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Multiple phosphorylation of α-synuclein by protein tyrosine kinase Syk prevents eosin-induced aggregation

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ABSTRACT

The presence of aggregated α-synuclein molecules is a common denominator in a variety of neurodegenerative disorders. Here, we show that α-synuclein (α-syn) is an outstanding substrate for the protein tyrosine kinase p72^{syk} (Syk), which phosphorylates three tyrosyl residues in its C-terminal domain (Y-125, Y-133, and Y-136), as revealed from experiments with mutants where these residues have been individually or multiply replaced by phenylalanine. In contrast, only Y-125 is phosphorylated by Lyn and c-Fgr. Eosin-induced multimerization is observed with wild-type α-syn, either phosphorylated or not by Lyn, and with all its Tyr to Phe mutants but not with the protein previously phosphorylated by Syk. Syk-mediated phosphorylation also counteracts α-syn assembly into filaments as judged from the disappearance of α-syn precipitated upon centrifugation at 100,000 × g. We also show that Syk and α-syn colocalize in the brain, and upon cotransfection in Chinese hamster ovary cells, α-syn becomes Tyr-phosphorylated by Syk. Moreover, Syk and α-syn interact with each other as judged from the mammalian two-hybrid system approach. These data suggest that Syk or tyrosine kinase(s) with similar specificity may play an antineurodegenerative role by phosphorylating α-syn, thereby preventing its aggregation.

Key words: Syk tyrosine kinase • tyrosine phosphorylation • α-synuclein multimerization • regulation by phosphorylation • protein kinase CK2

α-Synuclein (α-syn) is a 140-amino acid, highly conserved protein abundant in neurons, with special reference to the presynaptic terminals (1–3). It is composed of three discernible domains: a N-terminal amphipatic repeat region, which can form α-helices; a hydrophobic central segment; and a C-terminal acidic region. A fragment corresponding to the central domain was identified initially as a component of amyloid plaques isolated from brains with Alzheimer's disease (1, 4, 5). Additional and more stringent evidence that α-syn is implicated in neurodegenerative diseases was provided by the detection of α-syn mutations in families with autosomal dominant Parkinson's disease (6, 7) and by the finding that α-syn is the major component of Lewy bodies and Lewy neurites in Parkinson's disease, Lewy bodies in dementia, and the Lewy bodies variant of Alzheimer's disease (8, 9). In addition, α-syn also

appears to be a major component of glial and cytoplasmic inclusions in a wide variety of neurodegenerative disorders (10).

A common denominator of all these "synucleinopathies" is the multimerization of α-syn molecules to form insoluble aggregates that cause brain lesions. Although the precise mechanism by which insoluble aggregates are formed from soluble α-syn has yet to be understood, two structural elements have been implicated in α-syn self-oligomerization: a hydrophobic stretch in the middle of the protein (11), essential for eosine-induced aggregation (12), and three tyrosyl residues clustered near the C-terminal end, shown to form *o*,*o*'-dityrosine bridges (13, 14) (see Fig. 1). The residues are embedded in a very acidic context (see Fig. 1), making them potential targets for the nonreceptor tyrosine kinase $p72^{syk}$ (Syk) (15). At variance with most tyrosine kinases, whose targeting is mainly specified by adhesive modules outside the catalytic site, Syk displays a sharp site specificity determined by acidic residues surrounding the target tyrosine on both its N- and C-terminal sides (15, 16). Based on this consensus, it was predictable that all three C-terminal tyrosines of α-syn may function as good phosphoacceptor residues for Syk. These observations prompted us to start an investigation aimed at assessing whether α-syn can be phosphorylated by Syk, or by other tyrosine kinases, and, in this case, if this phosphorylation affects its ability to polymerize. Here, we show that α-syn is an outstanding substrate for Syk and that once it is tyrosine phosphorylated, it loses the ability to form oligomers.

MATERIALS AND METHODS

Materials

[γ^{32}P]ATP was purchased from Amersham Pharmacia Biotech (San Francisco, CA). Protease inhibitor cocktail was obtained from Boehringer (Mannheim, Germany). Anti-phosphotyrosine and anti-α-syn (raised against the sequence 111-131 of the protein) were obtained from ICN Biotechnology (Irvine, CA) and Chemicon (Temecula, CA), respectively. Porcine $p72^{syk}$ plasmid was kindly provided by Dr. H. Yamamura (Department of Biochemistry, University of Kobe, Japan). Other reagents were purchased from Sigma (Dorset, UK).

Protein kinases

Lyn, c-Fgr, Syk, and Csk were isolated from rat spleen (17–20), protein kinase CK1 and CK2 from rat liver (21), and G-CK from rat lactating mammary gland (22). One unit was defined as the amount of enzyme that transferred 1 pmol phosphate per min to either 0.1 mg/ml polyGlu/Tyr (4:1) (tyrosine kinases) or 0.1 mg/ml casein (protein kinases CK1 and CK2, and G-CK), under standard conditions.

Preparation of recombinant forms of human α-syn

Human brain cDNA library (CLONTECH, Heidelberg, Germany) has been used to amplify the α-*syn* gene by means of RACE-polymer chain reaction (RACE-PCR). PCR was performed using a specific primer complementary to the double-stranded cDNA based on the published sequence of the α-*syn* gene (2). The purified PCR product was cloned directly into the expression plasmid PRSETB (Life Technology, Karlsruhe, Germany), and the sequence was verified by double-stranded dideoxysequence by using the fluorescent terminator method (Applied Biosystems, Langen, Germany) and run on the ABI 377 sequencer (Applied Biosystems). Site-directed

mutagenesis at codon 125, 133, and 136 was performed using synthesized oligonucleotides to construct the mutants Y125F, Y133F, Y136F, and YY133,136FF as well as YYY125,133,136FFF. The constructs were sequenced in both strands to verify the proper mutations.

For the expression of the recombinant proteins, the correct plasmids were transferred onto the BL21 (DE3) *Escherichia coli* strain and cells were induced to produce recombinant proteins by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C. The bacterial pellet was recovered by centrifugation, and recombinant proteins were purified according to their relative resistance to heating (23). The boiled lysates were directly loaded onto a IMAC-NTA column (Qiagen, Milano, Italy) equilibrated with 10 mM Tris/HCl, pH 8.0, containing 150 mM NaCl. Recombinant proteins were eluted by 250 mM imidazole, pH 8.0, and further characterized by reverse-phase high-pressure liquid chromatography and Western blot probed with anti-α-syn antibody. The correct molecular weight of the recombinant proteins was verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. α-Syn recombinant proteins were dialyzed against 20 mM Tris/HCl, pH 7.0, containing 100 mM NaCl and were stored at –80°C.

Colocalization of α-syn and Syk tyrosine kinase cotransfected in both neuroblastoma and Chinese hamster ovary (CHO) cells

cDNA of human α-syn was fused at the N-terminus to the red fluorescence protein gene (DsRed1-N1) (CLONTECH, Basel, Switzerland) to obtain the plasmid pDSsyn. cDNA of Porcine $p72^{syk}$ was fused in a similar manner to a green fluorescence protein gene by using phEGFP (CLONTECH) vector to obtain the plasmid pEGFPSyk. In both cases, the stop codon for TGA was changed by PCR to TCA to create an in-frame fusion protein.

Human neuroblastoma (SH-N-BE) or CHO cells were cultured at 37°C in standard conditions and transfected using lipofectamine plus reagent (Life Technology) according to the manufacturer's instructions. To generate antibiotic-resistant clones, we placed cells in a medium containing 500 mg/ml Genetic (neomicin G418, Calbiochem, Darmstadt, Germany). Fluorescence was analyzed using an inverted fluorescence Olympus (Firenze, Italy) TM20 microscope. Images were recorded with a 1000 × 800 digital camera (Princeton Instruments, Monmouth Junction, NJ) at 12 bit and processed using Adobe (San Jose, CA) Photoshop 5.0 software. The fluorescence filter set was obtained from Chroma Technology (Brattleboro, VT; Excitation D470/20, dichroic 490DCLP, and emission D510/20 for green fluorescence protein and Texas red set for red fluorescence protein).

Analysis of α-syn and Syk immunolocalization in mouse brain by double-immuno-histochemistry and confocal microscopy

Brains from mice (strain C57) were isolated, frozen in liquid N_2, and stored at –80°C. Serial sagittal sections (10 µm) were prepared with a cryomicrotome on polylysine-coated glass microscope slides. Sections were fixed in formalin (30 min at 37°C), washed twice with PBS at room temperature for 2 min, permeabilized with 0.1% Triton X-100 (10 min at 4°C), and washed again with PBS. Sections were incubated with primary monoclonal anti-Syk (1/20) and polyclonal anti-α-syn (1/20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 37°C. Slides were washed twice with PBS for 10 min at room temperature and incubated with

anti-mouse fluorescein and anti-rabbit TRITC secondary antibodies (DAKO, Milano, Italy) for 10 min at 37°C. Slides were then incubated with Hoestch 33342 (Sigma, Dorset, UK;1 μg/ml) for 10 min at room temperature in order to visualize cell nuclei, washed in PBS, mounted in glycerol, and examined with a Zeiss (Oberkochen, Germany) Axiovert 100 STV microscope. Pictures were taken by a CCD digital camera (Princeton) with different filters, and images were overlapped using Adobe Photoshop 5.5 software.

Mammalian two-hybrid system

α-Syn was cloned by PCR in plasmid pM (CLONTECH), and Syk was cloned in plasmid pV16 (CLONTECH). Both genes were cloned between the BamHI and EcoRI in frame to DNA binding domain to give plasmids pMSyn and pV16Syk. Plasmid pG5CAT and the constructed plasmids were transfected alone or together in 100-mm-diameter dishes containing CHO cells at 70% confluence by lipofectamine. After 60 h, cells were recovered with 2.5 mM EDTA and cellular lysates were assayed for CAT activity by the ELISA method. Positive controls were also performed using pM53 and pV16T plasmids provided by CLONTECH.

Tyr-phosphorylation of α-syn in CHO cells

Human α-*syn* and porcine $p72^{syk}$ genes were cloned in eukaryotic expression vector pcDNA3 to obtain plasmids pcDNAsyn and pcDNASyk. CHO cells (5×10^6) were transfected with pcDNAsyn alone or pcDNAsyn plus pcDNASyk. After 48 h of transfection, cells were sonicated in 1 ml of 50 mM Tris/HCl, pH 7.5, containing 0.25 M saccharose, 1 mM orthovanadate, and a protease inhibitor cocktail (buffer A). Cell nuclei were isolated by 10 min of centrifugation at 900 × g (pellet I). The supernatant was then centrifuged for 1 h at 100,000 × g to separate the cytosol from the particulate fraction (pellet II). Both pellets were solved in 1 ml of buffer A, and 50 μl of each fraction were subjected to SDS-PAGE (15% gels) followed by Western blot with the appropriate antibody.

α-Syn phosphorylation assay

α-Syn (0.25 μM) was phosphorylated by Syk, Lyn, and G-CK protein kinases at 30°C in 30 μl of incubation medium containing 50 mM Tris/HCl, pH 7.5; 5 mM $MnCl_2$; 200 μM [γ^{32}P]ATP; and 10 U of protein kinases. In the case of protein kinases CK1, CK2, and c-Fgr, $MnCl_2$ was replaced by 5 mM $MgCl_2$. The reactions were stopped by the addition of 2% SDS, and the samples were subjected to SDS-PAGE (15% gels). The degree of protein phosphorylation was evaluated either by analysis on a Packard (Meriden, CT) Imager or by autoradiography and counting the identified radioactive bands. K_m and K_{cat} values for wild-type α-syn and its mutants were calculated from double-reciprocal plots constructed from initial rate measurements fitted linearly to the Lineweaver-Burk representation of the Michaelis-Menten equation.

Preparation of Syk- and Lyn-phosphorylated α-syn for self-association experiments

α-Syn (10 μg) was phosphorylated in 400 μl of phosphorylation medium in the presence of either 10 nM Syk or 50 nM Lyn as described previously. Phosphorylated α-syn was purified from the components of the incubation mixture by IMAC-NTA column as described previously. The eluted phospho-protein was then dialyzed against 50 mM ammonium bicarbonate, lyophilized, and solved in 20 mM MES, pH 6.5.

Self-oligomerization of α-syn in the presence of eosin

Self-oligomerization was performed according to Shin et al. (12). In brief, α-syn was preincubated with eosin at a molar ratio of 1:20 in 20 mM MES, pH 6.5, for 40 min at 37°C. The reactions were then incubated for l h at 37°C with 0.3 mM EEDQ stocked in dimethyl sulfoxide. After incubation, samples were subjected to 15% SDS-PAGE. Oligomerization was visualized by either immunostaining with anti-α-syn antibody or autoradiography.

Evaluation of α-syn assembly by centrifugation experiments

Self-aggregation of recombinant α-syn was induced by incubation of the protein (1 mg/ml) for 15 min at 37° C in 50 mM Tris/HCl, pH 7.5, with continuous shaking. The sample was divided in two aliquots that were incubated for 15 min in the previously described phosphorylation buffer in either the presence or the absence of 10 nM Syk. The two aliquots were then centrifuged at 100,000 × g for 20 min, and supernatants and pellets were loaded on SDS-PAGE. Gels were stained by Coomassie blue and proteins quantified by densitometry.

RESULTS

Phosphorylation of human α-syn by incubation with a series of acidophilic protein kinases, either Tyr- (Syk, Lyn, and c-Fgr) or Ser/Thr-specific (CK1, CK2, and G-CK), in the presence of [γ^{32}P]ATP is shown in Figure 2. To make the comparison meaningful, all the assays were run with identical units of the different kinases. Although some extent of radiolabeling was detectable with most kinases, except in the case of CK1 and Csk, phosphorylation catalyzed by Syk was by far superior, suggesting that α-syn is an especially good substrate for this kinase. This conclusion was corroborated both by time course experiments, in which a phosphorylation stoichiometry of almost 2 mol P/mol α-syn was reached with Syk, as opposed to much lower stoichiometry with the two Src family kinases, Lyn and c-Fgr (Fig. 3), and by the kinetic constants reported in Table 1. These show that α-syn is an outstanding substrate of Syk, with one of the lowest K_m values ever reported (50 nM), comparable to that of HS1 (hematopoietic lineage cell-specific protein 1), a recognized physiological substrate of Syk (19).

To map the phosphorylated residues, we used α-syn mutants in which the three tyrosines located in the C-terminal domain had been replaced by phenylalanine, either individually or in combination. From the data shown in Figure 4, it can be concluded that all three C-terminal tyrosines, but not the one located in the N-terminal domain (Y-39), are phosphorylated by Syk, albeit with different efficiencies. Although the triple mutant (YYY125,133,136FFF) is not able to be phosphorylated, all the individual mutants and the double mutant (YY133,136FF) undergo appreciable phosphorylation, but to varying extents. The relatively low phosphorylation of Y125F compared with the other two individual mutants would indicate that Tyr-125 is the main, but not the only, phosphoacceptor site. In fact, the other two individual mutants are defective in phosphorylation compared with the wild type, indicating that Tyr-136 and, to a lesser extent, Tyr-133 contribute to the overall phosphorylation of wild-type α-syn. Apparently, however, their phosphorylation is incomplete; we never succeeded in reaching stoichiometries significantly higher than 2 mol phosphate/mol protein. It is of interest that phosphorylation of α-syn by Src kinases appears to affect only Tyr-125, because the Y125F mutant is not phosphorylated and the two other individual mutants (Y133F and Y136F) and the double mutant (YY133,136FF) are

phosphorylated by either Lyn (Fig. 4) or c-Fgr (data not shown) even faster than the wild-type α-syn. Taken together, these data show that although α-syn is an outstanding substrate of Syk, which phosphorylates all three tyrosyl residues in its C-terminal tail, it is also phosphorylated, albeit less readily, by tyrosine kinases of the Src family, which affects only one of its C-terminal tyrosines (i.e., Y125).

To determine whether multiphosphorylation of α-syn by Syk alters its ability to self-oligomerize, we used the eosin-induced cross-linking method (12). As shown in Figure 5A, upon addition of eosin, both wild-type α-syn (lane 2) and its mutant, in which the three C-terminal tyrosines were replaced by phenylalanines (lane 4), give rise to several slow-migrating bands immunoreacting with the anti-α-syn antibody. This pattern is typical of self-oligomerization, whereas monomeric α-syn gives rise to the most mobile band of the expected relative molecular weight. In contrast, eosin-induced self-oligomerization disappears if α-syn is previously phosphorylated by Syk (Fig. 5B, lane 4), but not if it is phosphorylated by Lyn (lane 6).

To determine whether the different effects of Syk and Lyn might be accounted for merely by the different phosphorylation stoichiometry, which is higher with Syk (see Fig. 3 and Materials and Methods), the experiment was performed in the presence of [γ^{32}P]ATP to see how the phosphoradiolabeled molecules were distributed between the fast- and the slow-migrating bands upon eosin treatment. As shown by the autoradiogram in Figure 5C, although the whole radioactivity of α-syn stoichiometrically phosphorylated by Syk (lanes 2 and 4) was present in the fast-migrating band of the monomeric protein (which is the only one detectable by Western blotting in this case, as shown in Fig. 5C, panel B), the radioactivity incorporated upon incubation with Lyn (lane 6) was evenly distributed among the fast- and slow-migrating bands, ruling out any specific effect of phosphorylation on multimerization.

Therefore, phosphorylation of the individual tyrosyl residue 125 by Lyn is not sufficient to prevent eosin-induced multimerization of α-syn. The conclusions drawn from the eosin-induced cross-linking method were confirmed by measuring the amount of α-syn precipitated upon centrifugation at 100,000 × g (11). At an α-syn concentration of 1 mg/ml, after 15 min of incubation at 37°C, the amount precipitated was about 30%, but it was entirely abolished after extensive phosphorylation of α-syn by Syk (data not shown). Lyn- and c-Fgr-mediated phosporylation did not reduce the aliquot of α-syn precipitated (data not shown).

To corroborate the physiological relevance of these observations, the ability of Syk to interact with or to phosphorylate α-syn in a cellular environment was assessed by three distinct approaches. First, to determine whether the two proteins colocalize in the brain, we analyzed mouse brain sections by double-labeling immunofluorescence with antibodies to Syk (green) and to α-syn (red). We found that the two proteins colocalize in perinuclear areas (Fig. 6). Precise colocalization of the two proteins was confirmed by overlapping the two images, which generate the expected yellow color in the same compartments (see Fig. 6D). These data also corroborate the concept that α-syn is associated with granular cytoplasmic structures (14, 24), where Syk can also be detected. Similar colocalization of Syk and α-syn was also found in neuroblastoma and CHO cells cotransfected with α-syn and Syk fused to red fluorescent protein and green fluorescent protein, respectively (data not shown). Second, we examined Tyr-phosphorylation of α-syn in CHO cells transfected with either α-syn alone or with α-syn plus Syk (Fig. 7) In the latter case, a remarkable Tyr-phosphorylation of α-syn, localized in cell particulate fraction, was observed from Western blot with anti-P-Tyr antibody, providing clear-cut evidence that α-syn is

also a target for Syk inside the cell. Third, we performed a mammalian two-hybrid assay applicable to proteins that normally reside in the cytoplasm (25). Transcription of the reporter gene CAT occurs only if the two chimeric proteins can form a complex that reconstitutes the DNA-binding and transcriptional activity of GAL4 (see Materials and Methods). As shown in Figure 8, activation took place only when the plasmids pMSyn and pV16Syk and the reporter plasmid pG5CAT were present in the cells. This demonstrates a direct interaction between Syk and α-syn within the cell.

DISCUSSION

α-Syn belongs to a rather heterogeneous category of proteins normally expressed in a variety of tissues, whose common denominator is to undergo, under some circumstances, aggregation and formation of amyloid plaques, which are the hallmark of several neurodegenerative diseases (10, 26, 27). Therefore, it is a crucial challenge to decipher the factors that may influence the balance between the soluble, "normal," protein and its pathogenic form, by either preventing or triggering multimerization. Bearing in mind that about one-third of mammalian proteins contain covalently bound phosphate and that the possibility that such a reversible phosphorylation is responsible for the control of nearly all aspects of cell life (28), the possibility that α-syn might also undergo this kind of covalent modification has been studied in several laboratories. Previous investigations have shown that α-syn can be Ser-phosphorylated by protein kinases CK1 and CK2 at a residue (Ser-129) whose phosphorylation could be also observed in vivo if Ser/Thr protein phosphatases were inhibited by okadaic acid (29, 30). More recently, it has been shown that the same residue is also phosphorylated by G-protein-coupled receptor protein kinases (31). In that study, the ability of CK1 and CK2 to phosphorylate α-syn has been confirmed; however, the implication of Ser-129 has been reevaluated by showing that a mutant lacking this residue was also phosphorylated by CK1 and, to a lesser extent, by CK2 (31).

While this paper was in preparation, the possibility that α-syn may undergo tyrosine phosphorylation was assessed, by showing that it is a substrate for two Src family protein tyrosine kinases, c-Src and Fyn (32 ,33). The residue involved in this case is Tyr-125, whose mutation to Phe abrogates phosphorylation by c-Src and Fyn.

The data presented here show that Syk is by far superior to CK1, CK2, Lyn, and c-Fgr as an α-syn phosphorylating agent. If these kinases are matched under comparable conditions (i.e., using the same amounts of units of all enzymes, see Fig. 2), phosphorylation of α-syn by CK2, Lyn, c-Fgr, and, even more, CK1 appears to be almost negligible compared with the phosphoradiolabeling observed in the presence of Syk. This especially applies to the two Ser/Thr kinases, one of which, CK1, hardly displays any detectable α-syn phosphorylating activity, in accordance with the notion that peptides with just one carboxylic residue at the crucial n – 3 position, as is the case for Ser-129, are phosphorylated very poorly by CK1 (34). However, Ser-129 does not fulfill the consensus sequence of CK2 for lacking the crucial acidic residue at the n + 3 position. It does conform to the consensus sequence of the Golgi apparatus casein kinase (G-CK), whose targeting is specified by a glutamic acid at the n + 2 position. However, according to our data, phosphorylation of α-syn by G-CK is as poor as that observed with CK2 (see Fig. 2).

Neither the stoichiometry nor the kinetic constants of α-syn phosphorylation by CK1, CK2, GRKs, c-Src, and Fyn have been reported in previous studies. Phosphorylation by Lyn and c-Fgr is largely understoichiometric (reaching 0.2–0.3 mol P/mol protein after prolonged incubation,

see Fig. 3). The same applies to CK2- and G-CK-catalyzed phosphorylation. By sharp contrast, phosphorylation of α-syn by Syk approaches a stoichiometry of 2 mol P/mol protein, consistent with the outcome of experiments with Y→F α-syn mutants. These support the view that all the three tyrosyl residues of the C-terminal tail, Y125, Y136 and, to a lesser extent, Y133, are susceptible to phosphorylation by Syk. The very low K_m value of the reaction (50 nM) denotes high affinity and is compatible with physiological conditions. Such a performance was in some way expected considering the well-known affinity of Syk for tyrosyl residues embedded between acidic clusters (15) like those found in the C-terminal segment of α-syn. Phosphorylation by either Lyn or c-Fgr is conversely restricted to Tyr-125, in agreement with the data obtained by others with two different Src family kinases, c-Src and Fyn (32,33), and takes place with much less favorable kinetic constants (Table 1).

The most remarkable feature of Syk-mediated phosphorylation, however, is that it prevents multimerization of α-syn, which is believed to mimic the first step of the pathogenic event leading to the formation of amyloid plaques (35). Stoichiometric phosphorylation of α-syn by Syk in fact prevents the formation of eosin-induced slow-migrating bands of α-syn. These bands, moreover, are free of phosphoradiolabeled α-syn, which is entirely recovered in the fast-migrating band corresponding to monomeric α-syn (Fig. 5C). These data are consistent with the concept that multiphosphorylated α-syn molecules cannot participate in multimerization. By sharp contrast, α-syn phosphoradiolabeled by Lyn is present in the slow-migrating bands as well, suggesting that phosphorylation by Src family kinases does not suppress or enhance the tendency of α-syn to polymerize.

A possible explanation would be that in order to counteract multimerization, more than one tyrosyl residue in the C-terminal segment of α-syn must undergo phosphorylation. The mechanism by which the adverse effect of tyrosine phosphorylation on α-syn multimerization takes place is unknown. It has to be assumed that phosphorylation induces conformational alterations, rendering the central hydrophobic region of the molecule, which is responsible for the interactions that underlie the multimerization mechanism triggered by eosin less exposed (11, 12). This explanation is corroborated by the finding that previous phosphorylation by Syk causes the disappearance of polymeric α-syn, which can be precipitated upon 100,000 × g centrifugation. As a matter of speculation, another mechanism suspected to tighten α-syn multimerization, based on cross-linking via the oxidation of tyrosine to form *o*,*o*'-dityrosine (13), will also be deeply affected by phosphorylation. The phenyl group, in fact, is crucial for the formation of the tyrosyl radicals that are a prerequisite for subsequent dimerization and oligomerization (36), and its covalent linkage with a phosphate group would quench its reactivity by reducing the electronegativity of the oxygen. Moreover, considering that the C-terminal domain of α-syn is required for its chaperone-like activity (37), it is conceivable that the phosphorylation of these tyrosyl residues in this region would also affect this property of α-syn.

Whether in vivo Tyr-phosphorylation of α-syn is mediated by Syk or by another protein tyrosine kinase with overlapping site specificity remains an open question. Taken together, our data strongly suggest that Syk, which is abundant in the brain (38) where it is involved in neuronal differentiation (39), is the first choice candidate to perform this job. Not only does it phosphorylate α-syn in vitro with an efficiency by far superior to that of the Src family kinases, Lyn and c-Fgr, but it also colocalizes with α-syn both in the brain (Fig. 6) and upon cotransfection in neuroblastoma and CHO cells, where it also promotes the phosphorylation of α-syn (Fig. 7). Syk also physically interacts with α-syn, as judged from the mammalian two-

hybrid system approach (Fig. 8). The physiological implication of Syk in α-syn phosphorylation would also be consistent with the observation that the phosphorylation of α-syn in transfected cells is markedly impaired by the mutation of the two tyrosines, Y133 and Y136 (32), which are susceptible to Syk phosphorylation, whereas the Src family kinases c-Src (32), Fyn (32, 33), Lyn, and c-Fgr (as shown here) exclusively phosphorylate Tyr-125. The double mutation of tyrosines 133 and 136 to phenylalanines actually improves phosphorylation by Lyn (Fig. 4).

The finding that the Src-specific inhibitor PP2 prevents the phosphorylation of transfected α-syn in HEK293T cells (32) is not necessarily inconsistent with the implication of Syk in α-syn phosphorylation, because it may reflect an indirect inhibition. On one hand, in fact, the activation of Syk depends either directly or indirectly (through the phosphorylation of ITAM motifs) on the activity of the Src family kinases (40). On the other hand, it has been shown that Syk and Src kinases can cooperate in an interdependent manner to the sequential phosphorylation of protein targets such as HS1 (41, 42) and band-3 (43). The proximity of the three C-terminal tyrosyl residues may also suggest a hierarchical effect, in which the phosphorylation of one residue primes that of the others (44). Pertinent to this may be the observation that phosphorylated residues act as specificity determinants in Syk-mediated phosphorylation (16). This applies not only to phosphotyrosine, but also to phosphoserine: therefore, the seryl residue phosphorylated by CK2 (30) and GRKs (31) (Ser-129) is in a suitable position for enhancing the phosphorylation of Tyr-125 and/or Tyr-133 by Syk.

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Table 1

Kinetic constants of wild-type α-syn and its mutants for Syk and Lyn tyrosine kinases[a]

	Syk			Lyn		
	K_{cat} (min^{-1})	K_m (μM)	Efficiency K_{cat}/K_m	K_{cat} (min^{-1})	K_m (μM)	Efficiency K_{cat}/K_m
α-syn	1.5	0.050	30	0.25	0.50	0.5
α-syn (Y125F)	0.6	0.122	5	< 0.05	nm[b]	nm
α-syn (Y133F)	1.2	0.053	23	0.35	0.35	1.0
α-syn (Y136F)	1.1	0.050	22	0.40	0.40	1.0
α-syn (YY133,136FF)	0.9	0.053	17	0.40	0.40	0.9

[a]Data are means of four separate experiments. SE values were always less than 14%.

[b]nm, not measurable.

Fig. 1

MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVL**Y**V GSKTKEGVVH

GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL

GKNEEGAPQE GILEDMPV<u>D</u>P <u>D</u>N<u>E</u>A**Y**[125]<u>E</u>MPS<u>E</u> <u>E</u>G**Y**[133]Q<u>D</u>**Y**[135]<u>E</u>P<u>E</u>A

Figure 1. Sequence of human α-syn. Tyrosine residues are highlighted in bold. Acidic residues, which define the consensus sequence of Syk, are underlined.

Fig. 2

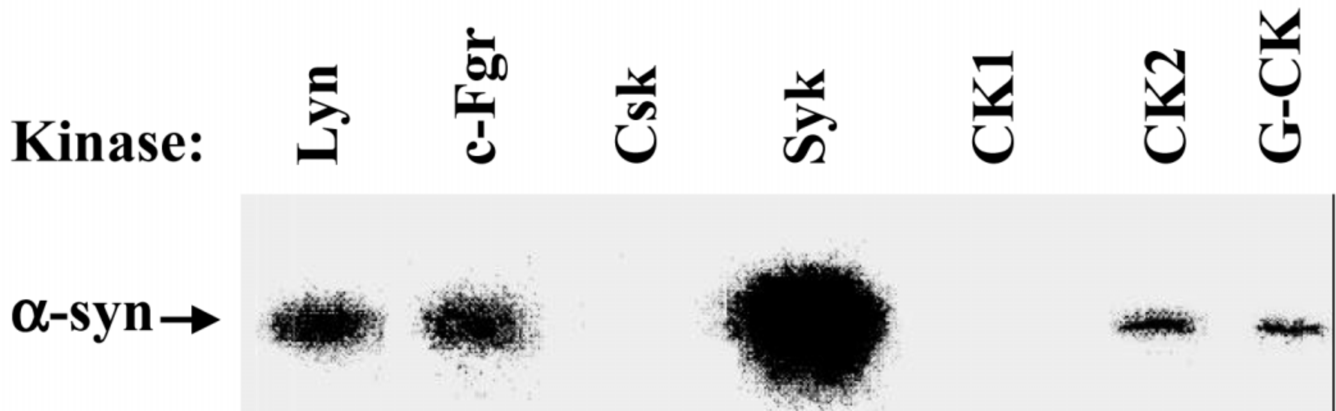

Figure 2. Phosphorylation of α-syn by different acidophilic protein kinases. α-Syn was phosphorylated by 10 U of the indicated protein kinases for 10 min as described in Materials and Methods. Autoradiography of dried gel is shown. At least four separate experiments are represented.

Fig. 3

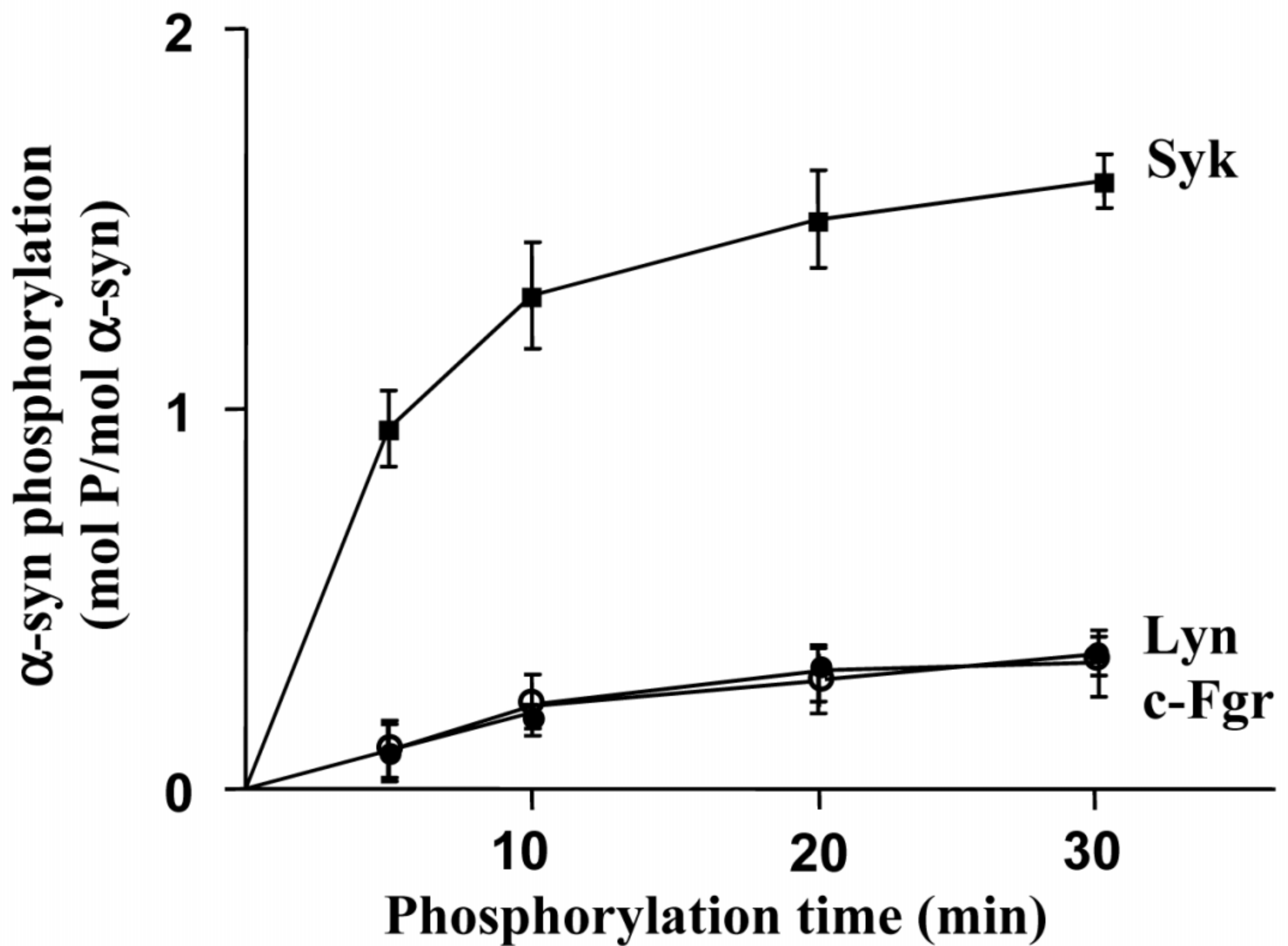

Figure 3. Time courses of α-syn phosphorylation catalyzed by Syk (solid squares), Lyn (solid circles), and c-Fgr (open circles) tyrosine kinases. α-Syn (0.25 μM) was phosphorylated in the presence of 200 μM [γ^{32}P]-ATP as described in Materials and Methods. Reported values represent the means of three separate experiments, with SE indicated by vertical bars.

Fig. 4

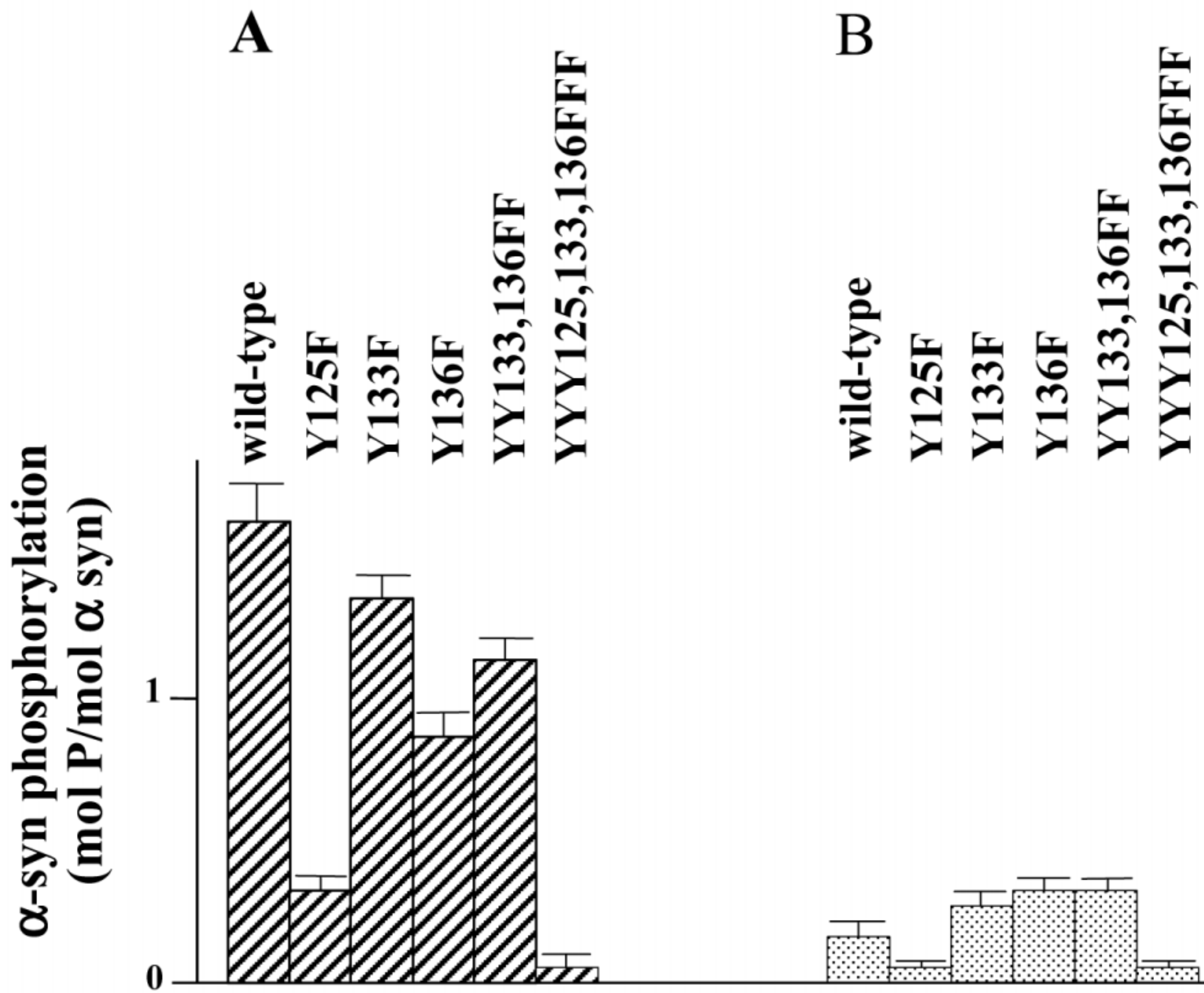

Figure 4. Phosphorylation of wild-type α-syn and its mutants by Syk and Lyn tyrosine kinases. Wild-type α-syn (0.25 μM) and α-syn mutants were phosphorylated for 30 min by either 10 nM Syk **(A)** or 50 nM Lyn **(B)**. Reported values represent the means of three separate experiments, with SE indicated by vertical bars.

Fig. 5

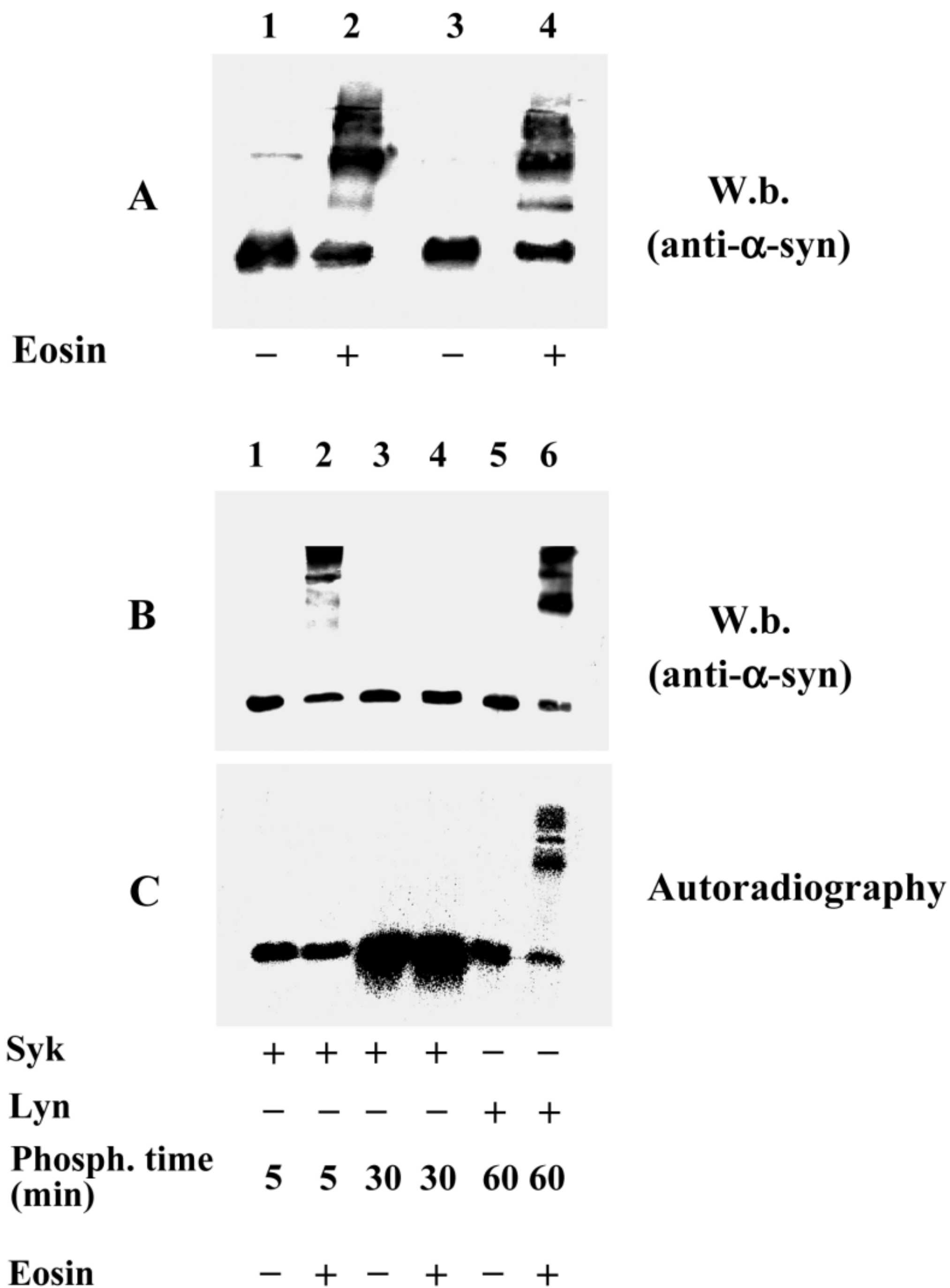

Figure 5. Tyr phosphorylation catalyzed by Syk prevents α-syn multimerization. (A) Anti-α-syn immunostaining of 50 pmol of either wild-type α-syn (lanes 1 and 2) or mutant α-syn (YYY125,133,136FFF) (lanes 3 and 4). Samples were incubated in the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of eosin, subjected to SDS-PAGE and analyzed by Western blot. **(B** and **C)** α-Syn (50 pmol) was phosphorylated by Syk or Lyn for the indicated times in the presence of $\gamma[^{32}P]$ATP. The stoichiometry of phosphorylation was 0.3 (lanes 1 and 2), 1.5 (lanes 3 and 4), and 0.3 (lanes 5 and 6) mol P/mol α-syn. Samples were then incubated in the absence (lanes 1, 3, and 5) or the presence (lanes 2, 4, and 6) of eosin, subjected to 15% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was either immunostained with anti-α-syn antibody **(B)** or analyzed by autoradiography **(C)**. Five separate experiments are represented.

Fig. 6

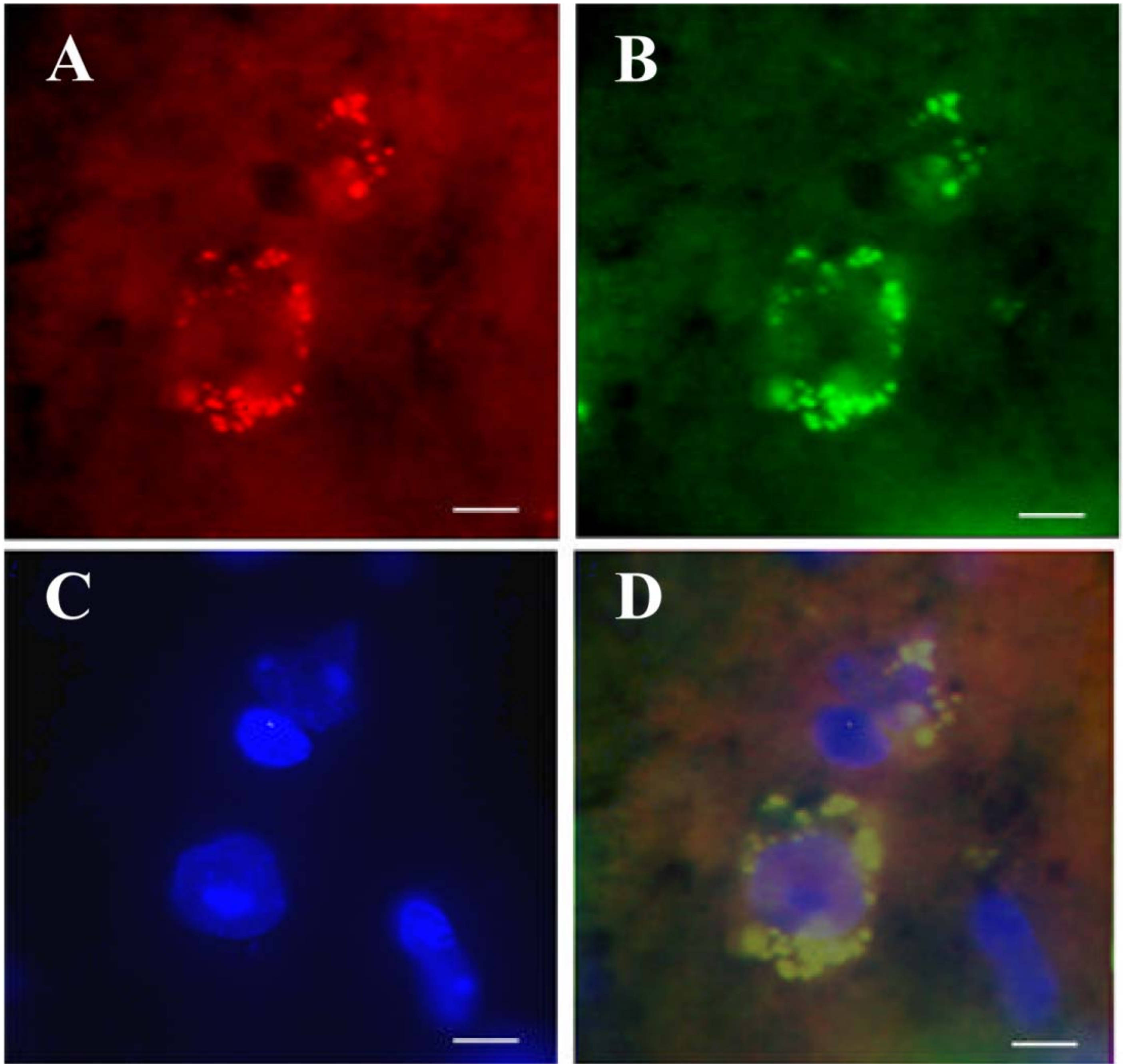

Figure 6. Colocalization of α-syn and Syk in mouse brain. Slices of mouse brain were analyzed by double-labeling immunofluorescence as described in Materials and Methods (scale bar, 10 μm). Detection was with anti-α-syn antibody **(A)**, anti-Syk antibody **(B)**, or both **(D)**. Nuclei were visualized by Hoechst 33342 staining **(C** and **D)**.

Fig. 7

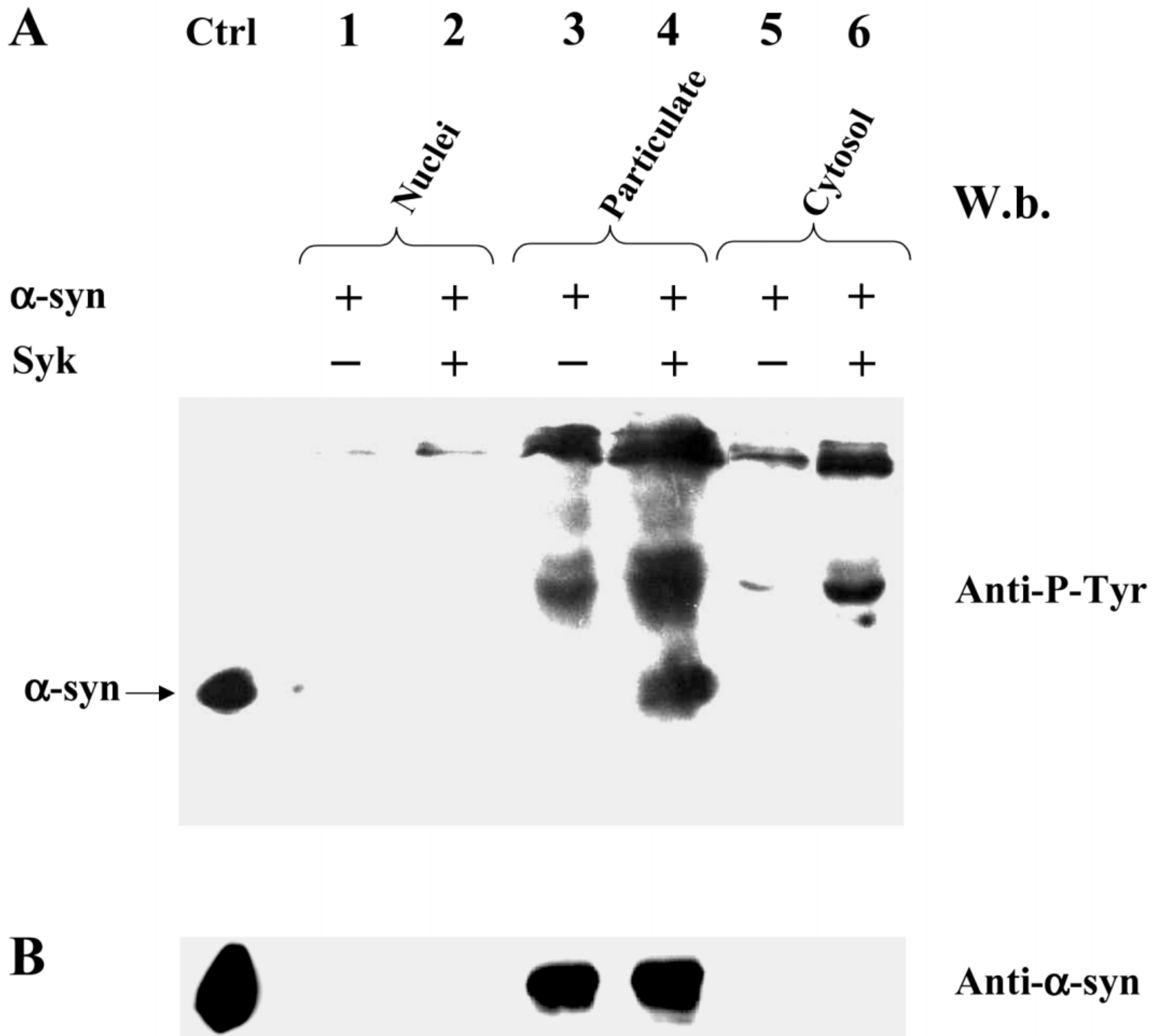

Figure 7. Tyr-phosphorylation of α-syn in CHO cells cotransfected with α-syn and Syk tyrosine kinase. CHO cells were grown and transfected either with pcDNAsyn alone (lanes 1, 3, and 5) or pcDNAsyn plus pcDNASyk (lanes 2, 4, and 6) as described in Materials and Methods. Cells were then sonicated in isotonic buffer and nuclei (lanes 1 and 2), particulate fraction (lanes 3 and 4), and cytosol (lanes 5 and 6) were separated by means of differential centrifugation. The different fractions (50 µl) and 5 ng of α-syn phosphorylated by Syk in vitro (Ctrl) were loaded on SDS-PAGE and immunostained with anti-P-Tyr antibody **(A)**. Blots were then stripped and reprobed with anti-α-syn antibody **(B)**. Three separate experiments are represented.

Fig. 8

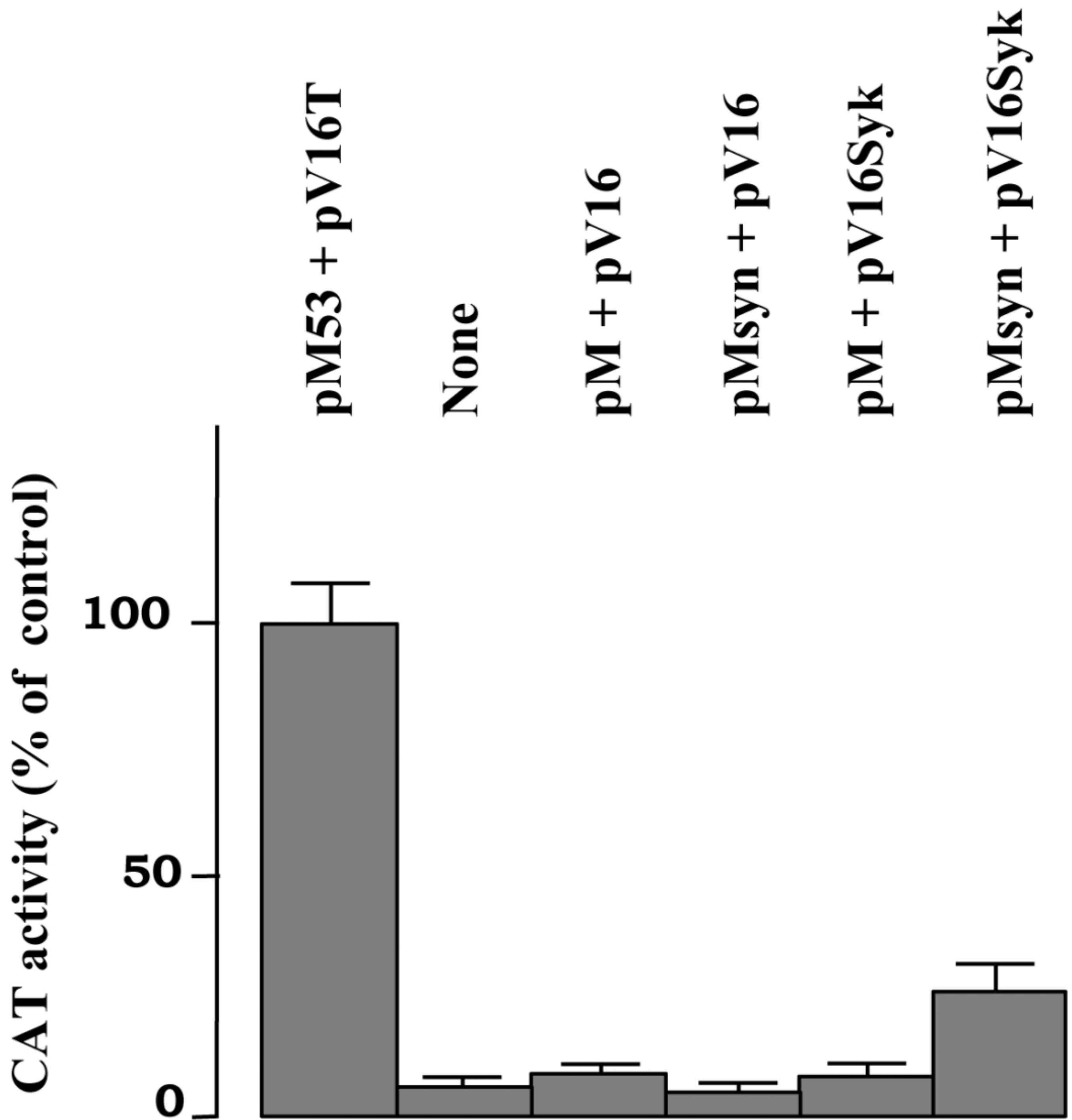

Figure 8. Interaction of human α-syn and porcine Syk tyrosine kinase in transiently transfected mammalian cells. Human neuroblastoma cells were transfected with pG5CAT plasmid (4 μg) and the indicated plasmids. Cell extracts were prepared and CAT activity was determined 48 h after transfection. CAT activity is reported relative to that found in control cells (100%) transfected with plasmids pM53 and pV16.