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Activation of Pyk2/RAFTK induces tyrosine phosphorylation of α-synuclein via Src-family kinases

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Abstract **α-Synuclein (αS) is a neuronal protein that has been implicated in the pathogenesis of Parkinson's disease. The present report demonstrates that the protein tyrosine kinase Pyk2/RAFTK is involved in cell stress-induced tyrosine phosphorylation of αS. Hyperosmotic stress induced tyrosine phosphorylation of αS via Pyk2/RAFTK at tyrosine residue 125. Pyk2/RAFTK-mediated phosphorylation of αS was primarily achieved with Src-family kinases. In addition, osmotic stress-induced phosphorylation of αS was dependent on Pyk2/RAFTK activation. Accordingly, such results indicate that Pyk2/RAFTK lies upstream of Src-family kinases in the signaling cascade by which osmotic stress induces tyrosine phosphorylation of αS.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: α-Synuclein; Tyrosine phosphorylation; Pyk2/RAFTK; Osmotic stress; Parkinson's disease

1. Introduction

α-Synuclein (αS) is a 140-residue soluble neuronal protein of unknown function that has been implicated in the pathogenesis of Parkinson's disease. Abnormal aggregates of αS are the main component of Lewy bodies, which represent a hallmark finding of Parkinson's disease [1]. Molecular genetic studies have identified two different point mutations, A30P and A53T, in the αS gene that causes familial Parkinson's disease [2,3]. In neuronal cells, αS is especially abundant in presynaptic terminals and is well known to be phosphorylated by several kinases [4–8]. Recently, another group and our own group have reported that αS is phosphorylated by members of the Src-family of protein tyrosine kinases (PTKs) [7,8]. Nonetheless, the mechanisms controlling phosphorylation of αS by Src-family kinases remain poorly understood.

Pyk2/RAFTK is a member of the focal adhesion kinase (FAK) family of PTKs, alternatively known as CAKβ, FAK-2, or CADTK [9]. Pyk2/RAFTK can be activated not only by a variety of extracellular signals that elevate the intracellular Ca^{2+} concentration, which results in protein kinase C activation, but also by stress signals [9]. In the central nervous system, Pyk2/RAFTK is present in abundance in synapses and appears to correlate with long-term potentiation [10]. Activation of Pyk2/RAFTK leads to modulation of ion channel function and activation of a mitogen-activated protein kinase (MAPK) signaling pathway [11]. Consequently, the action of Pyk2/RAFTK remains crucial for not only the control of differentiation and survival of neuronal cells, but also the regulation of neuronal excitability, plasticity, and memory [9]. In addition, Pyk2/RAFTK interacts with several proteins involved in integrin signaling, including paxillin and c-Src. The Src-binding site, tyrosine 402, represents an autophosphorylation site for Pyk2/RAFTK [9]. Following autophosphorylation, c-Src is recruited and the complex is able to phosphorylate several proteins. For example, in PC12 cells, c-Src binds to tyrosine 402 of Pyk2/RAFTK following stimulation with bradykinin, lysophosphatidic acid, and depolarization [12].

The present study demonstrates that αS is phosphorylated at a tyrosine residue via activation of Pyk2/RAFTK in response to cell stress. Moreover, it was also demonstrated that cooperation between Pyk2/RAFTK and Src-family kinases is required for αS phosphorylation.

2. Materials and methods

2.1. Antibodies, cells, and expression vectors

Anti-hemagglutinin (HA) and anti-c-Src antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine monoclonal antibody (P-Tyr) was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Pyk2/RAFTK and anti-αS antibodies were purchased from BD Transduction Laboratories (Lexington, KY, USA). M2 monoclonal antibody against the FLAG epitope was purchased from Sigma (St. Louis, MO, USA). COS7 cells and full-length αS cDNA were obtained from the American Tissue Culture Collection. cDNAs encoding wild-type and mutant (A30P, A53T, Y39F, Y125F, Y133F, and Y136F) αS were generated and subcloned into pcDNA3-HA vectors as previously described [6,7]. The c-Src expression vector was purchased from Upstate Biotechnology (Lake Placid, NY, USA). cDNAs encoding wild-type and mutant (Y402F and K457R) Pyk2/RAFTK were generated and subcloned into pcDNA3-FLAG vectors as previously described [13].

2.2. Expression of cDNA constructs in COS7 cells; cell stimulation with osmotic stress

COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. Trans-

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fection of expression vectors into cells was performed by FuGENE 6 (Roche, Indianapolis, IL, USA) according to the manufacturer's protocol. Cells were subjected to an assay 24 h post-transfection. Prior to stimulation, cells were starved for 18 h in DMEM containing 0.1% fetal bovine serum. Cells were then stimulated with osmotic stress by exposure to 300 mM D-sorbitol (Sigma) in culture medium at 37°C for indicated time periods. Ethylene glycol-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (BAPTA-AM) were obtained from Sigma. PP2 was obtained from Calbiochem (San Diego, CA, USA).

2.3. Immunoprecipitation and immunoblotting

After sorbitol treatment, cells were lysed with lysis buffer (50 mM Tris–HCl (pH 7.4), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 25 mM NaF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After the lysate protein contents were normalized using a protein assay kit (Bio-Rad, Hercules, CA, USA), the cell lysate (500 μg/sample) was immunoprecipitated with anti-FLAG or anti-HA antibodies using protein G-Sepharose beads (Pierce, Rockford, IL, USA). Each immunoprecipitate was divided into two parts, separated on SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany), and subjected to immunoblotting with anti-Pyk2/RAFTK, anti-αS, or anti-P-Tyr antibodies. The blots were developed using the ECL system (NEN, Boston, MA, USA). The degree of αS phosphorylation was quantitated by densitometric analysis of non-saturated radiographs with NIH Image software.

3. Results and discussion

3.1. Pyk2/RAFTK and c-Src are involved in osmotic stress-induced phosphorylation of αS

Other groups and our own group have reported that Src-family kinases phosphorylate αS [7,8]. Since Pyk2/RAFTK is highly expressed in the human brain, and is involved in various signaling pathways, to include cell stress signals in neuronal cells, the present study investigated whether or not hyperosmotic stress induced phosphorylation of αS. COS7 cells were transfected with HA-tagged expression vectors for αS and FLAG-tagged expression vectors for either PTK (c-Src or FAK) or Pyk2/RAFTK. After 24 h, the cells were starved for 18 h, and then subjected to hyperosmotic stress by treatment with 300 mM D-sorbitol for 10 min. Immunoblotting with anti-P-Tyr antibodies revealed that osmotic stress induced tyrosine phosphorylation of αS in both untransfected and transfected cells (Fig. 1A). In untransfected COS7 cells, the tyrosine phosphorylation level of αS was low after sorbitol treatment, despite endogenous expression of c-Src in COS7 cells (Fig. 3). Expression of c-Src or Pyk2/RAFTK increased the phosphorylation level of αS from 1.8 to 15.0 or 14.3 ($n = 4$), respectively; expression of FAK, however, had no effect on the tyrosine phosphorylation level of αS (Fig. 1A).

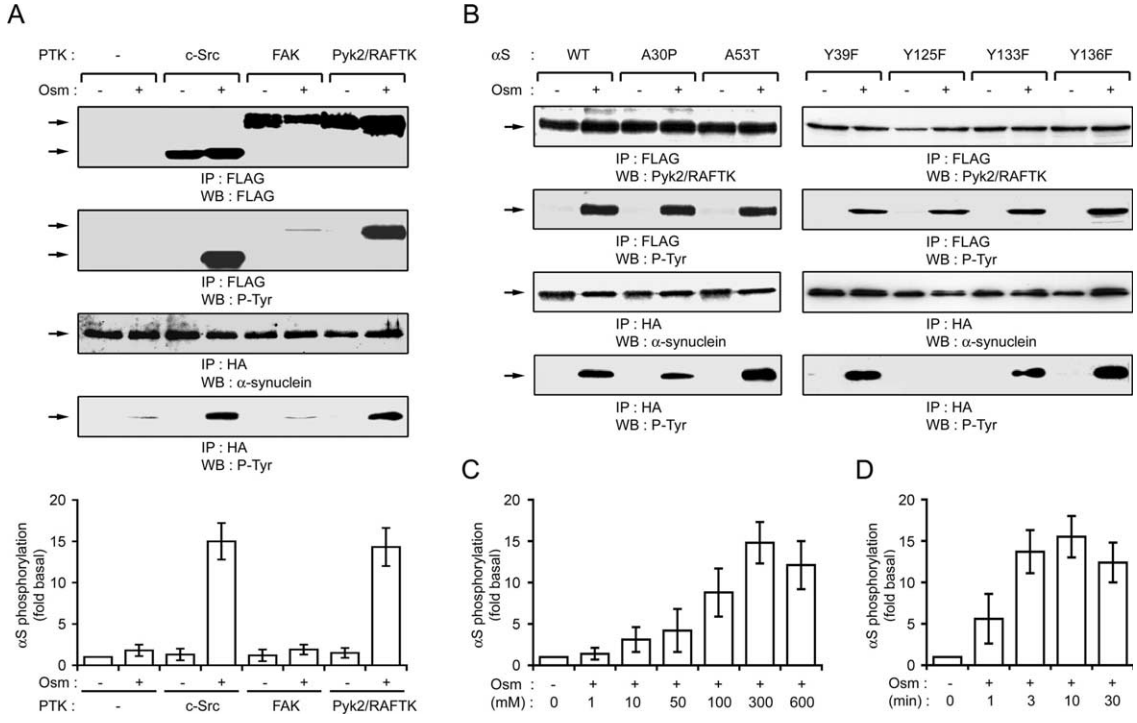

Fig. 1. Pyk2/RAFTK is involved in phosphorylation of αS at tyrosine residue 125 in response to osmotic stress. A: COS7 cells were cotransfected with HA-tagged expression vectors for αS and FLAG-tagged expression vectors for c-Src, FAK, or Pyk2/RAFTK, as indicated. Starved cells were treated with 300 mM D-sorbitol (Osm) for 10 min at 37°C and compared with untreated cells subjected to the same conditions. Each cell lysate was immunoprecipitated with anti-FLAG or anti-HA antibodies. The immunoprecipitates were then separated on 15% SDS–PAGE and immunoblotted with anti-FLAG, anti-αS, or anti-phosphotyrosine (P-Tyr). Densitometric analysis of αS phosphorylation is exhibited in the lower panel. Results are means ± S.E.M. for four independent experiments. B: COS7 cells cotransfected with expression vectors for FLAG-Pyk2/RAFTK and HA-αS (WT, A30P, A53T, Y39F, Y125F, Y133F, and Y136F) were treated with 300 mM D-sorbitol (Osm), lysed, and compared with untreated cells subjected to the same conditions. Each cell lysate was immunoprecipitated with anti-FLAG or anti-HA antibodies and analyzed by SDS–PAGE and immunoblotting with anti-FLAG, anti-αS, or anti-P-Tyr antibodies. C: Concentration dependence of osmotic stress-induced phosphorylation of αS. COS7 cells were treated with different concentrations of D-sorbitol (1, 10, 50, 100, 300, and 600 mM) for 10 min at 37°C and compared with untreated cells subjected to the same conditions. Cells were prepared and subjected to immunoprecipitation, immunoblotting, and densitometric analysis of αS phosphorylation. Results are means ± S.E.M. for four independent experiments. D: Time course of osmotic stress-induced phosphorylation of αS. COS7 cells were treated with 300 mM sorbitol for different periods of time (0, 1, 5, 10, 30 min) and compared with untreated cells. Cells were prepared and subjected to immunoprecipitation, immunoblotting, and densitometric analysis of αS phosphorylation. Results are means ± S.E.M. for four independent experiments.

In addition, c-Src and Pyk2/RAFTK, but not FAK, were phosphorylated and activated in response to osmotic stress. Such results suggest that both c-Src and Pyk2/RAFTK are involved in osmotic stress-dependent phosphorylation of αS. It should also be noted that it was found that COS7 cells do not endogenously express Pyk2/RAFTK. The above results suggest that other pathways might exist for αS phosphorylation via Src-family kinases.

3.2. Characterization of Pyk2/RAFTK-mediated tyrosine phosphorylation of αS

An additional experiment was performed to determine whether or not A30P and A53T mutations of αS could affect the phosphorylation state of αS under conditions of osmotic stress. COS7 cells expressing FLAG-tagged Pyk2/RAFTK and HA-tagged αS (wild-type, A30P, or A53T) were treated with sorbitol, lysed, and immunoprecipitated with anti-FLAG or anti-HA antibodies. Immunoblotting with anti-P-Tyr antibodies revealed that osmotic stress induced phosphorylation of not only wild-type αS, but also mutant αS, suggesting that A30P and A53T mutations do not affect Pyk2/RAFTK-mediated phosphorylation of αS (Fig. 1B). αS contains four tyrosine residues: Y39, Y125, Y133, and Y136. To determine the tyrosine phosphorylation site utilized following osmotic stress, COS7 cells were transfected with expression vectors for FLAG-Pyk2/RAFTK and HA-αS with Y–F single substitutions: Y39F, Y125F, Y133F, and Y136F. Immunoblotting with anti-P-Tyr antibodies revealed that osmotic stress induced phosphorylation of Y39F, Y133F, and Y136F, but not Y125F, suggesting that the tyrosine residue phosphorylated was 125 (Fig. 2B). This tyrosine residue has been reported to be phosphorylated by Src-family kinases [7,8]. c-Src and Fyn can directly phosphorylate αS, however phosphorylation of αS by Pyk2/RAFTK was not observed in vitro [7]. Although the issue of whether or not Pyk2/RAFTK directly phosphorylates αS in vivo remains a controversial topic of discussion, αS was found to be phosphorylated in response to osmotic stress when coexpressed with Pyk2/RAFTK.

Subsequently, the concentration dependence for osmotic stress-induced phosphorylation of αS was examined. COS7 cells transfected with expression vectors for FLAG-Pyk2/RAFTK and HA-αS were treated for 10 min with sorbitol at various concentrations: 1, 10, 50, 100, 300, and 600 mM. Treatment with sorbitol induced phosphorylation of αS in a concentration-dependent manner (Fig. 1C). Densitometric analysis demonstrated that phosphorylation of αS was maximal at ~14.8-fold above baseline ($n=4$) when cells were treated with 300 mM sorbitol; higher concentrations resulted in a decline of phosphorylation. Subsequently, the time course for αS phosphorylation was examined. Cells were stimulated with sorbitol for different periods of time: 0, 1, 5, 10, and 30 min. Phosphorylation of αS was rapid, becoming prominent at 1 min, reaching a maximum of ~15.5-fold above baseline ($n=4$) at 10 min after hyperosmotic stimulation, and subsequently declining (Fig. 1D). Such a result indicates that a dephosphorylation mechanism might exist for control of the αS phosphorylation state. Our group is presently searching for phosphatases that might potentially fulfill such a role. It is interesting to note that time-dependent translocation of αS oligomers from the plasma membrane to light vesicles via acetylcholine stimulation has been reported [14]. It remains possible that the αS phosphorylation state affects the intracellular localization of αS.

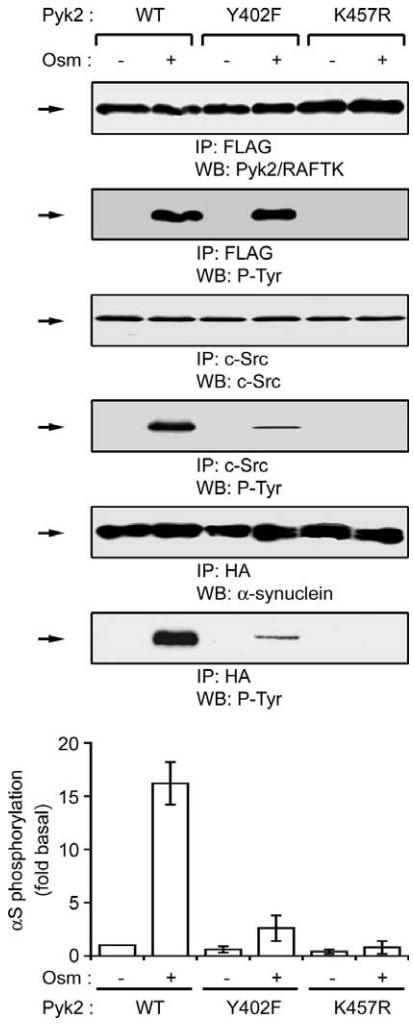

Fig. 2. Osmotic stress-induced in vivo phosphorylation of αS is dependent on Pyk2/RAFTK kinase activity. COS7 cells cotransfected with expression vectors for HA-αS and FLAG-Pyk2/RAFTK (Pyk2) (WT, Y402F or K457R) were starved and treated with 300 mM D-sorbitol for 10 min at 37°C. Cells were lysed and immunoprecipitated with anti-FLAG or anti-HA antibodies and analyzed by immunoblotting with anti-FLAG, anti-αS, or anti-P-Tyr antibodies. Densitometric analysis of αS phosphorylation is displayed in the lower panel. Results are means ± S.E.M. for four independent experiments.

3.3. Pyk2/RAFTK induces tyrosine phosphorylation of αS via Src-family kinases

It has been suggested that one autophosphorylation site of Pyk2/RAFTK, Y402, recruits c-Src to allow phosphorylation of substrates [9,13]. Alternatively, the binding of c-Src to Y402 of Pyk2/RAFTK might also lead to enhancement of Pyk2/RAFTK kinase activity. To characterize the role of Pyk2/RAFTK in the αS phosphorylation pathway in response to osmotic stress, the effects of expression of dominant-negative Pyk2/RAFTK mutants on tyrosine phosphorylation were examined. COS7 cells were cotransfected with expression vectors for HA-αS and FLAG-tagged wild-type Pyk2/RAFTK, the Src-binding site mutant Pyk2/RAFTK (Y402F), or the kinase-inactive Pyk2/RAFTK (K457R). After

the lysate protein contents were normalized, the cell lysate was divided into three aliquots and immunoprecipitated with anti-FLAG, anti-c-Src, or anti-HA antibodies. Osmotic stress-dependent phosphorylation of αS was markedly reduced by expression of Y402F Pyk2/RAFTK from 16.2 to 2.6-fold ($n = 4$) compared with baseline measurements with cells expressing wild-type Pyk2/RAFTK (Fig. 2). In cells expressing Y402F Pyk2/RAFTK, c-Src was not observed to be sufficiently phosphorylated in response to osmotic stress (Fig. 2). In addition, the effects of the Src-family kinase inhibitor, PP2, on RAFTK-mediated phosphorylation of αS were subsequently examined. COS7 cells were treated with PP2 at various concentrations for 1 h, stimulated with sorbitol and compared with PP2-untreated, sorbitol-stimulated controls. The osmotic stress-dependent phosphorylation of αS was significantly decreased by addition of PP2 in a dose-dependent manner (Fig. 3), suggesting that αS phosphorylation is primarily achieved

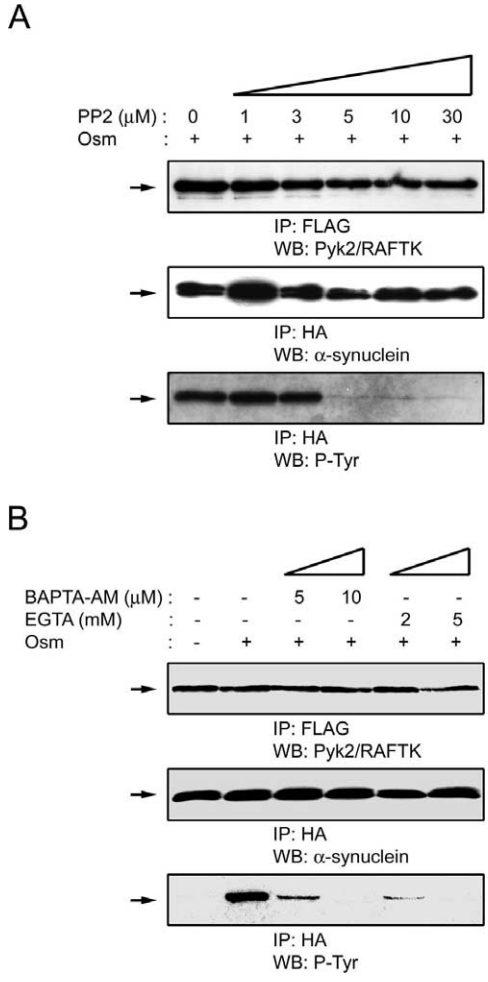

Fig. 3. Effects of a Src-family kinase inhibitor or calcium chelators on osmotic stress-induced phosphorylation of αS. A: COS7 cells cotransfected with HA-αS and FLAG-Pyk2/RAFTK constructs were starved and treated with 300 mM D-sorbitol for 10 min at 37°C in the presence or absence of PP2 at various concentrations. Cells were then lysed and immunoprecipitated with anti-FLAG or anti-HA antibodies and analyzed by immunoblotting. B: COS7 cells cotransfected with HA-αS and FLAG-Pyk2/RAFTK constructs were starved and treated with 300 mM sorbitol for 10 min at 37°C in the presence or absence of EGTA (1 mM, 3 mM) or BAPTA-AM (5 μM, 10 μM). Cells were lysed and immunoprecipitated with anti-FLAG or anti-HA antibodies and analyzed by immunoblotting.

with Src-family kinases. The results of the present work suggest that activated Pyk2/RAFTK recruits Src-family kinases, which could include c-Src, to phosphorylate tyrosine residue 125 of αS. Such findings indicate that Pyk2/RAFTK lies upstream of Src-family kinases in the signaling cascade by which osmotic stress induces tyrosine phosphorylation of αS.

It is also of note that osmotic stress-dependent phosphorylation of αS was completely abolished by expression of K457R Pyk2/RAFTK (Fig. 2). Moreover, c-Src was not phosphorylated in response to osmotic stress in the presence of K457R Pyk2/RAFTK. Such results suggest that Pyk2/RAFTK kinase activity is essential for osmotic stress-dependent phosphorylation of αS. It is of interest that such results also suggest the existence of a Src-family kinase-independent αS phosphorylation pathway that is mediated via Pyk2/RAFTK activation. One possibility is that Pyk2/RAFTK directly phosphorylates αS to a very low extent. Nonetheless, Pyk2/RAFTK-mediated phosphorylation of αS is primarily (~85%, Fig. 2) performed via Src-family kinases.

Pyk2/RAFTK is activated by a variety of stimuli that increase the intracellular Ca^{2+} level [9]. The effect of Ca^{2+} on osmotic stress-dependent phosphorylation of αS was further investigated by eliminating extracellular calcium with calcium chelators. The osmotic stress-dependent phosphorylation of αS was significantly decreased by addition of EGTA (2 mM, 5 mM) or BAPTA-AM (5 μM, 10 μM) in a dose-dependent manner, suggesting that Pyk2/RAFTK-mediated phosphorylation of αS was also dependent on extracellular calcium (Fig. 3). It is well established that osmotic stress affects intracellular free Ca^{2+} concentration [15]. It should be noted that MAPK pathways lie downstream of Pyk2/RAFTK-related signal transduction [11]; αS is known to interact with MAPK and affect its activity [16,17]. The present study demonstrates that extracellular Ca^{2+} is necessary for both activation of Pyk2/RAFTK and phosphorylation of αS following osmotic stress.

Recently, post-translational modifications of αS have been reported. Tyrosine nitration of αS by oxidative stress facilitates oligomer formation in both in vitro [18,19] and in vivo [20] experimentation. Furthermore, it is of note that αS protein in Lewy bodies is known to be nitrated [21]. Moreover, the Y125 residue of αS plays a critical role for oligomer formation following nitrative stress [19]. The present work has demonstrated that Pyk2/RAFTK is involved in phosphorylation of the Y125 residue of αS via Src-family kinases. Such a finding suggests that both extracellular signaling molecules and stresses not only activate Pyk2/RAFTK, but also regulate the phosphorylation state of αS.

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