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LRRK2 Inhibits FAK Activity by Promoting FERM-mediated Autoinhibition of FAK and Recruiting the Tyrosine Phosphatase, SHP-2

Insup Choi[1,2,3,4], Ji-won Byun[1], Sang Myun Park[1,2,4], Ilo Jou[1,2,4] and Eun-Hye Joe[1,2,3,4]*

[1]Department of Biomedical Sciences, Neuroscience Graduate Program, [2]Department of Pharmacology, [3]Department of Brain Science, [4]Chronic Inflammatory Disease Research Center, Ajou University School of Medicine, Suwon 16499, Korea

Mutation of leucine-rich repeat kinase 2 (LRRK2) causes an autosomal dominant and late-onset familial Parkinson's disease (PD). Recently, we reported that LRRK2 directly binds to and phosphorylates the threonine 474 (T474)-containing Thr-X-Arg(Lys) (TXR) motif of focal adhesion kinase (FAK), thereby inhibiting the phosphorylation of FAK at tyrosine (Y) 397 residue (pY397-FAK), which is a marker of its activation. Mechanistically, however, it remained unclear how T474-FAK phosphorylation suppressed FAK activation. Here, we report that T474-FAK phosphorylation could inhibit FAK activation via at least two different mechanisms. First, T474 phosphorylation appears to induce a conformational change of FAK, enabling its N-terminal FERM domain to auto-inhibit Y397 phosphorylation. This is supported by the observation that the levels of pY397-FAK were increased by deletion of the FERM domain and/or mutation of the FERM domain to prevent its interaction with the kinase domain of FAK. Second, pT474-FAK appears to recruit SHP-2, which is a phosphatase responsible for dephosphorylating pY397-FAK. We found that mutation of T474 into glutamate (T474E-FAK) to mimic phosphorylation induced more strong interaction with SHP-2 than WT-FAK, and that pharmacological inhibition of SHP-2 with NSC-87877 rescued the level of pY397 in HEK293T cells. These results collectively show that LRRK2 suppresses FAK activation through diverse mechanisms that include the promotion of autoinhibition and/or the recruitment of phosphatases, such as SHP-2.

Key words: Parkinson's disease, LRRK2, FAK, phosphatase, SHP-2

INTRODUCTION

Leucine-rich repeat kinase 2 (LRRK2) has been associated with an autosomal dominant, late-onset form of familial Parkinson's disease (PD). The encoded protein, LRRK2, is about 280 kDa in size and contains several functional domains, including a serine/threonine kinase domain [1]. Among the PD-related pathogenic mutations found throughout the entire *LRRK2* gene [2], the G2019S mutation, which enhances kinase activity [3], has been found in both sporadic and familial PD [4, 5]. Many studies have sought to identify the kinase substrates of LRRK2 to improve our understanding of LRRK2-mediated PD pathogenesis, and LRRK2 has been shown to govern various biological functions, including neurite outgrowth, cell migration, mRNA translation, protein synthesis, neurotransmitter release, and stem cell maintenance [6-12].

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*To whom correspondence should be addressed.
TEL: 82-31-219-5062, FAX: 82-31-219-5069
e-mail: ehjoe@ajou.ac.kr

Focal adhesion kinase (FAK) is a non-receptor kinase that controls the migration, proliferation, and survival of cells [13-15]. It consists of an N-terminal FERM domain, a kinase domain, and a C-terminal focal adhesion-targeting (FAT) domain [16, 17]. During cell migration, FAK is activated and recruited to the focal adhesion sites where lamellipodia are produced; this activates downstream signaling molecules that regulate the reorganization of cytoskeletal proteins, including the polymerization of actin [15]. FAK can be activated in response to cell-migration-promoting stimuli, such as the interaction between the extracellular matrix (ECM) and integrin [18], the activation of growth factor receptors or G protein-coupled receptors [19], and mechanical stress [20]. Upon activation of FAK demonstrated by autophosphorylation of Y397 (pY397), downstream signaling is activated for proper cell migration [15, 21].

We recently showed that G2019S-LRRK2 strongly inhibits FAK and attenuates microglial motility [9]. Our results revealed that microglia derived from G2019S-LRRK2 transgenic mice (TG-microglia) exhibited impaired FAK activation (decreased levels of pY397) when treated with ADP, which is a microglial activator that increases motility. TG-microglia produced unstable lamellipodia and exhibited reduce motility compared with wild-type (WT)-microglia. Moreover, we found that LRRK2 suppresses FAK activation by directly phosphorylating the Thr residue(s) in the Thr-X-Arg (TXR) motif(s) of FAK, which include Thr 474 (T474). In the present study, we further examined how T474-FAK phosphorylation prevents the activation of FAK. Our novel results suggest that T474 phosphorylation may promote the FERM-mediated autoinhibition of FAK and/or trigger the recruitment of SHP-2, which dephosphorylates pY397-FAK. Thus, LRRK2 appears to regulate FAK activity through diverse mechanisms.

MATERIALS AND METHODS

Cell culture

The HEK293T cell line was acquired from ATCC (Seoul, Korea), and maintained in DMEM supplemented with 10% (v/v) FBS and penicillin/streptomycin (50 U/mL).

DNA constructs

FLAG-FAK was prepared by inserting the human FAK gene into the p3xFLAG-CMV-7.1 vector (Sigma, St Louis, MO, USA) using AccuPrime Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and an infusion cloning kit (Clontech, Palo Alto, CA, USA). Mutations were introduced into FLAG-FAK using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Palo Alto, CA, USA). The FERM domain deletion mutant (Δ35~362) was prepared using AccuPrime Pfx DNA Polymerase. Plasmids encoding WT-SHP-2 were kindly provided by Prof. Young Ho Suh (Seoul National University College of Medicine, Seoul, Korea). The primers used for mutagenesis are listed in Table 1.

Transfection

HEK293T cells were transfected with DNA plasmids using the jetPEI transfection reagent (Polyplus-Transfection, San Diego, CA, USA) as described by the manufacturer. Briefly, cells were exposed to DNA plasmids and the jetPEI mixture for 4 hours, and then the media were replaced with fresh DMEM containing 10% FBS. Two days later, transfected cells were used for experiments.

Western blot analysis

Cells were lysed on ice in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 1 mM NaF, 0.25% Na-deoxycholate, 1 mM Na_3VO_4, and 150 mM NaCl) containing a protease/phosphatase inhibitor

Table 1. Sequences of the primers used for mutagenesis of FAK and SHP2

Mutation	Primer sequence(s)
FAK	
T474A	5'-CTTCAAGAAGCCTTAGCGATGCGTCAGTTTGACCATCCTC-3'
T219E	5'-GGATTCTGTCAAGGCCAAAGAGCTAAGAAAACTGATCC-3'
T227E	5'-CTAAGAAAACTGATCCAACAAGAGTTTAGACAATTTGCCAACC-3'
T284E	5'-AATCAGTTACCTAGAGGACAAGGGCTGCAATCCC-3'
T455E	5'-CGGTTGCAATTAAAGAGTGTAAAAACTGTACTTCGGACAGCG-3'
T474E	5'-CTTCAAGAAGCCTTAGAGATGCGTCAGTTTGACCATCCTC-3'
T979E	5'-TACCAGCCAGCGAGCACCGAGAGATTGAGATGGC-3'
Y180A/M183A	5'-CGGCGATCAGCCTGGGAGGCGCGGGGCAATGC-3'
V196D/L197D	5'-GAAAAGAAGTCTAACTATGAAGATGATGAAAAAGATGTTGGT TTAAAGCG-3'
F596D	5'- TGGCTCCAGAGTCAATCAATGATCGACGTT TTACCTCAGC-3'
FERM domain deletion (Δ35~362)	5'-CTCCATTGCACCAGGAGAACGTTCC-3' and 5'-CAGAAAGAAGGTGAACGGGCTTTGCC-3'

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cocktail (GenDEPOT, Barker, TX, USA). Lysates were centrifuged, and the proteins in the supernatant were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membranes (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were incubated with antibodies specific for FAK (1:1000, Santa Cruz, Dallas, TX, USA), pY397-FAK (1:1000, Cell Signaling Technology, Danvers, MA, USA), FLAG (1:2000, Sigma), SHP1 (1:1000, BD Bioscience, NJ, USA), SHP2 (1:1000, BD Bioscience), PTPD1 (1:1000, Thermo Scientific), and PTP-PEST (1:1000, Cell Signaling Technology). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with secondary antibodies, and the results were visualized using an enhanced chemiluminescence system (Daeil Lab Inc., Seoul, Korea).

Immunoprecipitation assay

WT- and T474E-FAK-encoding constructs were transfected to HEK293T cells. After 2 days, the cells were lysed with an immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 50 mM NaF, 1 mM EDTA, and 1 mM Na_3VO_4). Cell lysates (500 μg) were incubated with primary antibodies against FLAG (1 μg, F1804; Sigma) and then with Protein G agarose beads (20 μl per reaction; Millipore, Billerica, MA, USA). The protein-antibody-bead complexes were treated with 100 μM FLAG peptide (Sigma) in 50 mM Tris-HCl pH 7.4 to release FLAG-FAK proteins from the agarose beads. The antibody-protein G agarose complexes were removed by centrifugation, and equal volumes of supernatants were subjected to Western blotting.

In vitro kinase assay

FLAG-tagged WT and mutant FAK proteins were expressed in HEK293T cells, immunoprecipitated, and isolated from the agarose beads as described above. The released FLAG-FAK proteins were incubated in kinase buffer S (50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 0.01% Brij-35, and 1 mM EGTA; Invitrogen) containing a protease/phosphatase inhibitor cocktail (GenDEPOT) and 10 μM ATP and/or 1 μCi/mL ^{32}P-ATP (Perkin Elmer-Cetus, Norwalk, CT, USA).

Quantification and statistical analysis

The band intensities of the Western blots and Coomassie blue-stained gels were quantified using the Image J software (NIH, Bethesda, MD, USA). The statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test, as applied using the Graph Pad Prism 5 software package (GraphPad Software, San Diego, CA, USA).

RESULTS

Phosphorylation of T474-FAK decreases pY397 levels through FERM-mediated autoinhibition

We previously reported that FAK has six TXR consensus motifs that may be phosphorylated by LRRK2, and that the among phosphorylation-mimicking mutation of Thr (T) to Glu (E) (T→E), only T474 mutation attenuated the levels of pY397 in HEK293T cells [9]. Since the activation of FAK in these cells could be controlled by diverse mechanisms, we used *in vitro* kinase assays to examine the autophosphorylation capacities of FLAG-tagged proteins representing WT FAK (FLAG-FAK) and six (T→E)XR mutants. These proteins, WT and mutant FAKs, were immunoprecipitated from overexpressing HEK293T cells, and phosphorylation levels were compared by autoradiography. Our results showed that T474E-FAK exhibited far less phosphorylation compared with WT-FAK and the other (T→E)XR-mutant FAKs (Fig. 1), suggesting that T474 phosphorylation directly suppresses the endogenous kinase activity of FAK.

Next, we sought to determine how the phosphorylation of T474 suppresses FAK activity. A previous study showed that the FERM domain of FAK physically covers Y397, thereby blocking its autophosphorylation [22]. Conversely, point mutations of V180A/M183A, V196D/L197D, and/or F596D were shown to prevent the

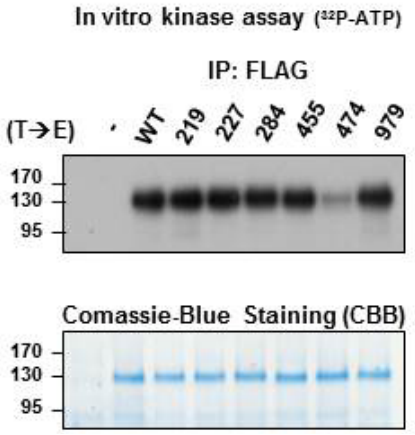

Fig. 1. Phosphorylation of T474-FAK suppresses FAK autophosphorylation. *In vitro* kinase assays were performed using FLAG-tagged WT or mutant proteins of which indicated threonine (T) residue were mutated to glutamate (E), (T→E), and [^{32}P]-ATP. FLAG-WT FAK (FLAG-FAK) or six FLAG-tagged mutants (T219, T227, T284, T455, T474, or T979→E) were overexpressed in HEK293T cells and collected by immunoprecipitation, and ^{32}P-labeled FAK was detected by autoradiography. Coomassie blue staining shows the amounts of proteins in each reaction mixture.

interaction between the FERM and kinase domains and increase Y397 autophosphorylation [23]. Here, we analyzed whether T474 phosphorylation decreased FAK activity through the FERM domain. Interestingly, all three point mutations, V180A/M183A, V196D/L197D, and F596D, rescued Y397 phosphorylation in T474E-FAK-overexpressing HEK293 T cells (Fig. 2). Furthermore, deletion of the FERM domain (ΔFERM) increased the pY397 levels for both WT-FAK and T474E-FAK (Fig. 2). These findings suggest that T474 phosphorylation induces FERM-mediated autoinhibition, thereby decreasing FAK activity.

T474 phosphorylation recruits SHP-2 to decrease FAK activity

FAK activity is known to be regulated by phosphatases that dephosphorylate pY397, such as protein tyrosine phosphatase (PTP)-PEST [24, 25], PTPD1 [26], SHP-1 [27], and SHP-2 [28, 29]. Accordingly, we treated T474E-FAK-overexpressing HEK293T cells with the broad-spectrum tyrosine phosphatase inhibitor, pervanadate [30], and examined whether phosphatases are involved in the T474-phosphorylation-induced decrease in pY397-FAK. Interestingly, we found that the level of pY397-FAK was rescued by pervanadate treatment (Fig. 3A). Previously, we reported that overexpression of G2019S-LRRK2 in HEK293T cells weakly increased pY397-FAK levels in response to ADP compared with overexpression of WT- or D1994A (a kinase dead mutant)-LRRK2 [9]. Here, we found that pervanadate rescued pY397-FAK levels in G2019S-LRRK2-overexpressing cells (Fig. 3B). These results suggest that tyrosine phosphatases contribute to the decreases in pY397 levels induced by T474 phosphorylation

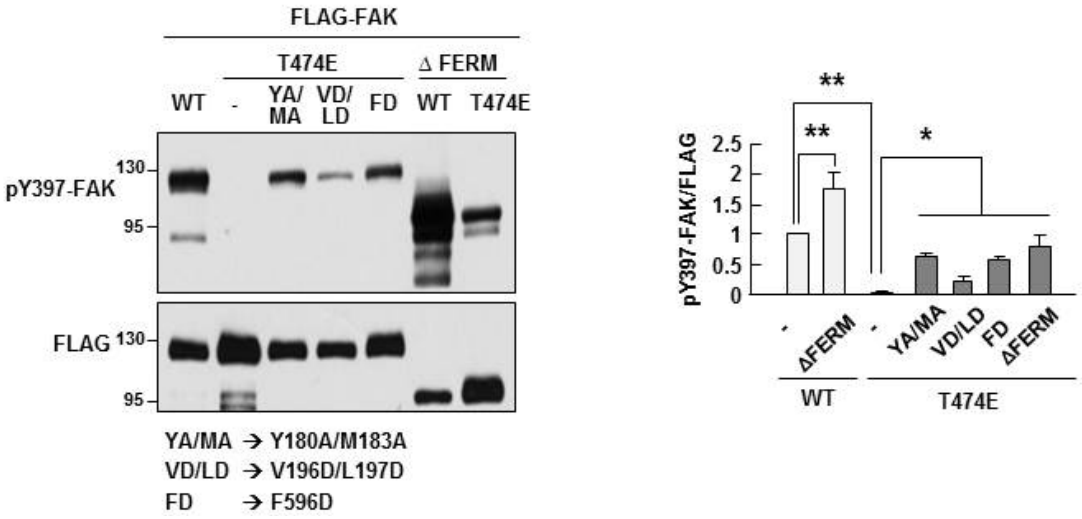

Fig. 2. T474 phosphorylation promotes FERM-mediated autoinhibition of FAK. FLAG-tagged FAK proteins (FLAG-FAK) harboring deletion of the FERM-domain (ΔFERM) or the mutations, Y180A/M183A (YA/MA), V196D/L197D (VD/LD), or F596D (FD) were expressed in HEK293T cells, and the levels of pY397 were assessed by Western blot analysis using pY397-FAK-specific antibodies. The total levels of overexpressed FLAG-FAK were determined using FLAG antibodies (left panel). The band intensities of pY397-FAK were quantified, normalized to that of FLAG, and plotted (right panel). Values shown represent the means+SEM of three separate experiments. *p<0.05; **p<0.01.

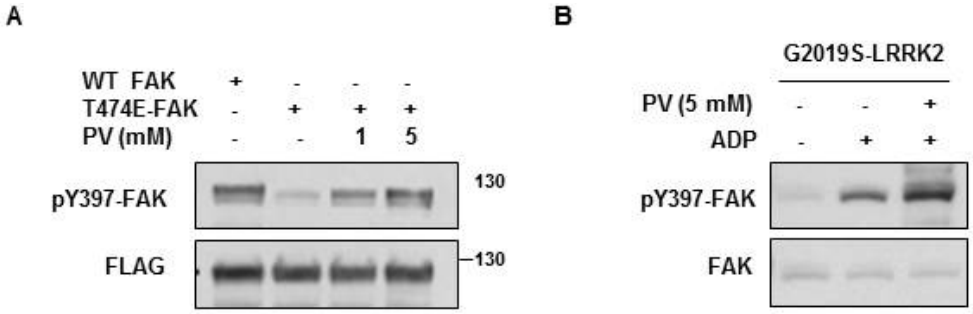

Fig. 3. The phosphatase inhibitor, pervanadate, rescues the level of pY397 in cells overexpressing T474E-FAK or G2019S-LRRK2. (A) T474E-FAK-overexpressing HEK293T cells were treated with indicated amounts of pervanadate (PV) for 1 h. (B) G2019S-LRRK2-overexpressing HEK293T cells were treated with100 μM ADP in the presence of PV. Western blot analysis was carried out using pY397-FAK-specific antibodies. FLAG and FAK were detected as loading controls.

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mimicked by overexpression of T474E-FAK or G2019S-LRRK2.

Next, we examined which tyrosine phosphatase(s) is/are responsible for dephosphorylating pY397-FAK. Our immunoprecipitation experiments revealed that T474E-FAK associated more strongly with SHP-2 than did WT-FAK (Fig. 4A). In contrast, PTPD1 and PTP-PEST both failed to bind WT- or T474E-FAK, while SHP-1 appeared to bind WT- and T474E-FAK with similar intensities (Fig. 4A). Our subsequent experiments revealed that the SHP-1/2 inhibitor, NSC-87877 (NSC), increased the level of pY397 in T474E-FAK-overexpressing HEK293T cells (Fig. 4B).

Taken together, our present findings suggest that the LRRK2-mediated phosphorylation of FAK at T474 suppresses FAK activity by inducing its autoinhibition and/or recruiting SHP-2 to dephosphorylate pY397.

DISCUSSION

FAK, which is known to regulate diverse and fundamental biological phenomena (e.g., cell survival, proliferation, and migration), is highly expressed in the brain, neurons, and glial cells, such as microglia and astrocytes [9, 31, 32]. Previous studies showed that conditional knock-out of FAK in neurons during postnatal brain development increased the number of axon terminals and synapses by increasing branch formation [33], while that in cultured hippocampal neurons triggered defects in spine formation [34]. FAK has been shown to mediate neurotrophin-induced neurite outgrowth in hippocampal neurons, and is known to regulate miniature excitatory postsynaptic currents (mEPSCs), long-term potentiation (LTP), and hippocampus-mediated spatial learning and memory [35]. In addition, FAK regulates the myelination of oligodendrocytes: conditional deletion of FAK in oligodendrocytes decreased the presence of myelinated fibers during development, but myelination became normal after birth, suggesting that FAK is involved in the initiation of myelination [36]. FAK inhibitors reportedly cause defects in microglial motility [9], which is important for the ability of these cells to scan the brain environment [37-40]. The PD-associated pathogenic LRRK2-G2019S mutant may affect neurons and non-neuronal cells in many ways through FAK. We previously showed that G2019S-LRRK2 directly phosphorylates FAK and decreases microglial motility [9], and other groups have reported that G2019S-LRRK2 suppresses neurite outgrowth by regulating ezrin/radixin/moesin (ERM) [6, 41], Rac1 [42], Rab5 [43], and actin-related molecules [7]. FAK functions as a platform for downstream cascades that regulate actin/microtubule polymerization/depolymerization [15]. FAK also mediates the translocation of Rac1 to adhesion sites to promote lamellipodia extension and cell spreading [44], and interacts with actin-related protein complex (Arp2/3) to induce actin nucleation as an initial step for actin polymerization. Therefore, it has been suggested that G2019S-LRRK2 affects the functions and/or properties of neurons or non-neuronal cells by regulating FAK.

The activity of FAK is regulated by its phosphorylation state. In response to stimuli that induce cell motility, FAK undergoes autophosphorylation at Y397, which triggers its Src-

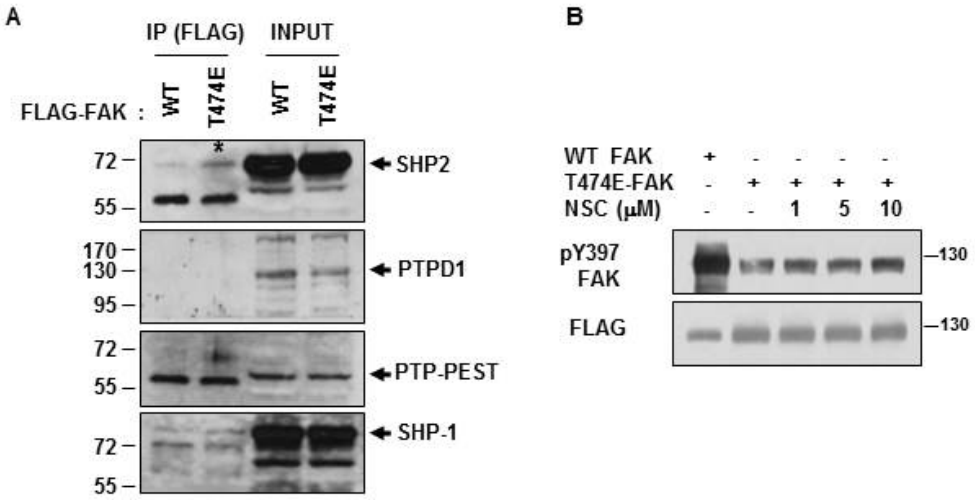

Fig. 4. T474 phosphorylation recruits the FAK phosphatase, SHP2. (A) FLAG-WT-FAK or T474E-FAK were overexpressed and immunoprecipitated using FLAG antibodies. The levels of SHP1, SHP2, PTPD1, and PTP-PEST bound to WT- or T474E-FAK were analyzed using antibodies specific for each protein. (B) Cells transfected with FLAG-WT-FAK or FLAG-T474E-FAK were treated with NSC-87877, an inhibitor of SHP2, and the levels of pY397 were determined by Western blot analysis.

mediated phosphorylation at Y576/577, Y861, Y863, and Y925 [45-48]. Previous studies have shown that: FAK Y576/Y577 phosphorylation is associated with maximum FAK activity [46]; the VEGF-mediated phosphorylation of Y861 promotes the formation of the FAK-integrin αvβ5 complex and facilitates migration in endothelial cells [49, 50]; Y861 phosphorylation enhances FAK Y397 autophosphorylation [45]; Y925 positively regulates lamellipodia formation and cell migration by promoting focal adhesion disassembly [47]; and the Cdk5-mediated phosphorylation of S732 is necessary for neuronal migration [51]. We previously added to this body of knowledge by showing that the LRRK2-mediated phosphorylation of T474 negatively regulates FAK activity by reducing Y397 phosphorylation [9]. Since LRRK2 is a Ser/Thr kinase, we speculated that one or more complicated mechanism(s) could be involved in the LRRK2-mediated inhibition of FAK Y397 phosphorylation. Here, we report for the first time that FERM domain point mutations and deletions previously shown to uncover the kinase domain [23] could rescue Y397 phosphorylation in T474E-FAK-overexpressing cells. Furthermore, the pY397 level of T474E-FAK was significantly reduced by SHP-2. Collectively, these results suggest a new mechanism for the regulation of FAK activation, wherein FAK T474 phosphorylation inhibits the phosphorylation of Y397 through FERM-mediated autoinhibition and/or SHP-2-mediated Y397 dephosphorylation.

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