Synucleins Are a Novel Class of Substrates for G Protein-coupled Receptor Kinases*

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G protein-coupled receptor kinases (GRKs) specifically recognize and phosphorylate the agonist-occupied form of numerous G protein-coupled receptors (GPCRs), ultimately resulting in desensitization of receptor signaling. Until recently, GPCRs were considered to be the only natural substrates for GRKs. However, the recent discovery that GRKs also phosphorylate tubulin raised the possibility that additional GRK substrates exist and that the cellular role of GRKs may be much broader than just GPCR regulation. Here we report that synucleins are a novel class of GRK substrates. Synucleins (α, β, γ, and synoretin) are 14-kDa proteins that are highly expressed in brain but also found in numerous other tissues. α-Synuclein has been linked to the development of Alzheimer’s and Parkinson’s diseases. We found that all synucleins are GRK substrates, with GRK2 preferentially phosphorylating the α and β isoforms, whereas GRK5 prefers α-synuclein as a substrate. GRK-mediated phosphorylation of synuclein is activated by factors that stimulate receptor phosphorylation, such as lipids (all GRKs) and Gγβ subunits (GRK2/3), suggesting that GPCR activation may regulate synuclein phosphorylation. GRKs phosphorylate synucleins at a single serine residue within the C-terminal domain. Although the function of synucleins remains largely unknown, recent studies have demonstrated that these proteins can interact with phospholipids and are potent inhibitors of phospholipase D (PLD2) in vitro. PLD2 regulates the breakdown of phosphatidylcholine and has been implicated in vesicular trafficking. We found that GRK-mediated phosphorylation inhibits synuclein’s interaction with both phospholipids and PLD2. These findings suggest that GPCRs may be able to indirectly stimulate PLD2 activity via their ability to regulate GRK-promoted phosphorylation of synuclein.

G protein-coupled receptor kinases (GRKs)† are involved in the regulation of G protein-coupled receptor (GPCR) signaling (1, 2). GRKs specifically recognize and phosphorylate agonist-occupied GPCRs. Receptor phosphorylation and subsequent binding of another protein, arrestin, uncouples activated receptor from G protein. These events can also promote receptor endocytosis. Internalized receptors are then either dephosphorylated and recycled back to the cell surface or targeted to lysosomes for degradation. The seven mammalian GRKs that have been identified can be divided into three subfamilies based on their overall structural organization and homology: GRK1 (rhodopsin kinase) and GRK7; GRK2 (βARK1) and GRK3 (βARK2); and GRK4, GRK5, and GRK6. Common features shared by the GRKs include a centrally localized catalytic domain of ~270 amino acids, an N-terminal domain of ~190 amino acids that has been implicated in receptor interaction and GRK regulation, and a variable length C-terminal domain of 105–233 amino acids that is involved in phospholipid association.

Until recently, GPCRs were considered to be the only natural substrates for GRKs. Common protein kinase substrates, such as casein, phosvitin, and synthetic peptides are relatively poor substrates for GRKs. Even inactive GPCRs are not phosphorylated well. These observations suggest that GRKs might be highly specialized kinases, which phosphorylate only activated receptors. However, several laboratories recently demonstrated that GRKs can also bind and effectively phosphorylate the cytoskeletal protein tubulin (3–5). Interestingly, GRK-mediated phosphorylation of tubulin appears to be stimulated by β2-adrenergic receptor activation (3). In addition, GRK6 appears to selectively phosphorylate the Na+/H+ exchanger regulatory factor via a PDZ domain-mediated interaction (6). Although the physiological role for GRK-mediated phosphorylation of tubulin and the Na+/H+ exchanger regulatory factor is unclear at present, these examples suggest that GRKs may have a broader substrate selectivity than previously assumed and thus play a wider role in signaling than previously appreciated.

In this study, we explored the possibility that additional nonreceptor GRK substrates exist. In our search for such substrates, we identified synucleins as a novel family of GRK substrates. Synucleins have been linked to the development of neurodegenerative diseases, such as Alzheimer’s and Parkinson’s diseases, and may be involved in regulating intracellular vesicular trafficking (7). We identified the site phosphorylated by GRKs in α-synuclein and demonstrated that this phosphorylation inhibits the ability of α-synuclein to interact with phospholipids and results in reduced inhibition of phospholipase D2 (PLD2).

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, the Expand High Fidelity PCR System, and other molecular biology reagents were purchased...
Phosphorylation of Synucleins by GRKs

Phosphorylation of Purified Synucleins—Human cDNAs for α-, β-, and γ-synucleins in Escherichia coli expression plasmids were purified from bovine brain as described (11) with several modifications. Bovine brain supernatant was prepared by sonicating the bovine brain homogenate (20 mM Tris-HCl, pH 8.0, 4 mM MgCl2) with 0.1 mM [γ-32P]ATP (1,000 cpm/pmol). Liposomes (1 mg/ml phospholipid) were included in some reactions. Reactions were carried out for 30 min at 30 °C, stopped with SDS sample buffer, and electrophoresed on a 13% SDS-polyacrylamide gel. Proteins were visualized by autoradiography.

Partial Purification and Sequencing of p19 GRK Substrate—All purification steps were performed at 4 °C. Ten ml of bovine protein extract was diluted to 20 ml with 20 mM Tris-HCl, pH 8, and loaded on a 3-ml Q Sepharose column equilibrated with 20 mM Tris-HCl, pH 8, 4 mM MgCl2 (with 0.1 mM [γ-32P]ATP (1,000 cpm/pmol). Liposomes (1 mg/ml phospholipid) were included in some reactions. Reactions were carried out for 30 min at 30 °C, stopped with SDS sample buffer, and electrophoresed on a 13% SDS-polyacrylamide gel. Proteins were visualized by autoradiography.

To confirm the identity of the residue phosphorylated by GRKs, we generated a mutant α-synuclein with Ser129 replaced with alanine using PCR. Homologous serine residues were similarly replaced in β-synuclein (Ser124) and γ-synuclein (Ser25). The PCR-derived portions of the constructs were then sequenced in their entirety using the dideoxy chain termination method. The mutant proteins were expressed in E. coli, purified, and compared with wild type synucleins in GRK-mediated phosphorylation assays.

Separation of Unphosphorylated and Phosphorylated Forms of α-Synuclein—Synucleins were phosphorylated using bovine brain as described above. Following the last centrifugation, the supernatant was brought to 0.5 M NaCl and heated in a boiling water bath for 10 min. Following further centrifugation (200,000 × g, 10 min), the pellet was resuspended in 0.5 M NaCl and heated in a boiling water bath for 10 min. Following further centrifugation (200,000 × g, 10 min), the supernatant was pooled and applied on a 1-ml Mono Q FPLC column equilibrated with 50 mM NaCl in buffer C at a flow rate of 1 ml/min, and 0.5-ml fractions were collected. An aliquot of each fraction (10 µl) was electrophoresed on a 13% SDS-polyacrylamide gel, and proteins were visualized by Coomassie Blue staining and autoradiography.

Transfection of COS-1 Cells and Assessment of α-Synuclein Phosphorylation—An expression plasmid for α-synuclein was constructed by cloning the coding sequence of human α-synuclein in the vector pcDNA3. COS-1 cells were grown to ~80–90% confluence in six-well dishes (35-mm wells) at 37 °C in a humidified atmosphere containing 5% CO2, 95% air in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% streptomycin. Cells were transiently transfected using FuGENE (Roche Molecular Biochemicals) with 2 µg of pcDNA3 containing cDNA for β-galactosidase (lacZ) or co-transfected with 1 µg of vector containing cDNA for α-synuclein and 1 µg of pcDNA3 with cDNA for either β-galactosidase, GRK2, or GRK5. For metabolic labeling, 42 h after transfection, 20 µl of [35S]methionine was added to 1 ml of the media directly above the cells. Cells were harvested as described above, and 1 ml of the cell lysate was analyzed by SDS-PAGE. The resolved α-synuclein was visualized by autoradiography.
Fig. 1. Identification and partial purification of a novel GRK substrate from brain protein extract. A, aliquots of bovine brain protein extract were incubated with [γ-32P]ATP either in the absence (none) or presence of recombinant GRK2 or GRK5. Liposomes (lipo) were added where indicated. Proteins were then separated using SDS-PAGE and visualized by autoradiography. Positions of protein molecular mass standards are indicated at the left. The position of autoposphorylated GRK5 is shown on the right. Note a protein (p19) whose phosphorylation is significantly increased in the presence of GRKs. A protein of ~50 kDa, which is also phosphorylated in the presence of GRKs, is most likely tubulin. B, proteins from bovine brain extract were separated on Q Sepharose. Aliquots of fractions were incubated with [γ-32P]ATP either in the absence or presence of recombinant GRK5. Proteins were then separated using SDS-PAGE and visualized by autoradiography.

RESULTS

Phosphorylation and Partial Purification of a Novel GRK Protein Substrate from Brain—To identify novel GRK substrates, we decided to use phosphorylation in the absence and presence of purified GRKs as a test assay. Because GRKs are known to be regulated by phospholipids, which activate phosphorylation of receptor substrates, liposomes were also included in some phosphorylation reactions. Brain protein extract prepared in the presence of a low detergent concentration was used as a potential source of protein substrates. As shown in Fig. 1A, many proteins are phosphorylated in brain extract even in the absence of added GRKs due to a large number of endogenous protein kinases and substrates. However, several protein bands can be identified that appear to be phosphorylated only in the presence of GRKs. Phosphorylation of one band (“p19”) by GRK2 and especially GRK5 was strikingly stimulated in the presence of liposomes (Fig. 1A). To purify this substrate, brain extract proteins were separated using two chromatographic resins. Eluted fractions from Q Sepharose were analyzed for the presence of proteins phosphorylated in a GRK-dependent manner (Fig. 1B). After the second purification step (gel filtration on Sephacryl S200, not shown), p19 protein could be sufficiently well separated from other proteins using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by Coomassie Blue staining. The N terminus of p19 was modified, precluding its direct amino acid sequencing; however, cyanogen bromide treatment of the protein generated one major fragment, which was isolated and successfully microsequenced. A data base search with this sequence (MKGL-SKAKEGV) yielded a 100% match with α-synuclein (11).

Phosphorylation of Purified Synucleins by GRKs and Other Kinases—Human α-synuclein belongs to a family of proteins, which currently consists of four members (α-, β-, and γ-synuclein and synoretin) (7, 10). To confirm that α-synuclein is a GRK substrate, human synuclein proteins were overexpressed and purified from E. coli. Purified synucleins were then incubated with GRK2 (+Gβγ) or GRK5 in the absence or presence of liposomes (Fig. 2). We also tested other kinases that have been shown to phosphorylate synucleins. Calmodulin-dependent protein kinase II (CaMKII) was reported to phosphorylate β-synuclein (17), whereas recent studies demonstrated that α-synuclein can be phosphorylated by the casein kinases CK1 and CK2 (18). Fig. 2 reveals that different kinases show different patterns of synuclein phosphorylation.

Both GRK2 and GRK5 phosphorylate α-synuclein very efficiently, and this phosphorylation is enhanced ~6~8-fold in the presence of liposomes. As reported (18), both CK1 and CK2 also phosphorylate α-synuclein (Fig. 2). Interestingly, α-synuclein phosphorylation by CK2 but not CK1 was stimulated by phospholipids. It appears that α-synuclein is not a substrate for CaMKII.

β-Synuclein is phosphorylated best by GRK2, whereas GRK5 phosphorylates it less efficiently. In both cases, β-synuclein phosphorylation is enhanced in the presence of phospholipids (Fig. 2). β-Synuclein appears to be a poor substrate for CK1, CK2, and CaMKII. GRKs can also phosphorylate γ-synuclein, albeit more slowly than α-synuclein (Fig. 2). This phosphorylation is increased in the presence of liposomes. Interestingly, in the case of GRK5,
phospholipids appear to stimulate phosphorylation of γ-synuclein residue(s) distinct from ones phosphorylated by other kinases. Phosphorylation of this site(s) noticeably reduces electrophoretic mobility of γ-synuclein (Fig. 2). CK1 does not phosphorylate γ-synuclein, whereas CK2 phosphorylates it very efficiently. Unlike phosphorylation of α-synuclein, γ-synuclein phosphorylation by CK2 is not enhanced by phospholipids. CaMKII phosphorylates γ-synuclein more efficiently than β-synuclein, and this phosphorylation appears to be inhibited in the presence of liposomes.

Synoretin, a synuclein that was originally cloned from a retinal library and is closely related to γ-synuclein, is phosphorylated by GRK2 and GRK5 in a manner similar to the phosphorylation of γ-synuclein (Fig. 2). In contrast to γ-synuclein, however, synoretin appears to be a very poor substrate for CK2 and CaMKII.

GRKs appear to interact with synucleins with a relatively high affinity. Both GRK2 and GRK5 phosphorylate all synucleins with a similar \( K_m \) of \(-1 \mu M\) (comparable with the 1–4 \( \mu M \) \( K_m \) for rhodopsin phosphorylation by GRKs). We have also tested the ability of GRK1 and GRK6 to phosphorylate synucleins. Both kinases can phosphorylate synucleins with a pattern of substrate preference similar to that of GRK5 (data not shown). Thus, it appears that synuclein phosphorylation can be facilitated by all members of the GRK family.

**Phosphorylation of α-Synuclein in COS-1 Cells**—To determine whether GRKs are able to phosphorylate synucleins in cells, α-synuclein was transiently expressed in COS-1 cells in the absence or presence of co-expressed GRKs. Cells were labeled with \(^{32}P\)orthophosphate, lysed, and radioactive proteins were immunoprecipitated with the α-synuclein antiserum. A weak signal that may represent endogenous α-synuclein was detected in control cells (Fig. 3). When α-synuclein was overexpressed, a significant increase in \(^{32}P\)-labeled α-synuclein was detected. This basal synuclein phosphorylation is similar to the one reported in 293 and PC12 cell lines (18) and might be attributed to CK1, CK2, GRK, or a yet to be identified protein kinase. Co-expression of either GRK2 or GRK5 caused a noticeable increase in synuclein phosphorylation (2.4 ± 0.3-fold compared with cells overexpressing α-synuclein alone), demonstrating that GRKs can phosphorylate synucleins in intact cells.

**Regulation of GRK-mediated Synuclein Phosphorylation**—As shown in Figs. 1 and 2, synuclein phosphorylation by GRKs in vitro is enhanced by phospholipids. The magnitude of this stimulation is dose-dependent and saturable (Fig. 4A). The concentration of phospholipids at which a half-maximal rate (EC\(_{50}\)) of α-synuclein phosphorylation by GRK5 is observed is \(-0.04 \text{ mg/ml}\).

Interestingly, phospholipids stimulate synuclein phosphorylation by GRK2 only in the presence of Gβγ (Fig. 4B). When added alone, either phospholipids or Gβγ actually inhibit GRK2-mediated synuclein phosphorylation by \(-50–60\%\). However, when liposomes and Gβγ are both included in the reaction, they activate synuclein phosphorylation \(-9\)-fold over basal level \((-20\%\) fold over phosphorylation in the presence of either phospholipids or Gβγ alone). Thus, the regulation of synuclein phosphorylation by GRK2 is similar to the regulation of receptor phosphorylation that appears to require synergistic action of Gβγ and phospholipids (1, 2).

Several laboratories have demonstrated that GRKs are regulated by the Ca\(^{2+}\)-binding protein calmodulin (8, 19, 20). Receptor phosphorylation by GRKs can be almost completely inhibited in the presence of sufficient calmodulin concentrations. The most sensitive GRK subtype is GRK5 with an IC\(_{50}\) for calmodulin inhibition of rhodopsin phosphorylation of \(-40\)
Phosphorylated by GRKs. As reported (18), S129A mutation phosphorylate exclusively serine residues in Phosphoamino acid analysis demonstrated that GRKs phosphorylate at least two separate sites in these proteins. Thus, it appears that calmodulin may have a dual effect on GRK5; it may inhibit phosphorylation of some substrates (receptors) while activating phosphorylation of others (autophosphorylation, α-synuclein). However, the stimulatory effect of calmodulin on GRK-mediated synuclein phosphorylation is not universal. Calmodulin had very little effect on γ-synuclein phosphorylation by GRK5, and it slightly inhibited phosphorylation of α-synuclein by GRK2 (data not shown).

Identification of the GRK Phosphorylation Site in α-Synuclein—Phosphorylation analysis revealed that GRK-mediated phosphorylation of α- and β-synucleins is saturable with a stoichiometry approaching 1 phosphate per molecule (data not shown). GRK5 phosphorylation of γ-synuclein and synoretin in the presence of lipids, although slower, can incorporate more than 1 phosphate per molecule, indicating that GRK5 can phosphorylate at least two separate sites in these proteins. Phosphoamino acid analysis demonstrated that GRKs phosphorylate exclusively serine residues in α-synuclein. In β-synuclein, over 95% of radioactivity is incorporated into phosphoserine, with a very small amount of phosphate also incorporated into phosphothreonine (data not shown). These data suggest that GRKs most likely phosphorylate a single serine residue in α- and β-synuclein, whereas GRK5 phosphorylates an additional residue in γ-synuclein and synoretin in the presence of lipids.

To identify specific residue(s) phosphorylated by GRKs, α-synuclein was incubated with GRK2 in the presence of GTPyS and liposomes either in the absence or in the presence of ATP. Samples then were digested with trypsin and analyzed using SELDI mass spectrometry. This analysis demonstrated that one additional peptide peak was present in the synuclein sample incubated with GRK2 and ATP (data not shown). The molecular mass of this fragment is exactly 79 Da (the mass of a phosphate residue) higher than the mass of the fragment in the control sample (no ATP). The mass of this peptide fragment (4289 Da) matches the calculated mass (4288.43 Da) of the extreme C-terminal fragment of α-synuclein generated by tryptic digest (last 38 amino acid residues). This fragment contains a single serine residue (Ser129).

To confirm the identity of the residue phosphorylated by GRKs, we generated a mutant α-synuclein with Ser129 replaced with alanine. The mutant protein was purified and compared with wild type α-synuclein for GRK-mediated phosphorylation. Fig. 5A demonstrates that mutation of Ser129 abolishes α-synuclein phosphorylation by both GRK2 and GRK5. This result confirms that Ser129 is the only residue in α-synuclein phosphorylated by GRKs. As reported (18), S129A mutation also significantly (~90%) inhibited phosphorylation of α-synuclein by CK2. However, this mutation reduced CK1-mediated phosphorylation of α-synuclein by only ~35%, indicating that the major CK1 phosphorylation site lies elsewhere.

Because the serine residue phosphorylated by GRKs in α-synuclein is conserved in all synucleins (Fig. 5B), we also replaced homologous serines in β- (Ser118) and γ-synuclein (Ser124) with alanines. Mutation of Ser118 practically abolishes β-synuclein phosphorylation by both GRK2 and GRK5 and inhibits phosphorylation by CK2 by ~80% (Fig. 5A). It is unclear whether this mutation has any effect on CaMKII activity, because phosphorylation of both wild type and mutant β-synucleins by CaMKII was very inefficient.

Mutation of Ser124 dramatically inhibits γ-synuclein phosphorylation by GRK2, CK2, and CaMKII (Fig. 5A). However, when phosphorylated by GRK5 in the presence of phospholipids, this mutation prevents generation of only the fastest moving phosphorylated γ-synuclein form (Fig. 5A), which is the same form observed when γ-synuclein is phosphorylated by GRK5 in the absence of lipids (Fig. 2). This confirms that in the presence of phospholipids, GRK5 phosphorylates γ-synuclein residues that are distinct from the residue phosphorylated by other kinases (Ser124).

Separation of Unphosphorylated and Phosphorylated Forms of Synucleins—It has been previously reported that recombinant α- and β-synucleins are eluted from a Mono Q FPLC column at different salt concentrations (11). While purifying synucleins from bovine brain, we noticed that in addition to the major peak of α-synuclein that elutes at ~230 mM NaCl, there is a smaller peak of α-synuclein that elutes with β-synuclein at a higher salt concentration (~260 mM NaCl, fractions 17 and 18, Fig. 6A). We reasoned that this second peak of α-synuclein might be binding more strongly to the anion exchange resin because of its higher negative charge due to phosphorylation. To confirm that unphosphorylated and phosphorylated forms of synuclein can be separated, α-synuclein was phosphorylated in the presence of GRK5 and [γ-32P]ATP and chromatographed on a Mono Q column. Fig. 6B shows that unphosphorylated recombinant α-synuclein is eluted as a single peak (at ~240 mM NaCl), whereas synuclein that was phosphorylated in the presence of GRK5 is eluted as two separate protein peaks (at ~240 and 270 mM NaCl). The peak eluting at a higher salt concent-
purified recombinant α-synuclein was eluted with a linear gradient from 150 to 350 mM NaCl. An aliquot of each fraction was electrophoresed on a 13% SDS-polyacrylamide gel, and proteins were visualized by Coomassie Blue staining. B and C, purified recombinant α-synuclein was incubated with [γ-32P]ATP and liposomes either in the absence (B) or presence (C) of GRK5. Reactions were then loaded on a Mono Q FPLC column. Bound proteins were eluted with a linear gradient from 150 to 350 mM NaCl. An aliquot of each fraction was electrophoresed on a 13% SDS-polyacrylamide gel, and proteins were visualized by silver staining and autoradiography.

Effect of Phosphorylation on α-Synuclein Function—One of the known biochemical properties of α-synuclein is its ability to interact with acidic phospholipids (21). To study synuclein interaction with phospholipids, we measured α-synuclein co-sedimentation with liposomes. We found that the amount of α-synuclein that was sedimented was dependent on the quantity of phospholipid in the incubation; we were thus able to determine EC50 values for α-synuclein binding to phospholipid. Half-maximal binding of α-synuclein occurred at ~0.2 mg/ml phospholipids (Fig. 7A). The GRK5-mediated phosphorylation of α-synuclein resulted in an ~2-fold loss of phospholipid binding affinity with an EC50 of ~0.4 mg/ml phospholipid.

It has been recently demonstrated that α- and β-synucleins are potent inhibitors of PLD2 in vitro (16). We thus tested the effect of phosphorylation on the ability of synucleins to inhibit PLD2 activity. At a concentration (~20 nM) of unphosphorylated recombinant α-synuclein that inhibited ~70 ± 3% of PLD2 activity, phosphorylated synuclein inhibited only ~47 ± 3% of PLD2 activity (Fig. 7B). Thus, the remaining PLD2 activity in the presence of phosphorylated synuclein was ~75% higher than in the presence of unphosphorylated synuclein (53 and 30%, respectively). Similar results were observed for β-synuclein phosphorylated by GRK2 (data not shown). Therefore, GRK5-mediated phosphorylation appears to inhibit 1) synuclein’s ability to interact with phospholipids and 2) synuclein-mediated inhibition of PLD2.

DISCUSSION

A significant role of GRKs in the regulation of GPCR-mediated signaling is well established (1, 2). Many experiments, which include gene-targeted knockouts and transgenic protein overexpression, demonstrated the importance of GRK-mediated phosphorylation in receptor desensitization. However, our understanding of GRK function is far from complete, and research continues to uncover novel ways of GRK regulation as well as new functions for these kinases. For example, the identification of an RGS homology domain in GRKs (22) led to the discovery that GRK2 and GRK3 can specifically interact with activated αvβ3 and inhibit αvβ3-mediated signaling (23).

Other recent studies have shown that GRK phosphorylation is not limited to activated receptors as substrates. GRKs can also efficiently phosphorylate nonreceptor protein substrates, such as Na+/H+ exchanger regulatory factor (6) and tubulin (3–5). Although the role for this phosphorylation is undetermined, phosphorylation of tubulin suggests that GRKs might be involved in regulating microtubule dynamics and cytoskeletal reorganization. The cytoskeleton plays an important role in assembly of signaling networks and is also involved in such processes as endocytosis and vesicular trafficking. It is possible that GRK-mediated tubulin phosphorylation during a signaling event activates rearrangement of microtubules, thus contributing to receptor internalization, recycling, or degradation. It is also possible that GRKs might be involved in the regulation of signaling and cytoskeleton dynamics by phosphorylating additional protein substrates.

In our search for novel nonreceptor GRK substrates, we identified α-synuclein as a protein phosphorylated by GRKs in

![Image](http://www.jbc.org/)

**Fig. 6.** Separation of unphosphorylated and phosphorylated forms of α-synuclein. A, synucleins were purified from bovine brain as described (13). Fractions after Q Sepharose that contained synucleins were pooled and applied on a Mono Q FPLC column. Bound proteins were eluted with a linear gradient from 150 to 350 mM NaCl. An aliquot of each fraction was electrophoresed on a 13% SDS-polyacrylamide gel, and proteins were visualized by Coomassie Blue staining. B and C, phosphorylated recombinant α-synuclein was incubated with [γ-32P]ATP and liposomes either in the absence (B) or presence (C) of GRK5. Reactions were then loaded on a Mono Q FPLC column. Bound proteins were eluted with a linear gradient from 150 to 350 mM NaCl. An aliquot of each fraction was electrophoresed on a 13% SDS-polyacrylamide gel, and proteins were visualized by silver staining and autoradiography.

**Fig. 7.** Effect of phosphorylation on α-synuclein activity. Purified α-synuclein was phosphorylated by GRK5, and unphosphorylated and phosphorylated forms were separated as described in Fig. 6. A, effect of phosphorylation on α-synuclein binding to liposomes. Unphosphorylated (syn) and phosphorylated (P-syn) synucleins were incubated with or without the indicated concentration of liposomes. The mixtures were then pelleted at 100,000 rpm, supernatant fractions were removed, and pellets were resuspended in the reaction buffer. Equal aliquots of both fractions were separated by SDS-PAGE, and proteins were visualized by silver stain. Optical density of the developed bands was determined by densitometry, and the amount of GRK5 pelleted was expressed as a percentage of the total. In the absence of liposomes ~10–15% of total α-synuclein was pelleted. B, effect of phosphorylation on the ability of α-synuclein to inhibit PLD2. PLD2 activity was measured in the presence of either unphosphorylated (syn) or phosphorylated (P-syn) α-synuclein. The data are presented as a percentage of PLD2 activity in the absence of synuclein.
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d a brain extract. α-Synuclein belongs to a family of small pro-
teins (127–140 amino acids), which currently includes four
members (α-, β-, and γ-synucleins and synoretin) (7, 10). Synucleins are expressed at high levels in the brain and at
lower levels in many tissues and common cell lines such as 293
d and COS. α-Synuclein has been linked to the development of
neurodegenerative disease. For example, a fragment of
α-synuclein is a component of senile plaques of Alzheimer’s
disease patients. α-Synuclein is also found within Lewy bodies
inside neurons of Parkinson’s disease patients. Indeed, two
independent autosomal mutations (A53T and A30P) in the
α-synuclein gene have been linked to the early onset inherited
forms of Parkinson’s disease in two families (24, 25). Moreover,
overexpression of human α-synuclein in transgenic mice re-
sulted in progressive accumulation of Lewy body-like struc-
tures in neurons and a loss of dopaminergic terminals in the
basal ganglia (26). Another member of the synuclein family,
γ-synuclein, may play a role in the etiology of breast cancer
(27). Whereas the biological role of synucleins is not yet de-
defined, recent studies have begun to shed some light on their
functions. For example, synucleins have the ability to form
fibrils in vitro (28). The accelerated fibril formation by mutant
synucleins may contribute to the development of neurodegen-
erative diseases. The intracellular localization of synucleins
is also not well defined. Although some studies have shown that
synucleins are cytosolic proteins, others have demonstrated that
synucleins are associated with synaptic vesicles (7) and
other membranes (29). α-Synuclein was shown to interact with
phospholipids. This interaction is facilitated mainly by a con-
served N-terminal domain (amino acid residues 1–94), which
changes its structure from “unfolded” to α-helical upon binding
to lipids (21). Because of synuclein’s ability to interact with
lipids and their association with synaptic vesicles, it has been
suggested that synucleins might be involved in intracellular
vesicular trafficking (7).

Recent studies have identified some proteins that can inter-
act with synucleins. Synucleins can bind to 14-3-3 proteins,
protein kinase C, BAD, and Erk (30). They are also potent
inhibitors of PLD2 in vitro (16), whereas overexpression of
synoretin activates Elk-1 transcription factor (10). It has been
also suggested that synucleins can be regulated by phosphor-
ylation (7, 16), since bovine β-synuclein occurs as a phospho-
protein and can be phosphorylated in vitro by CaMKII (17).
Recently, it has been shown that α-synuclein is phosphorylated in
cells and that phosphosynuclein is rapidly dephosphorylated by
okadaic acid-sensitive protein phosphatases (18). This study
also demonstrated that the casein kinases CK1 and CK2 can
phosphorylate α-synuclein in vitro. However, whether these
kinases phosphorylate other synuclein subtypes and what reg-
ulates phosphorylation and the consequences of synuclein
phosphorylation have not been determined.

Here we demonstrated that all four synucleins are sub-
strates for GRKs in vitro. All tested GRKs can phosphorylate
synucleins, albeit with different efficiency. α-Synuclein ap-
ppears to be the best substrate for all GRKs, whereas
γ-synuclein and synoretin are phosphorylated significantly
slower. Of all tested kinases, GRK2 phosphorylates β-synuclein
most efficiently. Other kinases can also phosphorylate synucle-
ins. As has been reported, both CK1 and CK2 phosphorylate
α-synuclein. In addition, CK2 can effectively phosphorylate
γ-synuclein but not β-synuclein or synoretin. CaMKII phos-
phorylates γ-synuclein best, although not very efficiently. Co-expression of α-synuclein with GRKs in COS-1 cells dem-
strated that GRK2 and GRK5 can also phosphorylate this
protein in cells.

We determined that synuclein phosphorylation by GRKs can
be regulated by some of the factors that are also known to
regulate receptor phosphorylation. Synuclein phosphoryla-
tion by all GRKs is enhanced by phospholipids. For GRK2, this
effect is dependent on the presence of Gβγ. Because Gβγ is
believed to be released after receptor activation, it is plausible
that activation of receptors by extracellular stimuli may lead to
synuclein phosphorylation via activated GRK2. Because both
GRK and synuclein can bind to liposomes, the mechanism for
the lipid-mediated increase in synuclein phosphorylation is
unclear. One possibility is that synucleins undergo a confor-
mational change when they bind to phospholipids, making them
a better substrate for GRKs. Another possibility is that phos-
pholipids directly activate the catalytic activity of GRKs. The latter
seems more likely, since the EC50 (0.04 mg/ml phospholipid) for
GRK5-mediated α-synuclein phosphorylation by liposomes
close matches the EC50 for GRK5 binding to liposomes (31)
and is significantly lower than the EC50 for α-synuclein binding
to phospholipids (Fig. 7A). Interestingly, liposomes also en-
harce the phosphorylation of α-synuclein by CK2. However,
this effect is not universal; phospholipids have no effect on
α-synuclein phosphorylation by CK1 or γ-synuclein phos-
phorylation by CK2.

Surprisingly, phosphorylation of α-synuclein by GRK5 is also
activated by Ca2+/calmodulin, which is known to potently in-
hbit GRK5-mediated receptor phosphorylation (8, 19). This
finding suggests that regulation of GRK5 by calmodulin may be
more complex than previously thought. Calmodulin may not
only regulate the catalytic activity of GRK5, but it may also
change the substrate specificity of the kinase. Calmodulin-de-
pendent activation of phosphorylation appears to be GRK and
synuclein subtype-specific. Synuclein phosphorylation by
GRK2 was weakly inhibited in the presence of calmodulin,
whereas GRK5 phosphorylation of γ-synuclein was virtually
unaffected. γ-Synuclein, on the other hand, is phosphorylated
by CaMKII in a calmodulin-dependent manner. Thus, calmod-
ulin activation in cells may lead to phosphorylation of different
synuclein subtypes depending on which kinase is expressed in
a particular cell type. Assuming that different synucleins have
distinct functions, calmodulin-dependent synuclein phos-
phorylation may have different functional effects.

GRKs appear to phosphorylate a single site in the C-tail of
synucleins (Ser129 in α-, Ser18 in β-, and Ser124 in γ-synuclein,
respectively). In α-synuclein, this is the same residue that is
degenerously phosphorylated in 293 cells (18). In some
synuclein subtypes, these sites can also be phosphorylated by
other kinases, such as CK1, CK2, or CaMKII. C-terminal re-

gions of synucleins vary significantly from each other. How-
ever, the serine residue phosphorylated by GRKs in synucleins
appears to be rather conserved (Fig. 5B). In all synucleins, this
residue is surrounded by several acidic residues. Interestingly,
earlier studies with peptide substrates suggested that GRKs
may prefer different environments for the residues they use as
substrates; GRK2 prefers an acidic environment, whereas
GRK5 prefers an uncharged or possibly basic environment (13).
Contrary to this notion, however, GRK5 phosphorylates
α-synuclein as efficiently as GRK2. Nevertheless, both the sur-
rounding residues and more distant parts of the molecule un-
doubtedly play an important role in determining substrate
specificity of the kinase. For example, β-synuclein, which is
most closely related to α-synuclein and which is a good sub-
strate for GRK2, is a very poor substrate for CK2. Interest-
gingly, phospholipids also stimulate GRK5 phosphorylation of
residues in γ-synuclein and synoretin that are distinct from the
residue phosphorylated by other kinases (Ser124). It seems
likely that these additional phosphorylation sites are exposed
when synucleins undergo a conformational change upon bind-

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ing to liposomes. The identity and function of these sites is under investigation.

Although the normal function of synuclein is not clear, some data indicate that synucleins may play a role in the regulation of intracellular vesicular trafficking and signaling. A recent report suggests that α-synuclein may be involved in desensitization of dopamine signaling (32). Mice lacking α-synuclein display a standard pattern of dopamine discharge and receptor levels in response to simple electrical stimulation. However, they exhibit an increased release with paired stimuli, supporting the idea that α-synuclein is a negative regulator of dopamine neurotransmission, possibly by modulating the refilling rate of the readily releasable synaptic vesicles. This modulation may involve either a direct interaction of synuclein with vesicles or regulation of an enzyme activity such as PLD2. Here we demonstrated that GRK-mediated phosphorylation inhibits both α- and γ-synuclein’s ability to interact with lipids and modulate the activity of PLD2. It is likely that the reduction in synuclein’s ability to inhibit PLD2 is due to the reduced affinity of phosphorylated synuclein for phospholipids. One can speculate about the possible role of GRK-mediated synuclein phosphorylation in signal regulation. PLD catalyzes the hydrolysis of phosphatidic acid to form phosphatic acid and diacylglycerol. Phosphatidic acid has been shown to stimulate vesicle formation aiding in receptor endocytosis. Activated GRKs can also phosphorylate synuclein, thus releasing inhibition of PLD2. Activation of PLD2 causes rearrangement of actin cytoskeleton and vesicle formation aiding in receptor endocytosis and/or recycling.

An additional mechanism by which synuclein phosphorylation might be linked to regulation of cytoskeleton comes from a recent report that α-synuclein can bind to tau protein and promote tau phosphorylation by protein kinase A (34). Tau is involved in the regulation of microtubule dynamics. Interestingly, the synuclein residue that is phosphorylated by GRKs and CKs lies inside the region (residues 87–140) that interacts with tau. Thus, it is possible that synuclein phosphorylation affects its interaction with tau and alters the dynamics of microtubule assembly. GRK phosphorylation of tubulin may also directly affect tubulin cytoskeleton formation. The findings that GRKs can directly interact with cytoskeletal proteins such as tubulin and actin (3–5) and the identification of synucleins as GRK substrates suggest an important role for GRKs in the regulation of cytoskeletal dynamics. Identification of novel GRK substrates may help us discover yet unknown cellular functions for these kinases.

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