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Contribution of endogenous G-protein-coupled receptor kinases to Ser129 phosphorylation of α-synuclein in HEK293 cells

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ABSTRACT

The majority of α-synuclein (αS) deposited in Lewy bodies, the pathological hallmark of Parkinson's disease (PD), is phosphorylated at serine 129 (Ser129). Ser129 phosphorylation of αS has been demonstrated to enhance the αS toxicity to dopaminergic neurons in a *Drosophila* model of PD. Phosphorylation of αS at Ser129 seems to play a crucial role in the pathogenesis of PD. Here, we assessed the contribution of ubiquitously expressing members of the G-protein-coupled receptor kinase family (GRK2, GRK3, GRK5, and GRK6) to Ser129 phosphorylation of αS in HEK293 cells. To selectively reduce the endogenous expression of each member of the GRK family in cells, we used small interfering RNAs. Knockdown of GRK3 or GRK6 significantly decreased Ser129 phosphorylation of αS; however, knockdown of GRK2 or GRK5 did not decrease αS phosphorylation. The results indicate that endogenous GRK3 and GRK6, but not GRK2 or GRK5, contribute to Ser129 phosphorylation of αS in HEK293 cells.

Sporadic Parkinson's disease (PD) is characterized pathologically by loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of intracytoplasmic inclusions (Lewy bodies, LBs) and dystrophic neurites (Lewy neurites, LNs) [1]. α-Synuclein (αS) is the major components of aggregates in LBs and LNs [2]. Immunohistochemical and biochemical studies have revealed that the majority of αS deposited in LBs is phosphorylated at serine 129 (Ser129) [3]. An *in vitro* fibrillization assay demonstrated that phosphorylated αS formed fibrils more readily than non-phosphorylated αS [3]. In cell culture models, Ser129 phosphorylation of αS has been reported to promote the formation of soluble oligomers [4], aggregates [4], and LB-like cytoplasmic inclusions [5]. Chen and Feany have shown that, in a *Drosophila* model of PD, Ser129 phosphorylation of αS by a *Drosophila* homolog of G-protein-coupled receptor kinase 2 (GRK2) significantly enhanced the toxicity of αS to dopaminergic neurons [6]. Therefore, phosphorylation of αS at Ser129 seems to play a crucial role in the pathogenesis of PD.

Previous reports have shown that αS is phosphorylated at Ser129 by casein kinases (CK1 and CK2) [7] and GRKs (GRK2 and GRK5) [8]. Chemical inhibitors specific for CKs inhibited Ser129 phosphorylation of αS in human embryonic kidney (HEK) 293 cells [7] and rat primary cortical neurons [9], indicating that endogenous CKs contributed to the phosphorylation of αS in these cells. On the other hand, Ser129 phosphorylation of αS by GRK2 or GRK5 was demonstrated by the experiments using overexpression of GRK2 or GRK5 in COS-1 cells [8]. Up to now, there has been no direct report showing that endogenous GRKs are involved in the phosphorylation of αS.

The present experiments were carried out to clarify the role of endogenous GRKs in Ser129 phosphorylation of αS in HEK293 cells. We focused on ubiquitously expressing members of the GRK family (GRK2, GRK3, GRK5, and GRK6) and evaluated the role of endogenous GRKs in the phosphorylation of αS by using small interfering RNAs (siRNAs) which selectively target each member of the GRK family.

Materials and methods

Plasmid construction. Human GRK2 cDNA (clone ID #5585846), human GRK6 cDNA (clone ID #4053197), and mouse GRK6 cDNA (clone ID #5031455) were obtained from Open Biosystems (Huntsville, AL, USA). Human GRK5 cDNA has been previously described [4]. Human GRK3 cDNA and mouse GRK5 cDNA were generated by PCR from reverse-transcribed human lymphocyte RNAs and mouse brain RNAs, respectively. All cDNAs were subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA).

Cell culture and transfection. HEK293 cells were maintained in Eagle's minimum essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM L-glutamine (Invitrogen). The HEK293 cell line stably expressing wild-type αS was selected against 1 mg/ml G418 (Invitrogen). For overexpression of each GRK, the cells in six-well plates

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were transfected with 1 μg cDNA using Lipofectamine Plus Reagents (Invitrogen) and harvested at 48 h after transfection. For RNA interference, approximately 30% confluent cells in six-well plates were transfected with 10 nM siRNA using RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocol. We used the 20-nucleotide-long siRNAs for the knockdown of GRK2 (5′-CCGGG AGATCTTCGACTCATA-3′, Qiagen, Hilden, Germany) and the *SMART* pool for a non-silencing control (GE Healthcare, Uppsala, Sweden). We also used the 25-nucleotide-long siRNAs (stealth RNAi, Invitrogen) for the knockdown of GRK3 (5′-CAAGAAACAAGUGACAUCAAC UCUU-3′), GRK5 (5′-UCAGGCAGUAUCGAGUGCUAGGAAA-3′), or GRK6 (5′-CCUGGAGAAAGUGAACAGUAGGUUU-3′). As a non-silencing control for stealth siRNAs, the stealth RNAi negative control medium GC (Invitrogen) was used. At 48 h after transfection, the medium was discarded, and the cells were incubated with a fresh medium containing 20 nM okadaic acid (Wako Chemicals, Osaka, Japan) for 3 h to block the dephosphorylation of αS.

Immunoblotting. For preparation of cell lysates, HEK293 cells were suspended in a buffer [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycerol, 1× protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany), 1 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4] and kept on ice for 30 min. After centrifugation at 12,000*g* for 30 min, the resultant supernatant was collected and stored at −80 °C until use. B6C3F1 mouse midbrain tissue was disrupted by brief sonication in the buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1× protease inhibitor cocktail, 10 mM NaF, 1 mM Na_3VO_4, 1 μM okadaic acid). After centrifugation at 12,000*g* for 30 min, the resultant supernatant was collected and stored at −80 °C until use.

For SDS–PAGE, protein samples were denatured by boiling for 5 min in a Laemmli's sample buffer containing 2.5% 2-mercaptoethanol. They were subjected to a 12.5% SDS–polyacrylamide gel and then transferred to a PVDF membrane (Millipore, Billerica, MA, USA). For the detection of GRK2 and GRK3, we used a 7.5% SDS–polyacrylamide gel. The membrane was probed with the antibodies specific for αS (Syn-1, monoclonal IgG, BD Transduction Laboratories, Franklin Lakes, NJ, USA), phosphorylated αS at Ser129 (psyn#64, monoclonal IgG, Wako), β-actin (AC-15, Sigma), GRK2 (#sc-562, polyclonal IgG, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), GRK3 (#sc-563, polyclonal IgG, Santa Cruz Biotechnology, Inc.), GRK2–3 (#C5/1.1, monoclonal IgG, Millipore), GRK5 (#139, monoclonal IgG, described in Results), GRK6 (#sc-566, polyclonal IgG, Santa Cruz Biotechnology, Inc.), or GRK4–6 (monoclonal IgG, Sigma). The membrane was treated with ECL (GE Healthcare) for the detection of β-actin and GRK2 or ECL plus (GE Healthcare) for the detection of other proteins. The membrane was then visualized using a CCD camera, VersaDog 5000 (Bio-Rad, Hercules, CA, USA). Relative intensities of detected signals were quantified with Quantity one software (Bio-Rad). Results were expressed as the means ± SD. Statistical comparisons were made by the unpaired Student's *t*-test.

Results

Ser129 phosphorylation of αS by overexpression of each member of the GRK family in HEK293 cells

In the present study, we first developed a hamster monoclonal anti-GRK5 antibody (GRK5 #139) against amino acid residues 94–157 of GRK5. As shown in Fig. 1, the #139 antibody identified endogenous GRK5 in both HEK293 cell lysates and mouse brain extracts. Preabsorption of #139 antibody with purified recombinant GRK5 (residues 94–157) protein abolished the staining of the GRK5 band in HEK293 cell lysates and mouse brain extracts. The antibody recognized human and mouse GRK5 with no cross-reactivity with GRK6 (Fig. 1), GRK2 (Figs. 3 and 4), or GRK3 (Figs. 3 and 4).

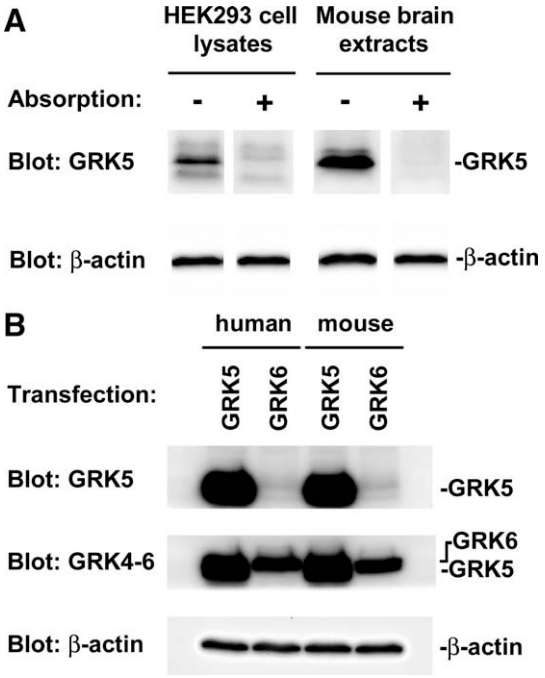

Fig. 1. Characterization of a newly developed anti-GRK5 monoclonal antibody (GRK5 #139). (A) Immunoreactivity of the GRK5 #139 antibody to endogenous GRK5 in HEK293 cells and mouse brain. The GRK5 #139 antibody was pre-incubated at 4 °C overnight with (+) or without (−) 1 mg/ml of purified GRK5 residues 94–157 and used to blot aliquots of HEK293 cell lysates (50 μg protein) and mouse brain extracts (50 μg protein). For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. (B) Cross-reactivity of the GRK5 #139 antibody to overexpressed GRK6. HEK293 cells were transfected with human GRK5, mouse GRK5, human GRK6, or mouse GRK6 cDNA. HEK293 cell lysates (50 μg protein) transfected with one of the GRK cDNAs were loaded on a SDS–polyacrylamide gel. Immunoblotting was performed with the GRK5 #139 antibody or the GRK4–6 antibody. The GRK4–6 antibody detected both GRK5 and GRK6. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody.

To test the contribution of GRKs to Ser129 phosphorylation of αS, we focused on ubiquitously expressing members of the GRK family (GRK2, GRK3, GRK5, and GRK6). First, we examined the effect of overexpressed GRKs on Ser129 phosphorylation of αS in HEK293 cells. In HEK293 cells, the expression level of endogenous αS was too low to detect its signal by immunoblotting (Fig. 2). For

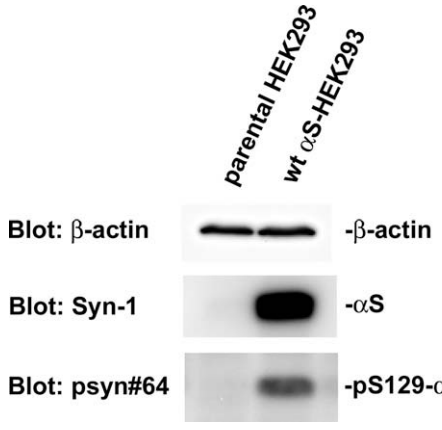

Fig. 2. The expression level of αS in parental HEK293 cells and a HEK293 cell line stably expressing wild-type αS (wt αS-HEK293). Cell lysates (50 μg protein) of parental HEK293 cells and the cell line stably expressing wild-type αS were loaded on a SDS–polyacrylamide gel. Immunoblotting was performed with the Syn-1 and psyn#64 antibodies. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. αS and phosphorylated αS were not detectable in parental HEK293 cells.