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Casein kinase 2 is the major enzyme in brain that phosphorylates Ser129 of human α-synuclein: Implication for α-synucleinopathies

Aasami Ishii[a,b,1], Takashi Nonaka[a,1], Sayuri Taniguchi[a,e,2], Taro Saito[b], Tetsuaki Arai[c], David Mann[d], Takeshi Iwatsubo[e], Shin-ichi Hisanaga[b], Michel Goedert[f], Masato Hasegawa[a,*]

[a] *Department of Molecular Neurobiology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan*
[b] *Molecular Neuroscience Laboratory, Graduate School of Science, Tokyo Metropolitan University, 1-1 Minami-Osawa, Hachioji-shi, Tokyo 192-0397, Japan*
[c] *Department of Psychogeriatrics, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan*
[d] *Greater Manchester Neurosciences Centre, University of Manchester, Hope Hospital, Salford M6 8HD, UK*
[e] *Department of Neuropathology and Neuroscience, Graduate School of Pharmaceutical Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[f] *Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*

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Abstract In Lewy body diseases and multiple system atrophy, α-synuclein is hyperphosphorylated at Ser129, suggesting a role in pathogenesis. Here, we report purification of the protein kinase in rat brain that phosphorylates Ser129 and its identification as casein kinase-2 (CK2). We show that most of the activity can be inhibited by heparin, an inhibitor of CK2. Phosphorylated Ser129 was detected in primary cultured neurons and inhibited by CK2 inhibitors. In some cases of Lewy body disease, CK2-like immunoreactivity was recovered in the sarkosyl-insoluble fraction, which was enriched in phosphorylated α-synuclein. Taken together, these findings suggest that CK2 may be involved in the hyperphosphorylation of α-synuclein in α-synucleinopathies.

Keywords: Aggregation; Parkinson's disease; Lewy body; Heparin; Mutation; CK2

1. Introduction

Filamentous α-synuclein (α-syn) inclusions in nerve cells or glial cells are the defining feature of a group of neurodegenerative diseases, which include Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [1–5]. Missense mutations (A30P, E46K and A53T) in the α-syn gene cause familial forms of PD and DLB [6–8]. Furthermore, multiplications (duplication and triplication) of a region on the long arm of chromosome 4 that encompasses the α-syn gene cause inherited forms of PD and DLB [9–11], indicating that overproduction of wild-type α-syn is sufficient to cause disease. We have previously used mass spectrometry and specific antibodies to show that filamentous α-syn in DLB and other α-synucleinopathies is phosphorylated at Ser129 [12–14]. Furthermore, by quantitative immunoblot analysis, >90% of insoluble α-syn from DLB brains was phosphorylated at Ser129, whereas only 4% of α-syn from mouse brain was phosphorylated, indicating hyperphosphorylation of this site in the human diseases. Casein kinase 1 (CK1), casein kinase 2 (CK2) and G protein-coupled receptor protein kinase 5 (GRK5) phosphorylate Ser129 of α-syn in vitro [15,16]. This residue is also constitutively phosphorylated in transfected 293 cells [15]. However, little is known about the protein kinases and protein phosphatases that regulate the phosphorylation at Ser129 of α-syn in vivo.

Here, we report that the α -and β-subunits of CK2 are the major protein kinases in brain that phosphorylate Ser129 in human α-syn. Furthermore, in primary cultures of rat cerebral cortex, CK2 inhibitors strongly reduced phosphorylation of α-syn.

2. Materials and methods

2.1. Chemicals and antibodies

Phosphorylation-dependent anti-α-syn antibody PSer129 and phosphorylation-independent antibody Syn102 were used as described previously [12]. An antibody to CK2 was purchased from Upstate Biotechnology. CK1 and CK2 were purchased from New England Biolabs. GRK5 was kindly provided by Dr. J.L. Benovic. CK2 inhibitors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), apigenin and emodin were purchased from Sigma. The CK1 inhibitor CKI-7 and okadaic acid were obtained from Seikagaku Company and Wako Chemicals, respectively.

2.2. Detection of brain protein kinase activity that phosphorylates Ser129 of human α-syn

Human α-syn cDNA in bacterial expression plasmid pRK172 was used [17]. Site-directed mutagenesis (Quik-Change, Stratagene) was used to produce A30P, E46K, A53T and S129A α-syn. All constructs were verified by DNA sequencing. Wild-type and mutant proteins were expressed in *E. coli* BL21 (DE3) and purified as described [18,19] and used for phosphorylation assay. Incubations (25 μl) were carried out at 37 °C and comprised 10 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM PMSF, 1 mM ATP, recombinant α-syn (1 mg/ml) and brain extract (5 μl) or fractionated sample (5 μl). Reactions were initiated by the addition of ATP and terminated at various times by boiling following the addition of 2.5 μl of 5 M NaCl and 1 μl 2-mercaptoethanol. The samples were then centrifuged for 5 min at 20000 × *g* and the

*Corresponding author. Fax: +81 3 3329 8035.
E-mail address: masato@prit.go.jp (M. Hasegawa).

[1]These authors contributed equally to the work.
[2]Present address: Dept. of CNS Pharmacology, Takeda Pharmaceutical Co. Ltd.

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supernatants diluted, followed by the addition of SDS–PAGE sample buffer. Aliquots were immunoblotted with antibody PSer129, the reactions developed by enhanced chemiluminescence (ECL) and quantified as described [18].

2.3. Purification of brain protein kinase activity that phosphorylates Ser129 of human α-syn

Brains of 6-week-old Wistar rats were homogenized in 4 vol. of buffer A (10 mM Tris–HCl, pH 7.5, 5 mM EGTA, 1 mM PMSF, 20 μg/ml pepstatin, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM DTT) and spun at $386000 \times g$ for 40 min at 4 °C. The supernatant was the brain extract used in kinase assays. For purification, the brain extracts were fractionated by ammonium sulfate precipitation. The fractions were dialyzed against buffer B (10 mM Tris–HCl, pH 7.5, 5 mM EGTA, 1 mM DTT) and tested for protein kinase activity. Active fractions were applied to a Q-Sepharose column equilibrated in buffer C (50 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM DTT) and eluted using increasing concentrations of NaCl. The active fractions were collected, dialyzed against buffer C, applied to a phosphocellulose column and eluted using a linear gradient of NaCl.

2.4. In gel digestion and LC/MS/MS analysis of purified proteins

The 45 kDa, 42 kDa and 24 kDa bands from the purified kinase fractions were excised, cut into 1×1 mm cubes, washed twice with 50% acetonitrile in 0.1 M ammonium bicarbonate and dried in a vacuum centrifuge. The gel pieces were rehydrated in buffer containing 1 μg/ml trypsin (Promega Inc.) and 0.1 M ammonium bicarbonate, and incubated for 16 h at 37 °C. The digested peptides were extracted using 50% acetonitrile and 5% formic acid, and dried. They were dissolved in 0.1% trifluoroacetic acid, 2% acetonitrile and an aliquot was separated on a Develosil ODS-HG-5 column (0.15×50 mm, Nomura Chemical Company) at a flow rate of 300 nl/min using nano-flow HPLC (Dina, TYK Tech Company) and analyzed ion-trap mass spectrometry (LCQ Advantage, Thermo).

2.5. Inhibition of CK1, CK2 and GRK5 by heparin

Recombinant α-syn (1 mg/ml) was phosphorylated with CK1 (0.5 μg; 1000 U), CK2 (1 μg; 500 U) or GRK5 (1 μg) in the presence or absence of heparin (10–100 μg/ml) and analyzed by immunoblotting with PSer129. Inhibition of the protein kinase activity from brain extract (2 μl) and the 0–33% ammonium sulfate fraction (1 μl) by heparin (10–100 μg/ml) was also examined. PSer129 immunoreactivity was quantified and expressed as % activity in the absence of heparin (taken as 100%).

2.6. Primary culture of rat brain neurons and immunoblot analysis of α-syn

Cortical neurons were prepared from 16-day-old rat embryos, as described [20], and cultured for up to 28 days. Cells were collected at days 3, 6, 9, 12, 15, 18, 21, 25 and 28, sonicated in 50 mM Tris–HCl, pH 7.5, 1 mM EGTA, 0.5 M NaCl, 1 mM DTT and centrifuged at $20000 \times g$ for 10 min. Supernatants were boiled for 5 min and cleared by centrifugation, followed by SDS–PAGE and immunoblotting. For the analysis of phosphorylation of α-syn by CK2, cells were treated with 200 μM DRB, 200 μM apigenin and 50 μM emodin or 100 μM CK1-7 for 6 h at day 19, followed by a 30 min treatment with 1 μM okadaic acid. Neurons were collected and lysed in SDS-sample buffer. Homogenates (20 μg/lane) were analyzed by immunoblotting with Syn102 and PSer129.

2.7. Immunoblot analysis of fractionated DLB brain extracts

Frozen cerebral cortex (0.5 g) from control and DLB brains was homogenized in 4 vol. of buffer D (10 mM Tris–HCl, pH 7.5, 1 mM EGTA, 10% sucrose, 0.8 M NaCl) and spun at $386000 \times g$ for 20 min at 4 °C. The resulting supernatant was retained (as the Tris-soluble fraction) and the pellet homogenized in 4 vol. of buffer D containing 1% Triton X-100. After a 20 min spin at $386000 \times g$, the supernatant was retained (as the Triton X-100-soluble fraction) and the pellet homogenized in buffer D containing 1% sarkosyl. The homogenate was left for 30 min at 37 °C, followed by a 20 min spin at $386000 \times g$. The supernatant was retained (as the sarkosyl-soluble fraction) and the pellet solubilized in one volume of 50 mM Tris–HCl, pH 7.5, containing 1% SDS, followed by a 20 min spin at $386000 \times g$. The supernatant was retained (as the sarkosyl-insoluble, SDS-soluble fraction). Each fraction was run on SDS–PAGE and analyzed by immunoblotting using Syn102, PSer129 and the anti-CK2α antibody.

3. Results

3.1. Detection of brain protein kinase activity that phosphorylates Ser129 of human α-syn

α-Syn phosphorylated at Ser129 was detected in the Tris-soluble and Triton X-100-soluble fractions prepared from 6-week-old rat brains. The brains had to be processed rapidly, since reactivity with PSer129 was lost, when the extracts were prepared more than 30 min after the death of the animal (Fig. 1A and B). To detect kinase activity that generates the PSer129 epitope, wild-type and S129A α-syn were incubated with rat brain extract for 30 min at 37 °C in the presence or absence of ATP. Reactivity with PSer129 was only detected when wild-type α-syn was incubated in the presence of ATP (Fig. 1C and D), indicating that the brain extract contained a kinase activity able to phosphorylate Ser129. It was enhanced with increasing $MgCl_2$ (2–20 mM), but was not changed in presence of $CaCl_2$ or phosphatidylcholine (data not shown). We next proceeded to purify this activity. Since most of the kinase

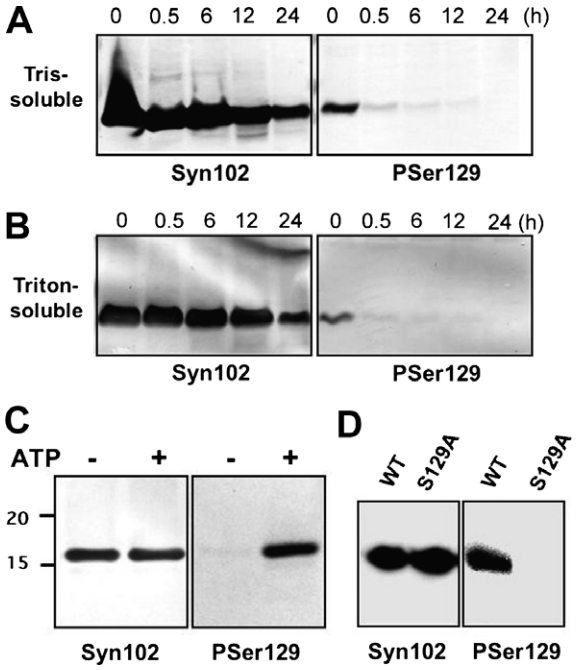

Fig. 1. (A,B) Dephosphorylation of rat brain α-syn after death. Brains were removed and frozen on dry ice 0, 0.5, 6, 12 and 24 h after the rats had been killed by cervical dislocation under deep anesthesia. Tris-soluble (A) and Triton-soluble (B) fractions were prepared and analyzed by immunoblotting with Syn102 and PSer129. (C) Detection of kinase activity generating the PSer129 epitope in rat brain extract. Recombinant α-syn was incubated with rat brain extract for 30 min at 37 °C in the presence or absence of ATP and analyzed by immunoblotting with antibodies Syn102 and PSer129. (D) Wild-type and S129A α-syn were incubated with rat brain extract for 30 min at 37 °C in the presence of ATP and analyzed by immunoblotting with Syn102 and PSer129. Note that the PSer129 epitope was generated following incubation of wild-type α-syn with brain extract in the presence of ATP.

activity was precipitated by 33% ammonium sulphate (data not shown), the 0–33% ammonium sulphate fraction was applied to a Q-Sepharose column and the bound kinase activity eluted with 0.4–0.5 M NaCl (Fig. 2A and B). The activity was then separated from most other proteins on a phosphocellulose column and eluted with 0.5–0.7 M NaCl (Fig. 2C–E). The purified fractions consisted of one major band of 42 kDa molecular mass and two minor bands of 45 kDa and 24 kDa, respectively (Fig. 2D). These bands were in-gel digested with trypsin and analyzed by mass spectrometry.

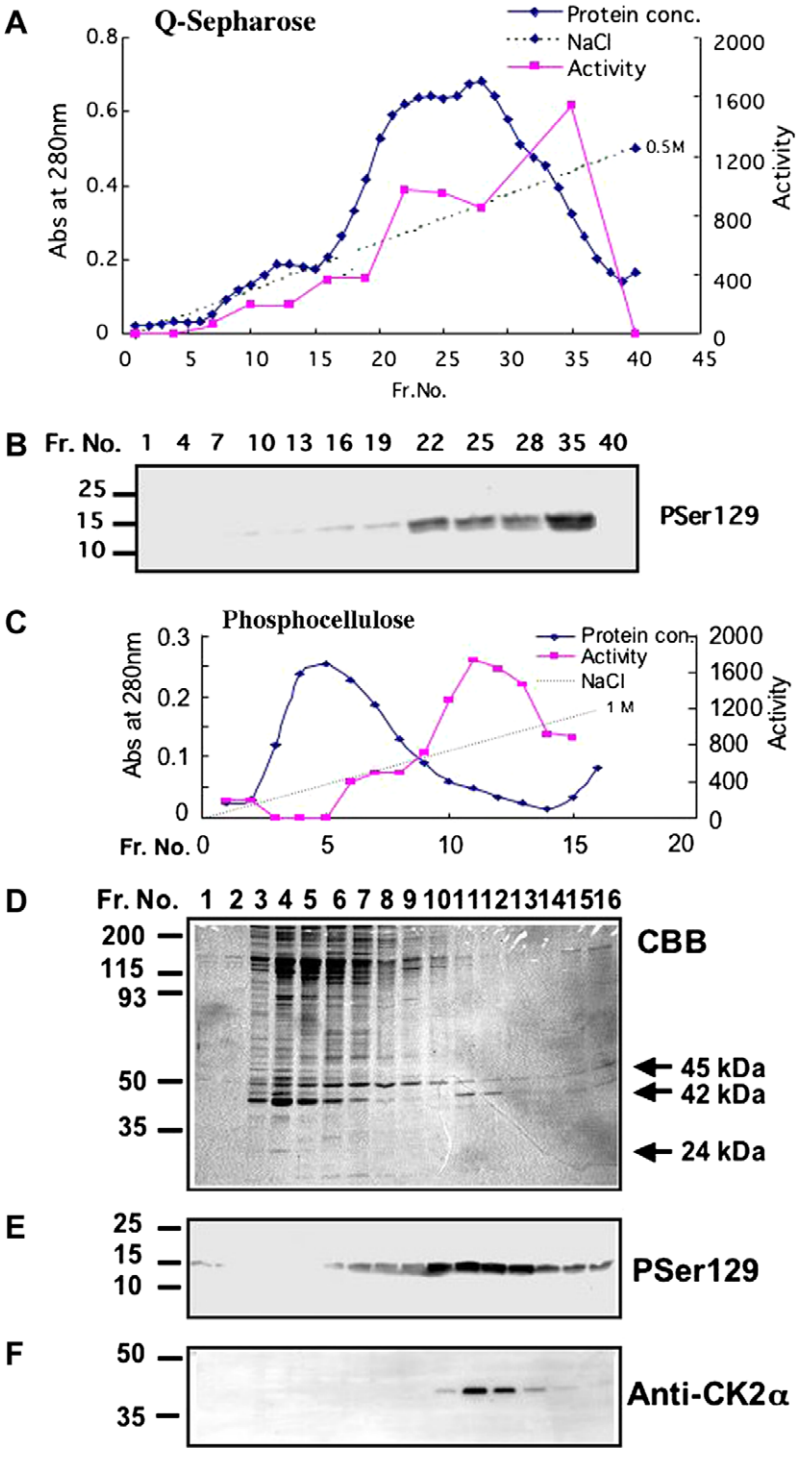

Fig. 2. Purification of kinase activity generating the PSer129 epitope. (A) Elution profiles of proteins (absorbance at 280 nm) and kinase activity (PSer129 immunoreactivity in presence of ATP) from a Q-Sepharose column. (B) Kinase activities of the column fractions (the PSer129 immunoreactivity in the presence of ATP) are shown. (C) Elution profiles of proteins from a phosphocellulose column. (D) Column fractions were run on SDS–PAGE and stained with Coomassie Brilliant Blue (CBB). The major band of 42 kDa and two minor bands of 45 kDa and 24 kDa in fractions 11 and 12 were analyzed by LC/MS/MS analysis following in-gel digestion with trypsin. (E) Kinase activities of the phosphocellulose column fractions (the PSer129 immunoreactivity in the presence of ATP) are shown. (F) Column fractions were run on SDS–PAGE and immunoblotted with an anti-CK2α antibody.

LC/MS/MS analysis revealed that the 42 kDa was the α-subunit and the 24 kDa band the β-subunit of CK2. The 45 kDa was identified as β-actin. Immunoblot analysis using an antibody specific for the α-subunit of CK2 confirmed that CK2α purified with the kinase activity that phosphorylated Ser129 in human α-syn (Fig. 2E and F).

3.2. Inhibition of kinase activity by heparin

To further examine the nature of the kinase activity phosphorylating Ser129, we used heparin, a known inhibitor of CK2 [21]. First, the ability of recombinant CK1, CK2 and GRK5 to phosphorylate Ser129 in the presence of heparin was investigated. Phosphorylation by CK1 and GRK5 was partially inhibited by 1–100 μg/ml heparin, retaining approximately 40% of activity at 100 μg/ml heparin (Fig. 3A). This contrasted with the phosphorylation of Ser129 by CK2, which was completely inhibited at 10–100 μg/ml heparin. We next added heparin (0.1–10 μg/ml) to the fractions with kinase activity purified from brain and found that this activity was almost completely inhibited, with an IC_{50} value of 0.6 μg/ml heparin (data not shown). The inhibition of kinase activities in crude brain extract and the 0–33% ammonium sulphate fraction by heparin was also investigated. In the presence of 10 μg/ml heparin, these activities were reduced to ~40% and ~20% of control values with crude extracts and 0–33% ammonium sulphate fraction, respectively (Fig. 3B and C). These results suggested that at least 60% of the kinase activity in crude brain extract was due to CK2. However, heparin may have

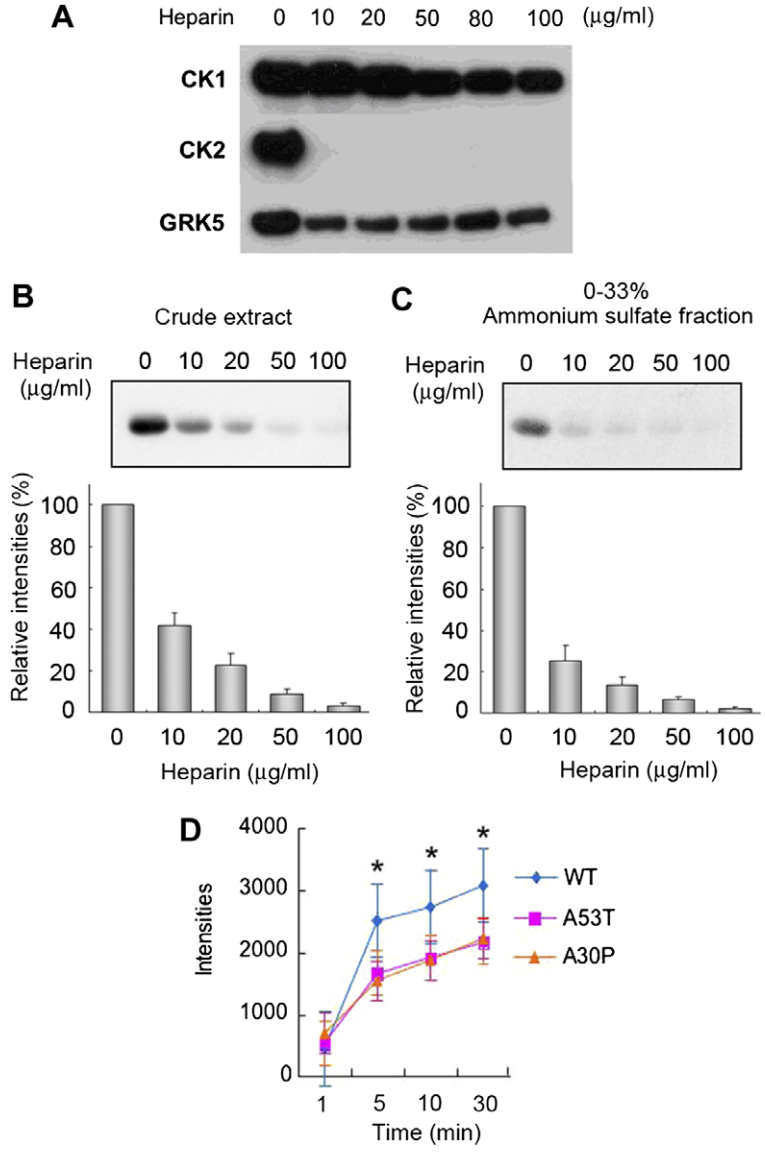

Fig. 3. (A) Inhibition of the phosphorylation of Ser129 of α-syn by heparin. Recombinant α-syn was phosphorylated with CK1, CK2 and GRK5 in the absence or presence of heparin (10, 20, 50, 80 or 100 μg/ml) and analyzed by immunoblotting with antibody PSer129. (B) Inhibition of the protein kinase activity that generates the PSer129 epitope in crude brain extract by 10, 20, 50 or 100 μg/ml heparin. (C) Inhibition of the protein kinase activity in the 0–33% ammonium sulphate fraction by 10, 20, 50 or 100 μg/ml heparin. Quantitation of the kinase activity in the presence and absence (taken as 100%) of heparin. (D) Effects of A30P and A53T mutations of α-syn on the phosphorylation of Ser129 by CK2. Time course of Ser129 phosphorylation of wild-type (WT), A30P and A53T α-syn by recombinant CK2. Intensities of Pser129 immunoreactivity were quantitated and expressed as means ± S.E.M. ($n = 3$) ($^{*}P < 0.05$).

bound a number of additional proteins, reducing the amount available to inhibit CK2. In the presence of 100 μg/ml heparin, the kinase activities present in crude brain extract and the 0–33% ammonium sulphate fraction were reduced by over 95% (Fig. 3B and C), consistent with CK2 being the major kinase able to phosphorylate Ser129 in α-syn.

3.3. Effects of α-syn mutations A30P and A53T on the phosphorylation of Ser129 by CK2

Recombinant wild-type α-syn, A30P α-syn and A53T α-syn (1 mg/ml) were phosphorylated with CK2 (500 U) for 1, 5, 10 and 30 min. Reactions were terminated as described above and Ser129 phosphorylation analyzed by immunoblotting with PSer129. As shown in Fig. 3, generation of the PSer129 epitope was slower for A30P and A53T α-syn than for the wild-type protein. Immunoreactivities were quantified and expressed as means ± S.E.M. ($n = 3$) (Fig. 3D). Statistical analysis was carried out by unpaired t-test using Kai plot software.

3.4. Phosphorylation of Ser129 of human α-syn in primary cultures of rat brain neurons

Primary cultures were prepared from cerebral cortex of rat embryos. They were cultured for 3–28 days and immunoblotted with Syn102 and PSer129 (Fig. 4A). Immunoreactivity with Syn102 was first detected at day 15 in vitro and was present at high levels until day 28. Reactivity with PSer129 was also present at days 15–28. The cultured neurons were exposed to the CK2 inhibitors DRB, apigenin and emodin or the CK1 inhibitor CKI-7 for 6 h, followed by a 30 min exposure to okadaic acid (Fig. 4B). In the presence of DRB, apigenin and emodin, immunoreactivity with PSer129 was markedly reduced, in the absence of a reduction in total α-syn. In contrast, CKI-7 only slightly reduced the production of PSer129 immunoreactivity. Exposure to okadaic acid increased phosphorylation of Ser129.

3.5. Distribution of CK2α in fractionated DLB brains

Cerebral cortex from controls and DLB patients was homogenized differentially using Tris–HCl, Triton X-100, sarkosyl and SDS, followed by immunoblotting of the soluble fraction from each extraction step with Syn102, PSer129 and anti-CK2α antibodies. In control brain, α-syn and CK2α immunoreactivities were recovered only in the Tris-soluble and Triton-soluble fractions (Fig. 4C). In fractions extracted from DLB brain, α-syn immunoreactivity was detected in the sarkosyl-insoluble, SDS-soluble fraction, as described [12,22]. In some of these cases, CK2α immunoreactivity was detected in the insoluble fraction (Fig. 4C).

4. Discussion

We previously showed that α-syn is phosphorylated at Ser129 in the filamentous deposits of Lewy body diseases and MSA [12]. The C-terminal region of α-syn is negatively charged and contains several potential phosphorylation sites. CK1, CK2, GRK2 and GRK5 are known to phosphorylate Ser129 of α-syn in vitro [15,16]. The related proteins β-syn and γ-syn are also phosphorylated by some of these kinases in their negatively charged C-termini [16]. However, nothing is known about the protein kinases that phosphorylate α-syn in brain.

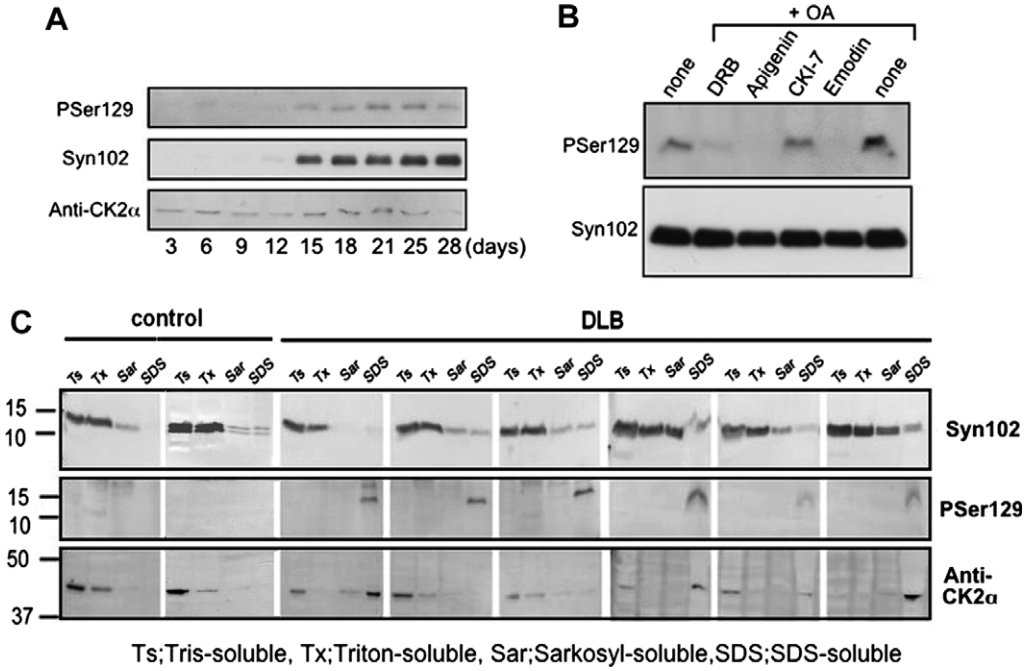

Fig. 4. (A) Immunoblot analysis of α-syn in cultured cortical neurons. Primary neurons of rat cortex were cultured for 3–28 days and the cell lysates analyzed by immunoblotting with Syn102, PSer129 and anti-CK2α. (B) Primary cultures (day 19) were exposed to 200 μM DRB, 200 μM apigenin, 100 μM CK1-7 or 50 μM emodin for 6 h, treated with okadaic acid for 30 min and analyzed by immunoblotting with Syn102 and PSer129. (C) Immunoblot analysis of α-syn and CK2α extracted from control and DLB brains. α-Syn and CK2α were serially extracted with Tris–HCl (Ts), Triton-X100 (Tx), Sarkosyl (Sar) and SDS from cerebral cortex of six patients with DLB and two controls. They were probed with antibodies Syn102, PSer129 and anti-CK2α.

We report here that the α- and β-subunits of CK2 were the major proteins purified from rat brain capable of phosphorylating Ser129 of human α-syn. CK2 is a constitutively active protein kinase that exists mainly as a holoenzyme composed of two catalytic α-subunits of 42–44 kDa and two regulatory β-subunits of 24–26 kDa molecular mass [23,24]. To further examine the involvement of CK2 in the phosphorylation of Ser129, we incubated crude rat brain extract in the presence of low concentrations of heparin. At these concentrations, heparin is a potent and relatively specific inhibitor of CK2 [21]. Consistent with the above, heparin inhibited phosphorylation of Ser129 by the brain extract. Furthermore, phosphorylation of Ser129 in primary cultures of cortical neurons was inhibited by the CK2 inhibitors DRB, apigenin and emodin. These findings establish that CK2 is the major protein kinase in brain that phosphorylates Ser129 of α-syn. Much less is known about the protein phosphatases that dephosphorylate α-syn phosphorylated at Ser129. In primary cultures of rat cortical neurons, exposure to okadaic acid resulted in increased phosphorylation of Ser129, suggesting that protein phosphatases 1 and/or 2A may be involved.

Pathogenic mutations A30P and A53T reduced the ability of CK2 to phosphorylate α-syn in vitro, suggesting that the previously described [25,26] conformational changes resulting from these single amino acid changes reduced the phosphorylation of α-syn. It remains to be determined whether the same is true in cases with familial PD and DLB. These findings are reminiscent of work on tau protein, where the presence of pathogenic mutations P301L, P301S and R406W resulted in lower levels of phosphorylation by cyclin-dependent kinase-5 [27].

The relevance of CK2 and phosphorylation of Ser129 for the pathogenic process in α-synucleinopathies is only incompletely understood. We found that in some cases of DLB, CK2 was present in the sarkosyl-insoluble fraction, indicating reduced solubility and a possible interaction with filamentous α-syn. It remains to be determined whether CK2 levels and activity are abnormal in human Lewy body diseases. It is interesting to note that activating mutations in another protein kinase, LRRK2, are a relatively common cause of inherited PD [28–31]. Currently, the in vivo substrates of LRRK2 are unknown; it will be interesting to see whether phosphorylation of Ser129 of α-syn lies downstream of the activation of LRRK2. In a *Drosophila* model of PD, phosphorylation or pseudophosphorylation of Ser129 of soluble α-syn species was an essential determinant of neurotoxicity, while correlating negatively with inclusion body formation [32]. This negative correlation stands in apparent contrast to PD, DLB and MSA, where the filamentous α-syn deposits are hyperphosphorylated at Ser129 relative to the soluble protein [12]. Phosphorylation of Ser129 by CK2 has been shown to increase filament formation of α-syn both in vitro and in transfected cells, consistent with a disease-promoting role [12,33].

Although the major deleterious species of α-syn in human diseases remain to be identified, current evidence suggests that inhibition of CK2 to reduce the phosphorylation of Ser129 may represent a promising therapeutic target in α-synucleinopathies.

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