Identification and Characterization of a Novel Pyk2/Related Adhesion Focal Tyrosine Kinase-associated Protein That Inhibits α-Synuclein Phosphorylation*

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α-Synuclein is a presynaptic protein involved in the pathogenesis of several neurodegenerative diseases, such as Parkinson's disease. Pyk2/related adhesion focal tyrosine kinase (RAFTK) tyrosine kinase is an upstream regulator of Src family kinases in the central nervous system that is involved in α-synuclein phosphorylation. The present study reports the cloning and characterization of a novel adaptor protein, Pyk2/RAFTK-associated protein (PRAP), that specifically binds to Pyk2/RAFTK and inhibits α-synuclein tyrosine phosphorylation. PRAP contains a coiled-coil domain, a pleckstrin homology domain, and a SH3 domain; the SH3 domain binds to the proline-rich domain of Pyk2/RAFTK. PRAP was observed to be present throughout the brain, including substantia nigra dopaminergic neurons, in which it localized to the cytoplasm. PRAP was found to function as a substrate for Src family kinases, such as c-Src or Fyn, but not for Pyk2/RAFTK. Hyperosmotic stress induced phosphorylation of tyrosine 125 of α-synuclein via Pyk2/RAFTK, which acted through Src family kinases. Such phosphorylation was inhibited by PRAP expression, suggesting that PRAP negatively regulates α-synuclein phosphorylation following cell stress. In conclusion, PRAP functions as a downstream target for Pyk2/RAFTK and plays a role in α-synuclein phosphorylation.

Parkinson's disease is a common disabling neurodegenerative disorder that can present as both familial and nonfamilial conditions. Abnormal aggregates of α-synuclein constitute the primary component of Lewy bodies, which represent a hallmark finding of Parkinson's disease (1). α-Synuclein is a soluble neuronal protein that is especially abundant in presynaptic terminals. It has been shown that α-synuclein knockout mice display a functional deficit in the nigrostriatal dopamine system that is involved in α-synuclein phosphorylation. It has been shown that α-synuclein is involved in the modulation of synaptic vesicle release, which subsequently leads to dopaminergic neurotransmission regulation. Pyk2/RAFTK1 is a nonreceptor protein-tyrosine kinase (PTK) that is expressed to a high degree in human brain, specifically in presynaptic terminals (4, 5). Pyk2/RAFTK is activated by either stress signals or a variety of extracellular signals that elevate the intracellular calcium concentration, leading to neurotransmitter release (4, 6, 7). Recently, we have reported that activation of Pyk2/RAFTK tyrosine kinase induces α-synuclein phosphorylation via Src family kinases in response to cell stress (8). Both our group and another group have reported that with in vitro experiments, members of the Src family of PTK, which include c-Src and Fyn, phosphorylate α-synuclein at tyrosine residue 125 (9, 10). Pyk2/RAFTK lies upstream of Src family kinases in the signaling cascade by which cell stress induces tyrosine phosphorylation of α-synuclein (8). Such findings suggest that both extracellular signaling molecules and cell stress not only activate Pyk2/RAFTK but also regulate the phosphorylation state of α-synuclein.

In an attempt to study the mechanisms involved in Pyk2/RAFTK-mediated α-synuclein phosphorylation, the yeast two-hybrid cloning system was used in the present study to identify proteins that interact with Pyk2/RAFTK. This work reports the identification of a novel Pyk2/RAFTK-associated protein (PRAP) that inhibits Pyk2/RAFTK kinase activity. The results of the present work suggest that PRAP is a downstream target for Pyk2/RAFTK that regulates α-synuclein tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System and PRAP cDNA Cloning—The DNA fragment encoding the C-terminal proline-rich domain (amino acids 845–967) of the Pyk2/RAFTK protein was generated with PCR and subcloned into the yeast two-hybrid vector pAS2-1 (Clontech, Palo Alto, CA), which was fused in-frame to the GAL4-binding domain (pAS2-Pyk2/RAFTK-C). The recombinant plasmid was introduced into the yeast strain HF7c; yeast cell expression of the Pyk2/RAFTK-C terminus fusion protein was confirmed by immunoblotting with anti-GAL4BD antibodies (Clontech). A human brain cDNA library contained in the yeast two-hybrid vector pACT2 was obtained (Clontech). The library DNA was introduced into the yeast strain expressing the Pyk2/RAFTK

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¶ This abbreviation used is: RAFTK, related adhesion focal tyrosine kinase; PTK, protein-tyrosine kinase; PRAP, Pyk2/RAFTK-associated protein; CC, coiled-coil; PH, pleckstrin homology; SH3, Src homology 3; GST, glutathione S-transferase; SUMO, small ubiquitin-related modifier; HA, hemagglutinin.

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C terminus fusion protein, and transformants were selected by culture on SD-/Trp- His- Leu plates. Positive clones were detected by a β-galactosidase assay. To eliminate false positives, plasmid DNA from positive clones was purified, amplified, and retransformed into the yeast strain expressing the Pyk2/RAFTK C terminus fusion protein. The positive clones in this second screening were subjected to DNA sequencing.

To obtain the full-length cDNA of the positive clone of interest, a library screening was performed as previously described (9, 11). A specific probe was generated by restriction digestion of the positive clone insert with EcoRI and PstI. The DNA fragment was gel-purified and radiolabeled with [α-32P]dCTP (PerkinElmer Life Sciences) using a random primer labeling kit (Stratagene, La Jolla, CA). With this probe, the mouse Pyk2/RAFTK cDNA library contained in the bacteriophage λZAPII (Stratagene) was screened via filter hybridization. Positive clones were isolated, plaque-purified, excised, subcloned into pBluescript SK (Stratagene), and sequenced for both complementary strands.

cDNA was obtained for the novel Pyk2/RAFTK target protein, PRAP. The coding region of PRAP cDNA was amplified by PCR using a Pfu DNA polymerase (Stratagene) and the following forward and reverse primers with the attached restriction enzyme sites BamHI and XhoI, respectively: 5′-CGC GGA TCT ATG CCC AAC CCC AGC AGC AC-3′ (F1) and 5′-CGC GGC TCG ATG CAA ATA ATC TAC ATC TCC-3′ (R1). The DNA fragment obtained from PCR (1080 bp) was subcloned into the pcDNA3 vector (Invitrogen) together with the HA tag sequence (pcDNA3-HA-PRAP).

**Vectors and Antibodies**—Human cDNA encoding wild type and mutant (A53T, Y39F, Y125F, Y133F, and Y136F) α-synuclein were generated and subcloned into pcDNA3 vectors together with the Myc tag sequence (pcDNA3-Myc-α-synuclein) as previously described (8). Human cDNA encoding wild type and mutant Pyk2/RAFTK immunoprecipitated with anti-FLAG antibodies and precipitated with GST fusion proteins, the bound proteins were immunoblotted with anti-Pyk2/RAFTK antibodies.

**Immunohistochemical Analysis**—Immunohistochemical analysis was performed as previously described (42). Sections were sequentially processed as follows: 1) the sections were washed with ice-cold phosphate-buffered saline and lyzed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After the lysis protein content were normalized using a protein assay kit (Bio-Rad), the cell lysis (500 μg/sample) was immunoprecipitated with normal rabbit IgG, anti-Pyk2/RAFTK, anti-FLAG, anti-HA, or anti-c-Src antibodies using protein G-Sepharose beads (Pierce). Each immunoprecipitate was divided into two parts, separated on SDS-PAGE, and transferred onto nitrocellulose membranes (Schleicher & Schuell). Bound proteins were immunoblotted with anti-Tyr(P), as well as the appropriately indicated antibodies. The blots were developed using enhanced chemiluminescence reagents (PerkinElmer Life Sciences). The degree of α-synuclein phosphorylation was quantitated by densitometric analysis of nonsaturated radiographs with NIH Image software. The data were calculated from four independent experiments. Statistic analysis was performed by analysis of variance.

**Confluent Immunofluorescent Staining**—Immunofluorescence was performed as previously described (9). Briefly, COS7 cells transfected with expression vectors for FLAG-Pyk2/RAFTK were lysed and precipitated with c-Src antibodies and anti-Pyk2/RAFTK antibodies, and then the precipitated proteins were immunoblotted with anti-FLAG antibodies. The immunoblotted proteins were immunoprecipitated with anti-FLAG antibodies, and then the precipitated proteins were immunoassayed with anti-α-synuclein antibodies. The blots were developed as described.

**Northern Blot Analysis**—Blots containing poly(A)+ RNA from brain tissues were purchased (Clontech). A generic-specific probe for PRAP or α-synuclein was generated. The spanning regions were as follows: for α-synuclein, nucleotides 1–420 (420 bp in length); and for PRAP, nucleotides 345–906 (562 bp in length). These probes were radiolabeled with [α-32P]dCTP (PerkinElmer Life Sciences). The reactions were terminated by adding 1 μl of 50% deoxycytoine. The blots were washed with neutral buffered 4% (w/v) paraformaldehyde and permeabilized with 0.2% Triton X-100. Double immunofluorescent staining was performed by adding rabbit anti-HA antibodies, followed by anti-rabbit IgG-Cy3. Pyk2/RAFTK was visualized with anti-FLAG antibodies, followed by anti-mouse IgG-fluorescein isothiocyanate. Immunostained preparations were examined with a Zeiss LSM510 confocal laser scanning microscope corresponding to the circulus-RAFTK fusion.
PRAP Inhibition of α-Synuclein Phosphorylation

RESULTS

Identification of a Pyk2/RAFTK-binding Protein with a Yeast Two-hybrid System—During investigation of the α-synuclein kinase-related activity of Pyk2/RAFTK, Pyk2/RAFTK-interacting proteins were sought using a yeast two-hybrid system. A human brain cDNA library was screened using the C-terminal proline-rich domain (amino acids 845–967) of Pyk2/RAFTK as a bait, which allowed isolation of three independent clones (Fig. 1, A and B). Two of these clones contained fragments nearly identical to the Cas (Crk-associated substrate) gene, which has already been reported to associate with Pyk2/RAFTK (15). The third clone contained a sequence partially encoding for a novel protein possessing an SH3 domain. This clone was used to probe a human hippocampus cDNA library, which yielded four clones. The longest cDNA (~2.4 kilobase pairs, not including the poly(A) stretch) encoded a protein consisting of 359 amino acids with an estimated molecular mass of ~41 kDa. This protein was designated PRAP. PRAP primary structure analysis was conducted by comparing the predicted amino acid sequence with all available data bases. Such a search demonstrated that PRAP contains a coiled-coil domain (amino acids 75–202), a PH domain (amino acids 115–214), and a C-terminal SH3 domain (amino acids 303–356) (Fig. 1, B and C). Analysis in the yeast two-hybrid system suggested that PRAP could interact with the C terminus of Pyk2/RAFTK (Pyk2/RAFTK-C). The SH3 domain of PRAP (PRAP-SH3) alone also interacted with Pyk2/RAFTK-C (Fig. 1, A and B). Nonetheless, PRAP could not interact with the N terminus of Pyk2/RAFTK (Pyk2/RAFTK-N; amino acids 1–202) (Fig. 1, A and B). Pyk2/RAFTK-C could associate with the SH3 domain (PRAP-SH3) but not the NH domain (PRAP-NH) of PRAP.

Despite the fact that PRAP had a predicted molecular mass of ~41 kDa when it was expressed in E. coli, PRAP was collected as a ~55-kDa mass of protein in COS7 cells transfected with expression vectors for PRAP (Fig. 2A). To evaluate for potential post-translational modification, such as ubiquitination or SUMOylation, COS7 cells transfected with expression vectors for PRAP fused at its N terminus with the HA tag (HA-PRAP) were grown in the presence of lactacystin or iodoacetamide, lysed, and immunoprecipitated with anti-HA antibodies. Nonetheless, immunoblotting with anti-HA antibodies.
anti-SUMO antibodies demonstrated that PRAP lacked ubiquitin or SUMO conjugation (data not shown).

**PRAP and Pyk2/RAFTK Interaction in Mammalian Cells—**
PRAP binding with Pyk2/RAFTK was confirmed using an immunoprecipitation assay. To examine the interaction of PRAP with Pyk2/RAFTK in mammalian cells, Pyk2/RAFTK fused at the N terminus with the FLAG tag (FLAG-Pyk2/RAFTK) was expressed together with HA-PRAP by transient cotransfection of COS7 cells. The immunoprecipitates from the lysate of these cells were analyzed by immunoblotting. When both FLAG-Pyk2/RAFTK and HA-PRAP were expressed in cells, an anti-FLAG antibody (data not shown) and an anti-Pyk2/RAFTK antibody immunoprecipitated HA-PRAP (Fig. 2A). In addition, an anti-HA antibody immunoprecipitated FLAG-Pyk2/RAFTK in the same manner (data not shown). It was concluded that PRAP and Pyk2/RAFTK proteins interact with each other in mammalian cells.

Analysis in the yeast two-hybrid system suggested that the SH3 domain of PRAP could interact with the C terminus of Pyk2/RAFTK (Fig. 1A). Potential involvement of other PRAP domains in the interaction with Pyk2/RAFTK was then assessed. COS7 cells transfected with an expression vector for FLAG-Pyk2/RAFTK were lysed and precipitated with various GST fusion PRAP proteins, such as GST-PRAP, GST-CC-PH, GST-PH, GST-PH-SH3, and GST-SH3, as well as the GST protein alone as a control. Anti-Pyk2/RAFTK immunoblotting revealed that GST fusion proteins containing the PRAP SH3 domain exclusively precipitated Pyk2/RAFTK (Fig. 2B). The results suggest that PRAP binds to Pyk2/RAFTK with its SH3 domain.

**Potential interaction between α-synuclein and PRAP was also assessed with both immunoprecipitation and precipitation assays with GST fusion PRAP proteins. Nonetheless, interaction between α-synuclein and PRAP was not detected by either assay (data not shown).**

**PRAP Localization to the Perinuclear Cytoplasm—**
PRAP localization was determined by a double staining immunofluorescence study. Confocal microscopic analysis revealed that both PRAP (Fig. 3A) and Pyk2/RAFTK (Fig. 3B) were present in the cytoplasm. In contrast to PRAP, Pyk2/RAFTK was relatively sparse around the perinuclear region. An overlay of PRAP and Pyk2/RAFTK staining revealed colocalization of the proteins (Fig. 3C).

**PRAP Expression in the Brain—**Northern blot analysis was performed to examine the mRNA expression levels of PRAP in the brain. PRAP mRNA was expressed in all regions in the

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**Fig. 2.** Pyk2/RAFTK and PRAP interaction. A, Pyk2/RAFTK and PRAP coimmunoprecipitation. Plasmids pcDNA3-FLAG and pcDNA3-HA-PRAP (FLAG vector + HA-PRAP) or pcDNA3-FLAG-Pyk2/RAFTK and pcDNA3-HA-PRAP (FLAG-Pyk2/RAFTK + HA-PRAP) were cotransfected into cells, and cell lysates were immunoprecipitated (IP) using anti-Pyk2/RAFTK antibodies and normal rabbit IgG as the control antibody. Each sample was subjected to 15% SDS-PAGE and Western blot analysis (WB). Part of each precleared lysate obtained before immunoprecipitation was also loaded to show equivalence of HA-PRAP in the lysates. HA-PRAP was detected using anti-HA antibodies. Bottom, specific FLAG-Pyk2/RAFTK immunoprecipitation with anti-Pyk2/RAFTK antibodies was confirmed by re-probing the membrane with anti-FLAG antibodies. B, the PRAP SH3 domain was found to specifically bind to Pyk2/RAFTK. COS7 cells transfected with pcDNA3-FLAG-Pyk2/RAFTK were lysed and precipitated (PT) with GST fusion proteins (10 μg) containing full-length PRAP (GST-PRAP), the coiled-coil and PH domains (GST-CC-PH), the PH domain (GST-PH), the PH and SH3 domains (GST-PH-SH3), or the SH3 domain (GST-SH3), with GST alone as a control. Top panel, the amount of GST fusion proteins used for the precipitation assay was detected by Coomassie staining. Middle panel, bound proteins with various GST fusion proteins were separated by 15% SDS-PAGE and immunoblotted with anti-Pyk2/RAFTK antibodies. Bottom panel, a portion of the cell lysate used for the precipitation assay was loaded to demonstrate equivalence of FLAG-Pyk2/RAFTK in the lysates. The molecular mass markers are indicated on the left (kDa).
c-Src was not strongly phosphorylated, the autophosphorylation site of Pyk2/RAFTK, Tyr 402, recruits the tyrosine phosphorylation of PRAP was increased with expression of wild type Pyk2/RAFTK (Fig. 5A). In cells expressing Y402F Pyk2/RAFTK, c-Src was not strongly phosphorylated, as had been previously described (8). The Y402F Pyk2/RAFTK mutant was not able to increase PRAP tyrosine phosphorylation over base-line phosphorylation of untransfected cell lysate was divided into three aliquots and immunoprecipitated with anti-FLAG, anti-c-Src, and anti-HA antibodies. After the lysate protein contents were normalized, the cell fractionation was performed. Immunoblotting with anti-Tyr(P) antibodies revealed that mutant Pyk2/RAFTK also bound to PRAP (data not shown). Such a result suggests that these mutations might not affect the Pyk2/RAFTK binding capacity to PRAP.

To ascertain PRAP distribution in the brain, immunohistochemical studies were performed. Similar to the expression of PRAP mRNA, luche images of sagittal sections of mice brains demonstrated that PRAP was present in all regions throughout the brain and was relatively abundant in the Purkinje cell layer (Fig. 4B), hippocampal gyrus, and cortex (Fig. 4C). Sagittal sections near the isthmus demonstrated PRAP immunoreactivity in the mesencephalic nucleus (Fig. 4D), which was defined as the region caudal to the medial lemniscus and ventrolateral to the red nucleus. With serial sectioning, the same population of neurons was also calbindin-immunoreactive (Fig. 4E), indicating that such neurons rich with PRAP immunoreactivity were dopaminergic neurons, a finding that had been previously described (16). The specificity of PRAP immunoreactivity was confirmed by demonstrating that the reactivity was eliminated by preadsorption of the antiserum with immunizing His6-PRAP at a concentration of 10 μg for 2 h (Fig. 4F). Higher magnification examination of the mesencephalic nucleus revealed the presence of PRAP in perikarya and dendrites (Fig. 4, G and H).

PRAP was found to be a substrate for Src family kinases but not for Pyk2/RAFTK. It has been suggested that one autophosphorylation site of Pyk2/RAFTK, Tyr 402, recruits c-Src (17). The binding of c-Src to Tyr 402 on Pyk2/RAFTK leads to enhancement of c-Src kinase activity. Because Pyk2/RAFTK associates with PRAP, the present study investigated whether or not PRAP is a substrate for Pyk2/RAFTK. COS7 cells were cotransfected with expression vectors for HA-PRAP, FLAG-tagged wild type Pyk2/RAFTK, the Src-binding site mutant Pyk2/RAFTK (Y402F), and the kinase inactive Pyk2/RAFTK (K457R). Immunoprecipitation assay revealed that mutant Pyk2/RAFTK also bound to PRAP (data not shown). Such a result suggests that these mutations might not affect the Pyk2/RAFTK binding capacity to PRAP. After the lysate protein contents were normalized, the cell lysate was divided into three aliquots and immunoprecipitated with anti-FLAG, anti-c-Src, and anti-HA antibodies. Immunoblotting with anti-Tyr(P) antibodies revealed that tyrosine phosphorylation of PRAP was increased with expression of wild type Pyk2/RAFTK (Fig. 5A). In cells expressing Y402F Pyk2/RAFTK, c-Src was not strongly phosphorylated, as had been previously described (8). The Y402F Pyk2/RAFTK mutant was not able to increase PRAP tyrosine phosphorylation over base-line phosphorylation of untransfected...
cells, despite being able to associate with PRAP. Furthermore, phosphorylation of PRAP was still detected with expression of the kinase-inactive Y402F Pyk2/RAFTK. The phosphorylation level of PRAP with K457R Pyk2/RAFTK was similar to that with Y402F Pyk2/RAFTK, suggesting that Pyk2/RAFTK does not directly phosphorylate PRAP (Fig. 5B).

To further investigate in vitro PRAP phosphorylation, an immunocomplex kinase assay was performed. FLAG-tagged c-Src, Fyn, and Pyk2/RAFTK were expressed in COS7 cells. Such PTK were immunoprecipitated with an anti-FLAG antibody and subjected to an immunocomplex kinase assay using a purified GST-PRAP protein as a substrate. Pyk2/RAFTK did not phosphorylate GST-PRAP, but c-Src phosphorylated GST-PRAP (Fig. 5C). Such results suggest that PRAP is a substrate of c-Src and Fyn but not Pyk2/RAFTK.

**PRAP Inhibits α-Synuclein Phosphorylation**

To further investigate in vitro PRAP phosphorylation, an immunocomplex kinase assay was performed. FLAG-tagged c-Src, Fyn, and Pyk2/RAFTK were expressed in COS7 cells. Such PTK were immunoprecipitated with an anti-FLAG antibody and subjected to an immunocomplex kinase assay using a purified GST-PRAP protein as a substrate. Pyk2/RAFTK did not phosphorylate GST-PRAP, but c-Src phosphorylated GST-PRAP (Fig. 5C). Such results suggest that PRAP is a substrate of c-Src and Fyn but not Pyk2/RAFTK. As a control, the same assay was performed using GST alone as a substrate, which led to no GST phosphorylation (data not shown).

**PRAP Inhibits Pyk2/RAFTK-mediated α-Synuclein Tyrosine Phosphorylation**—Pyk2/RAFTK is involved in α-synuclein phosphorylation in response to cell stress (8). The present study investigated whether or not hyperosmotic stress induced α-synuclein phosphorylation. COS7 cells expressing FLAG-Pyk2/RAFTK and Myc-α-synuclein were starved for 18 h and subsequently subjected to hyperosmotic stress by treatment with 300 mM d-sorbitol for 10 min. Immunoblotting with anti-Tyr(P) antibodies revealed that osmotic stress induced α-synuclein tyrosine phosphorylation in both untransfected and transfected cells (Fig. 6A). In untransfected COS7 cells, the α-synuclein tyrosine phosphorylation level was low after sorbitol treatment, despite endogenous expression of c-Src in COS7 cells (Fig. 6A). Pyk2/RAFTK expression significantly \((p < 0.05; \text{analysis of variance})\) increased the α-synuclein phosphorylation level from 2.3-fold to 8.4-fold above baseline (\(n = 4\)) (Fig. 6C). It should also be noted that it was found that COS7 cells do not endogenously express Pyk2/RAFTK (data not shown). It also deserves mention that c-Src and Pyk2/RAFTK both were found to be phosphorylated and activated in response to osmotic stress. Pyk2/RAFTK expression markedly increased the c-Src phosphorylation level from 3.8-fold to 14.8-fold (\(n = 4\)) (Fig. 6B). Such results suggest that both c-Src and its upstream regulator Pyk2/RAFTK are involved in osmotic stress-dependent α-synuclein phosphorylation. An additional experiment was performed to determine whether or not PRAP could affect the α-synuclein phosphorylation state under conditions of osmotic stress. PRAP coexpression markedly reduced Pyk2/RAFTK-mediated α-synuclein phosphorylation following osmotic stress (Fig. 6, A and C). PRAP coexpression also reduced Pyk2/RAFTK-mediated c-Src activation following osmotic stress (Fig. 6, A and B). Such results indicate that PRAP functions as a downstream target for Pyk2/RAFTK, inhibiting Pyk2/RAFTK-mediated α-synuclein tyrosine phosphorylation. In addition, the Pyk2/
RAFTK-mediated \( \alpha \)-synuclein phosphorylation site was determined. \( \alpha \)-Synuclein contains four tyrosine residues at amino acids 39, 125, 133, and 136. Expression vectors for Myc-\( \alpha \)-synuclein with tyrosine-phenylalanine single substitutions (Y39F, Y125F, Y133F, and Y136F), as well as the A53T mutation, which has been linked to familial Parkinson’s disease (1), were prepared. COS7 cells expressing FLAG-Pyk2/RAFTK and various mutant Myc-\( \alpha \)-synuclein expression vectors were starved and subsequently subjected to hyperosmotic stress.

Immunoblotting with anti-\( \alpha \)-synuclein antibodies revealed that hyperosmotic stress induced phosphorylation of both wild type and A53T \( \alpha \)-synuclein, suggesting that the A53T mutation did not affect Pyk2/RAFTK-mediated \( \alpha \)-synuclein phosphorylation (Fig. 6D). Osmotic stress induced phosphorylation of Y39F,
Y133F, and Y136F but not Y125F, suggesting that tyrosine 125 was the Pyk2/RAFTK-mediated α-synuclein phosphorylation site in response to osmotic stress (Fig. 6D).

**DISCUSSION**

α-Synuclein is a presynaptic protein that is involved in the pathogenesis of several neurodegenerative diseases, including Parkinson’s disease, Alzheimer’s disease, and Lewy body dementia. The present study has identified and characterized a novel protein PRAP that inhibits α-synuclein phosphorylation. PRAP was found to contain a coiled-coil domain, a PH domain, and a SH3 domain. The primary structure of PRAP appears to be that of an adaptor protein. PRAP and Pyk2/RAFTK proteins interact with each other in mammalian cells, i.e. PRAP was found to bind to the proline-rich domain of Pyk2/RAFTK via its SH3 domain. In contrast, PRAP does not appear to bind to α-synuclein. Recently, novel Pyk2/RAFTK target proteins, namely Nir1, Nir2, and Nir3 (18) as well as PAP (19, 20) and PSGAP (21) have been identified. Both PAP and PSGAP exhibit a similar domain structure, with a centrally located PH domain flanked by an SH3 domain at the C terminus and expression of GAP activity for ADP-riboseylation factors and the Rho family of small GTPases (19, 20, 21). Similar to such proteins, the SH3 domain of PRAP provides a binding site for the proline-rich domain of the Pyk2/RAFTK C terminus. Although PRAP had been previously reported as SKAP55R (22), SKAP-HOM (23), and RA70 (24), the physiological function of this adaptor protein had yet to be elucidated.

Although PRAP had a predicted molecular mass of ~41 kDa based on measurements made with expression in *E. coli*, PRAP was collected as a ~55-kDa mass of protein in COS7 cells transfected with expression vectors for PRAP. The difference in molecular size suggests possible post-translational modification. PRAP has two potential acceptor motifs (FYKXE) for SUMOylation (25) at lysines 33 and 275 and three possible PEST sequences, which represent potential signal sites for SUMOylation (26–28). Nonetheless, immunoblotting revealed an absence of SUMO or ubiquitin conjugation with PRAP. Accordingly, the mobility shift of the PRAP protein might be due to modification other than SUMOylation or ubiquitination.

Confocal microscopic analysis revealed *in vivo* cytoplasmic colocalization of PRAP and Pyk2/RAFTK, consistent with observed *in vitro* interactions. Furthermore, immunohistochemical analysis revealed that PRAP was distributed throughout the central nervous system, including substantia nigra dopaminergic neurons, and localized to the perikarya and dendrites. Such an expression pattern is similar to that observed for Pyk2/RAFTK (4, 5, 29). In contrast, immunohistochemical studies of the central nervous system demonstrated a more localized distribution of α-synuclein (30).

Autophosphorylation site Tyr102 of Pyk2/RAFTK recruits c-Src to allow substrate phosphorylation (17). PRAP phosphorylation was increased with wild type Pyk2/RAFTK expression. PRAP phosphorylation was significantly reduced with Y402F Pyk2/RAFTK expression, despite its known positive kinase activity. Furthermore, PRAP phosphorylation remained detectable with expression of the kinase-inactive K457R Pyk2/RAFTK. The PRAP phosphorylation level with K457R Pyk2/RAFTK was similar to that seen with Y402F Pyk2/RAFTK, suggesting that Pyk2/RAFTK does not directly phosphorylate PRAP. It is also possible that the observed phosphorylation activity results from residual wild type RAFTK present in the cells or PRAP phosphorylation by c-Src without Pyk2/RAFTK activation. In addition, an immunocomplex kinase assay revealed that Pyk2/RAFTK did not phosphorylate PRAP; however, c-Src was able to phosphorylate PRAP. Such results suggest that PRAP is a substrate for Src family kinases, such as c-Src and Fyn, but not for Pyk2/RAFTK. Nonetheless, it appears that Pyk2/RAFTK recruits Src family kinases to phosphorylate PRAP.

Recently both our group and another group have demonstrated that Src family kinases (9, 10) and their upstream regulator Pyk2/RAFTK (8) act as a α-synuclein kinase that phosphorylates α-synuclein following induction of cell stress (8). To investigate the involvement of PRAP in cell stress-dependent α-synuclein phosphorylation, the present study induced hyperosmotic stress by treating cells with 300 mm D-sorbitol. Both Pyk2/RAFTK and c-Src were phosphorylated and activated in response to osmotic stress. In addition, Pyk2/RAFTK expression markedly enhanced c-Src tyrosine phosphorylation, consistent with previous reports that Pyk2/RAFTK lies upstream of c-Src in the signaling cascade by which stress signals, such as UV light, tumor necrosis factor-α, and osmotic stress, induce c-Src and JNK/SAPK activation (8, 28, 31). The fact that osmotic stress induced α-synuclein tyrosine phosphorylation coupled with the fact that Pyk2/RAFTK expression markedly enhanced α-synuclein phosphorylation suggests that Pyk2/RAFTK is involved in α-synuclein phosphorylation in response to osmotic stress. The Pyk2/RAFTK-mediated phosphorylation site for α-synuclein in response to osmotic stress was tyrosine 125, one of four tyrosine residues. It is also of note that PRAP coexpression markedly reduced Pyk2/RAFTK-mediated α-synuclein tyrosine phosphorylation. Such results suggest that Pyk2/RAFTK-mediated α-synuclein phosphorylation was primarily carried out by Src family kinases. Moreover, it would appear that PRAP negatively regulates this process, resulting in decreased α-synuclein phosphorylation. To summarize, PRAP appears to function as a downstream target for Pyk2/RAFTK that is involved in α-synuclein phosphorylation.

The meaning of cell stress-dependent phosphorylation of tyrosine 125 is not fully understood. Nonetheless, recent studies revealed that α-synuclein tyrosine nitration following oxidative stress facilitates oligomer formation in both *in vitro* and *in vivo* experimentation (32–34). Increasing evidence suggests that extensive and widespread accumulation of nitrated-tyrosine α-synuclein following oxidative injury is involved in inclusion body formation in Parkinson’s disease (35). Recently, we demonstrated that tyrosine 125 on α-synuclein plays a critical role for oligomer formation following nitric stress (13). α-Synuclein oligomerization facilitated by nitration of tyrosine 125 is affected by phosphorylation of this residue with Src family kinases and Pyk2/RAFTK activation. Accordingly, PRAP regulation of Pyk2/RAFTK activity plays an important role for not only phosphorylation but also α-synuclein nitration and accumulation following oxidative stress. The present study suggests that inhibition of Pyk2/RAFTK activity by PRAP might increase α-synuclein oligomer formation, a process considered to be crucial for the early stages of Lewy body formation.

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