several clues have also indicated the involvement of a tyrosine phosphatase(s) in the focal contact formation (21, 29, 30). Vanadate, the inhibitor of general tyrosine phosphatase, could induce the focal contact formation accompanying with the enhancement of tyrosyl phosphorylation on FAK and paxillin (21). Although the FAK-specific tyrosine phosphatase(s) is still elusive, the widely expressed LAR/LIP.1 present in focal adhesion (30) and p130^{cas}-associated PTP1B (31) and PTP-PEST (32, 33) may be involved in the regulation of FAK activity and cytoskeleton organization.

In summary, the results presented in this paper demonstrate that Src-mediated phosphorylation on FAK Tyr-576/577, -863, and possibly Tyr-407 is phosphatase-labile. The vanadate-induced enhancement of FAK tyrosyl phosphorylation is at least attributed by sustained FAK Tyr-576/577, -863, and possibly Tyr-397/407 phosphorylation and the elevation of FAK kinase activity is due to Tyr-576/577 phosphorylation. Interestingly, when FAK autophosphorylation site (i.e. Tyr-397) is mutated, Src family kinase can no longer mediate the phosphorylation of FAK. These data support a model that interaction between autophosphory-

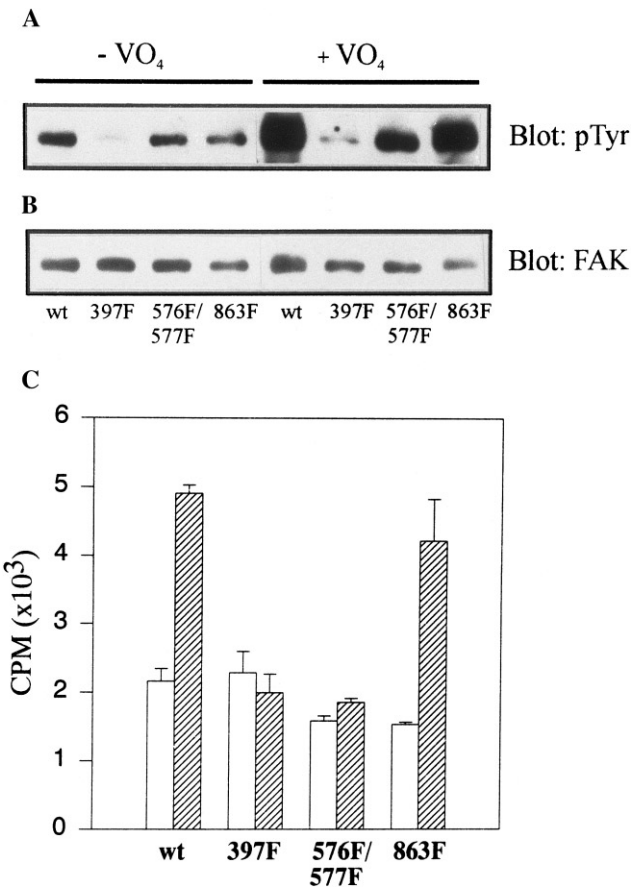

FIG. 4. Phosphorylation of FAK Tyr-397, -576/577 is required for sodium vanadate-induced FAK activation. FAK immunocomplexes were prepared from cells overexpressing wt-, 397F-, 576F/577F-, and 863F-FAK pretreated with (hatched boxes) or without 500 μM sodium vandate (open box) for 1 h. One-quarter of these immunocomplexes was resolved by SDS–PAGE and immunoblotted with (A) anti-pTyr antibody or (B) anti-FAK antibody. Also, the remaining immunocomplexes were subjected to *in vitro* kinase assays and the ^{32}P-labeled poly(Glu-Tyr) were directly quantified from SDS–PAGE as described under Experimental Procedures. The results are shown in mean ± S.D. for three independent experiments performed in triplicate.

lated FAK and Src SH2 is important for FAK activation which may lead to the assembly and disassembly of focal contacts. Further identification and characterization of the tyrosine phosphatases responsible for FAK dephosphorylation may dissect the mechanisms of FAK-participated integrin signal transduction.

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