Siah-1 Facilitates Ubiquitination and Degradation of Synphilin-1*

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Parkinson’s disease is a common neurodegenerative disorder characterized by loss of dopaminergic neurons and appearance of Lewy bodies, cytoplasmic inclusions that are highly enriched with ubiquitin. Synphilin-1, α-synuclein, and Parkin represent the major components of Lewy bodies and are involved in the pathogenesis of Parkinson’s disease. Synphilin-1 is an α-synuclein-binding protein that is ubiquitinated by Parkin. Recently, a mutation in the synphilin-1 gene has been reported in patients with sporadic Parkinson’s disease. Although synphilin-1 localizes close to synaptic vesicles, its function remains unknown. To investigate the proteins that interact with synphilin-1, the present study performed a yeast two-hybrid screening and identified a novel interacting protein, Siah-1 ubiquitin ligase. Synphilin-1 and Siah-1 proteins were endogenously expressed in the central nervous system and were found to coimmunoprecipitate each other in rat brain homogenate. Confocal microscopic analysis revealed colocalization of both proteins in cells. Siah-1 was found to interact with the N terminus of synphilin-1 through its substrate-binding domain and to specifically ubiquitinate synphilin-1 via its RING finger domain. Siah-1 facilitated synphilin-1 degradation via the ubiquitin-proteasome pathway more efficiently than Parkin. Siah-1 was found to not facilitate ubiquitination and degradation of wild type or mutant α-synuclein. Synphilin-1 inhibited high K+-induced dopamine release from PC12 cells. Siah-1 was found to abrogate the inhibitory effects of synphilin-1 on dopamine release. Such findings suggest that Siah-1 might play a role in regulation of synphilin-1 function.

Parkinson’s disease (PD),† which is characterized by tremor, bradykinesia, rigidity, and postural instability, represents the second most common neurodegenerative disorder. PD is pathologically characterized by loss of dopaminergic neurons in the substantia nigra pars compacta and appearance of Lewy bodies (LBs), cytoplasmic inclusions that are highly enriched with ubiquitin (1–3). α-Synuclein is a presynaptic protein of undetermined function that was found to be the main component of LB (4, 5). Two rare missense mutations in the α-synuclein gene (A53T and A30P) cause autosomal dominant familial PD (6, 7). α-Synuclein has been implicated in the pathogenesis of several neurodegenerative diseases, including PD, multiple system atrophy, and dementia with LBs (8). In addition, α-synuclein knockout mice display increased dopamine release under stimulated conditions (9). Moreover, α-synuclein inhibits dopamine biosynthesis (10), suggesting that α-synuclein is a negative regulator of dopamine neurotransmission.

Synphilin-1 represents a cytoplasmic protein that interacts with α-synuclein (11) and localizes close to synaptic vesicles (12). Synphilin-1 has been found to constitute an intrinsic component of LBs in PD, indicating that it might be involved in the pathogenesis of PD (13). Synphilin-1 contains six ankyrin (ANK) repeats (Swiss Protein Database number Q9Y6H5), a coiled-coil domain, and an ATP/GTP-binding site (11). Nonetheless, the physiological function of synphilin-1 remains unclear. Coexpression of synphilin-1 and α-synuclein in cells was found to lead to the deposition of eosinophilic inclusions that resembled LBs (11, 14), supporting the hypothesis that the interaction might be related to LB formation. Recently, a mutation in the synphilin-1 gene leading to an amino acid substitution of cysteine for arginine in position 621 was reported in two apparently sporadic PD patients (15). The number of inclusions in cells expressing this mutant synphilin-1 was significantly reduced compared with wild type synphilin-1 (15).

Proteins fated to degrade in proteasomes are subjected to ubiquitination. Ubiquitination proceeds through a sequential enzymatic reaction composed of ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). The exquisite specificity for proteins destined for ubiquitination is usually determined by a diverse family of E2s with specific E2s. Parkin is a RING finger-type E3 that contains two RING finger domains and an IBR (in between RING fingers) domain. One type of autosomal recessive juvenile parkinsonism, which represents the major cause of juvenile PD, results from mutations of the Parkin gene (16). Autosomal recessive juvenile parkinsonism-linked Parkin mutations have been demonstrated to disrupt E3 activity (17–19). Parkin has been shown to interact with and ubiquitinate synphilin-1 (20). Coexpression of synphilin-1, α-synuclein, and Parkin elicits matography; IP, immunoprecipitation; WB, Western blotting; aa, amino acids; HA, hemagglutinin; HEK, human embryonic kidney.

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formation of ubiquitin-positive cytoplasmic inclusions that resemble LBs, suggesting that synphilin-1 might link α-synuclein and Parkin to a common pathogenic mechanism (20). A recent report demonstrated that Dorfin, an E3 for mutant superoxide dismutase-1, also interacts with and ubiquiti- nates synphilin-1 (21). Like Parkin, Dorfin contains two RING finger domains and an IBR domain. Furthermore, Dorfin is colocalized with ubiquitin in LBs of PD, suggesting that Dorfin is also implicated in the pathogenesis of PD.

Siah/Sina family proteins represent mammalian homologues of the Drosophila Sina (seven in absentia) protein. Sina is also a RING finger-type E3 that is critically involved in neuronal development of the R7 photoreceptor cell in Drosophila (22). Sina functions downstream of the tyrosine kinase receptor Sevenless and the Ras/Raf mitogen-activated protein kinase pathway (23–25). Sina is required for targeting the transcriptional repressor Traumtrack for proteasome-mediated degradation, which is a requisite step for neuronal differentiation of R7 photoreceptor cells (26). In humans, there are two highly conserved Sina homologues, Siah-1 and Siah-2, which are abundantly expressed in the central nervous system, as well as other tissues (27, 28), and are associated with synaptophysin (12). Like Parkin and Dorfin, both Siah-1 and Siah-2 are also RING finger-type E3s. Siah proteins facilitate ubiquitination and proteasome-dependent degradation of multiple proteins, such as DCC (29), Nco-R (30), c-Myb (31), BOB1/BOBF1 (32, 33), Peg3/Fr1 (34), APC (35), Kid (36), Numb (37), synaptophysin (12), and group 1 metabotropic glutamate receptors (38). The present report demonstrates that Siah-1 interacts with and ubiquitinates synphilin-1 in vivo, resulting in facilitation of synphilin-1 degradation via the ubiquitin-protea- some pathway.

**EXPERIMENTAL PROCEDURES**

**YEAST TWO-HYBRID SCREENING**—The full-length human synphilin-1 cDNA was cloned by a library screening as described previously (39). The coding region of synphilin-1 cDNA was subcloned into the yeast two-hybrid vector pGBKT7 (Clontech), which is in-frame fused to the GAL4-binding domain sequence. The recombinant plasmid was introduced into the yeast strain AH109. A rat brain cDNA library constructed in pGAD10 (Clontech) was introduced into the yeast strain expressing the synphilin-1 fusion protein, and ~1.0 × 10⁶ transformants were screened for growth on SD plate media lacking tryptophan, leucine, histidine, and adenine. Positive clones were detected by a β-galactosidase assay. An α-Synuclein construct in pGAD10 was used as a positive control for the screening. To eliminate false positives, plasmid DNA from positive clones was purified, amplified, and retransformed into the yeast strain expressing synphilin-1 protein fused to the GAL4-binding domain. The positive clones in this second screening were subjected to DNA sequencing. A BLAST search revealed that one of the isolated positive clones contained a fragment nearly identical to the Siah-1a gene.

**VECTORS AND ANTIBODIES**—Full-length human Siah-1 cDNA was amplified from a human brain cDNA library (Stratagene) by PCR using the following forward and reverse primers: 5’-GAA TTC TCG AGA CAT GTT GTC CAA CTT GCC GG-3’ and 5’-GCG ATC TAG ATC AGG TTG TAA TGG ACT TAT GCT G-3’ from various tissues (50). A rat brain cDNA library constructed in a pcDNA3 vector (Invitrogen) was used as an expression host for the transfection of yeast cells. A Tet-repressible Siah-1 expression strain was established by transfecting vectors into HEK 293 cells. The expression plasmids were transfected with pTet-sil-Tet-Siah or pTet-sil-Myc-Siah-1 using LipofectAMINE 2000 (Invitrogen). After transfection, cells were grown in a medium containing 10% fetal bovine serum, 5% horse serum, and 0.1 mg/ml hygromycin (Roche Applied Science). To establish a Tet-repressible Siah-1 expression system, HEK 293 cells were transfected with pTet-sil-Myc-Siah-1 or pTet-sil-Myc-Siah-1C using LipofectAMINE 2000 (Invitrogen). After transfection, cells were grown in a medium containing 10% fetal bovine serum, 5% horse serum, and 0.1 mg/ml hygromycin, 500 ng/ml Tet (Sigma), and 400 μg/ml G418 (Sigma). For 3–4 weeks, colonies were selected by hygromycin and G418. Among the selected colonies, multiple monoclonal cell lines that exhibited Tet-repressible expression of Siah-1 (PC12-Tet-Siah) or Siah-1C (PC12-Tet-AC) were established.

**IMMONOPRECIPITATION**—HEK 293 cells were transfected with pcDNA3-Myc-Siah-1, pcDNA3-Myc-Siah-1N, pcDNA3-HA-synphilin-1, and pcDNA3-HA-α-synuclein. After 24 h, the cells were cultured with dimethyl sulfoxide (DMSO) (0.5% final concentration) and 8 h and then treated with ice-cold phosphate-buffered saline and lysed in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, pH 8.0) with protease inhibitors (1 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After the lysate protein contents were normalized using a protein assay kit (Bio-Rad), the cell lysate (500 μg/sample) was immunoprecipitated with NRI or anti-HA antibodies using protein G-Sepharose beads (Pierce). For immunoprecipitation of the endogenous proteins from rat brains, adult rat brains were homogenized in 5 volumes of Tris-HCl (50 mM; pH 7.4), KCl (140 mM), EDTA (3 mM), and 0.5% Triton X-100 supplemented with protease inhibitors. The tissue homogenate was centrifuged at 5,000 × g at 4 °C for 20 min. For immunoprecipitation, the supernatant was incubated with the following antibodies: normal rabbit IgG, anti-GST, or anti-Siah-1 antibodies. Each immunoprecipitate was divided into two parts, separated on SDS-PAGE, and transferred onto nitrocellulose membranes. Bound proteins were visualized using immunoblotting.

**Siah-1 Protein Expression in Rat Tissues**—Rat brain protein lysate from various tissues (50 μg/homogenate) was subjected to SDS-PAGE and immunoblotted with anti-Siah-1, anti-synphilin-1, anti-α-synuclein, and anti-α-tubulin antibodies. To determine the specificity of anti-Siah-1 antibody, identical blots of rat protein lysate from various tis- sues were incubated with anti-Siah-1 antibody preabsorbed with anti-galactosidase (GST) Siah-1 fusion protein.

**Confocal Immunofluorescent Staining**—Immunofluorescence was performed as described previously (41). Briefly, HEK 293 cells cotransfected with pcDNA3-HA-synphilin-1 and pcDNA3-Myc-Siah-1 or SH-SY5Y cells were seeded and grown on glass coverslips in medium. Adherent cells were fixed with neutral buffered 4% (w/v) paraformal-
dehydrate and permeabilized with 0.2% Triton X-100. To assess the distribution of endogenous Siah-1 and synphilin-1, untransfected SH-SY5Y cells were treated with anti-Siah-1 and anti-synphilin-1 antibodies. Anti-Siah-1 and anti-synphilin-1 antibodies preabsorbed with each antigen were also used. To assess the colocalization of synphilin-1 and Siah-1, a double-labeling immunofluorescent staining was performed with a combination of anti-HA and anti-Myc antibodies. Anti-Siah-1, anti-synphilin-1, and anti-HA antibodies were visualized by Alexa Fluor488 anti-rabbit antibody (Molecular Probes). Anti-Myc antibody was visualized by Alexa Fluor 568 anti-mouse antibody (Molecular Probes). Immunostained preparations were examined with the Zeiss LSM510 confocal microscope.

Pulse-Chase Assay—A pulse-chase assay was performed as described previously (42, 43). HER 293 cells were transfected with pcDNA3-HA-synphilin-1 and pcDNA3-Myc-Siah-1, pcDNA3-Myc-SiahN, pcDNA3-Myc-Parkin, pcDNA-Myc-beta-TrCP/FWD1, or empty pcDNA3-Myc vectors. After 24 h, cells were cultured with 20 μM MG132 for 8 h; cycloheximide (Sigma) was subsequently added to the medium to yield a final concentration of 40 μM, which would inhibit new synthesis of synphilin-1. The cells were cultured for chase intervals of 0, 2, 4, 6, 8, 12, 18, and 24 h and harvested in the lysis buffer after the appropriate chase time. An equal amount of protein from each lysate was separated on SDS-PAGE and immunoblotted with an anti-HA or anti-Myc antibody. The degree of synphilin-1 expression was quantified by densitometric analysis with NIH Image software.

Ubiquitination Assay—An in vivo ubiquitination assay was performed as described previously (12, 20, 44). HER 293 cells were transfected with pcDNA3-Myc-Siah-1, pcDNA3-Myc-SiahN, pcDNA3-Myc-SiahΔC, pcDNA3-Myc-Parkin, pcDNA-Myc-beta-TrCP/FWD1, pcDNA3-HA-synphilin-1, pcDNA3-HA-α-synuclein, or pcDNA3-FLAG-Ub. After 24 h, cells were cultured for 8 h with 20 μM MG132. Cells were lysed and immunoprecipitated with an anti-HA antibody. Each precipitate was divided into two parts, separated on SDS-PAGE, and analyzed by immunoblotting with anti-FLAG and anti-HA antibodies to detect ubiquitin-conjugated synphilin-1.

Preparation of GST Fusion-Siah Proteins and in Vitro Binding Assays—To generate Siah-1 protein fused with GST at the N terminus (GST-Siah), the coding region of Siah-1 cDNA was amplified by PCR using the following forward and reverse primers: 5'-CTC GAA TTC TTC TTG GCG AGT CTT TTC CCC TGT AAA TAT GCG-3' (GF4) and 5'-CTC CTC GAG TCA GTG TAT GGA CTG CTG CAG-3' (GR4) for GST-RING-ZF; 5'-CTC GAA TTC ACA TGT TGT CCA ACT TGC CCG-3' (GF5) and GR1 for GST-SBD, 5'-CTC GAA TTC CAT CTC GAG TCA GTG TAT GGA CTG CTG CAG-3' (GF6) and GR1 for GST-SBD; and 5'-CTC GAA TTC CCT CAT CTG ATG CAT CAG AAG-3' (GF7) and 5'-CTC GAA TTC CCT CAT CTG ATG CAT CAG AAG-3' (GR2) for GST-SBD-S. DNA fragments obtained from PCR were cloned into a pGEX-4X-1 vector (Amersham Biosciences). The sequences of all constructs were confirmed by DNA sequencing. GST fusion synphilin-1 proteins that contained the N terminus (aa 1–202; GST-synphilin-N), the ANK repeats 1–3 (aa 87–458; GST-synphilin-N2), the ANK repeats 1–4 and the coiled-coil domain (aa 349–617; GST-synphilin-ANK), or the ANK repeats 5–8 and the C terminus (aa 611–919; GST-synphilin-C), were constructed as described previously (39). GST fusion proteins were produced in Escherichia coli BL21 via isopropyl-beta-D-thiogalactopyranoside induction and purified using glutathione-Sepharose 4B (Amer- sham Biosciences) as described previously (8, 39).

For the precipitation assay, HER 293 cells transfected with pcDNA3-HA-synphilin-1, pcDNA3-Myc-Siah-1, pcDNA3-HA-α-synuclein were lysed and precipitated with various GST fusion proteins or GST alone as described previously (39). Each binding assay was conducted with 10 μg of GST fusion protein bound to glutathione-Sepharose 4B. After the lysate protein content was normalized and precipitated with GST fusion proteins, bound proteins were separated on SDS-PAGE and immunoblotted with an anti-HA or anti-Myc antibodies. GST fusion proteins used for the binding assays were stained by Coomassie Brilliant Blue R250 (Sigma).

Dopamine Release Assay—PC12-Tet-Siah cells, PC12-Tet-ΔC cells, or PC12-Tet cells were plated onto 35-mm dishes at a density of 10^5 cells per dish. In a standard experiment, cells were subsequently transfected with the indicated plasmid DNA using LipofectAMINE 2000 and grown with or without Tet for 24 h. For a dopamine release assay, cells were washed twice with a low K+ solution (20 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 11 mM glucose) and incubated in the low K+ solution for 2 min. The medium was subsequently replaced with 1 ml of the low K+ solution plus 1 ml of a high K+ solution (20 mM HEPES-NaOH, pH 7.4, 85 mM NaCl, 60 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 11 mM glucose) for 0, 2, 4, 6, 10, and 16 min; the solution was then collected. Concentrations of dopamine, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were determined by high pressure liquid chromatography (HPLC) using a reverse-phase column and...
an electrochemical detector system (Eicom) as described previously (45). Dopamine, DOPAC, and HVA concentrations for each sample were normalized for the total cell number for each condition. Dopamine release was calculated as a percentage of the total intracellular dopamine contents. Data were calculated from four independent experiments. 

Statistic analysis was performed with one-way analysis of variance.

RESULTS

Identification of Siah-1 as a Protein That Interacts with Synphilin-1—To identify proteins that interact with synphilin-1, yeast two-hybrid screening of a rat brain cDNA library was performed using full-length synphilin-1 as bait. From 1.0 × 10⁶ library transformants, a positive clone that contained a fragment nearly identical to the Siah-1a gene was obtained. Rat Siah-1a is a RING finger-type E3 that shares 99.6% amino acid identity with human Siah-1. The full-length human Siah-1 cDNA was isolated from a human brain cDNA library by PCR.

Interaction of Siah-1 with synphilin-1 was examined in mammalian cells using an immunoprecipitation assay. From lysates of HEK 293 cells coexpressing HA-tagged synphilin-1 (HA-synphilin-1) and Myc-tagged Siah-1 (Myc-Siah-1), an anti-HA antibody immunoprecipitated Myc-Siah-1 (Fig. 1A). In addition, an anti-Myc antibody immunoprecipitated HA-synphilin-1 in a similar manner (data not shown). It was concluded that Siah-1 and synphilin-1 proteins interact with each other in mammalian cells. Similarly, HA-synphilin-1 and Myc-SiahΔN, a deletion-mutant Siah-1 that lacks the RING finger domain, were also immunoprecipitated, suggesting that the RING finger domain of Siah-1 is not required for association with synphilin-1 (Fig. 1A). To determine whether endogenous synphilin-1 and Siah-1 interact in brain in vivo, an immunoprecipitation assay using antibodies against Siah-1, synphilin-1, GST, or normal rabbit IgG was performed in rat brain homogenate followed by immunoblotting with antibodies against synphilin-1 or Siah-1. Synphilin-1 and Siah-1 immunoprecipitated each other in vivo, suggesting physiologic relevance of the interaction (Fig. 1B).

Identification of Domains Involved in Siah-1-Synphilin-1 Association—To define the specific domain of Siah-1 responsible for interaction with synphilin-1, a series of GST fusion proteins containing various truncations of the Siah-1-conserved region were generated. HEK 293 cells transfected with expression vectors for HA-synphilin-1 were lysed and precipitated with various GST fusion Siah-1 proteins, such as GST-Siah, GST-RING-ZF, GST-ZF, GST-SBD, GST-SBD-S, and GST-SBD-SS, as well as GST alone as a control (Fig. 2A). Anti-HA immunoblotting revealed that GST fusion Siah-1 proteins containing the SBD exclusively precipitated synphilin-1 (Fig. 2B). Such results suggest that Siah-1 binds to synphilin-1 through its SBD. Furthermore, the centrally located polypeptide in the SBD (aa 180–240; SBD-SS) was found to be necessary and sufficient for binding to synphilin-1 (Fig. 2B).

To identify the binding site of synphilin-1 to Siah-1, HEK 293 cells transfected with expression vectors for Myc-Siah-1 were lysed and precipitated with various GST fusion synphilin-1 proteins (Fig. 2A). Anti-Myc immunoblotting revealed that only the N-terminal residues 1–202 of synphilin-1 (synphilin-N1) could precipitate Siah-1 (Fig. 2C). In contrast, other regions of synphilin-1, including the ANK repeats or C-terminal domain, were unable to bind to Siah-1. It was concluded that the SBD of Siah-1 and the N terminus of synphilin-1 interact with each other.

Siah-1 and Synphilin-1 Proteins Are Widely Expressed in the Brain—To investigate expression of Siah-1, synphilin-1, and α-synuclein, rat protein lysate from various tissues were analyzed by immunoblotting using antibodies against Siah-1, synphilin-1, α-synuclein, or α-tubulin. Siah-1 was enriched in brain, heart, pancreas, kidney, and skeletal muscle (Fig. 3).
Siah-1 was expressed in all regions in the central nervous system, including the cerebral cortex, hippocampus, striatum, cerebellum, medulla, and spinal cord. In the rat brain, the anti-Siah-1 antibody recognized a single band of 35 kDa, similar to the predicted molecular mass of Siah-1 protein (32 kDa), that disappeared when the antibody was preincubated with antigen (GST-Siah protein) (Fig. 3). Synphilin-1 was enriched in all regions in the brain, whereas α-synuclein was enriched in the cerebral cortex, hippocampus, and striatum. Such results demonstrate that both Siah-1 and synphilin-1 are coexpressed in the brain.

**Colocalization of Siah-1 with Synphilin-1 in the Cytoplasm**—Because Siah-1 interacted with synphilin-1 in the brain, endogenous Siah-1 and synphilin-1 localization was investigated with an immunofluorescent study. Confocal microscopic analysis revealed that Siah-1 localized in the cytoplasm of human dopaminergic SH-SY5Y cells. Synphilin-1 was observed as a cytoplasmic ring-like appearance as described previously (11) (Fig. 4A). Such immunofluorescence disappeared when the antibodies were preincubated with each antigen. Furthermore, a double-staining immunofluorescent study was performed to determine whether Siah-1 colocalized with synphilin-1. Myc-Siah-1 was concomitantly expressed with HA-synphilin-1 by transient transfection of HEK 293 cells. Confocal microscopic analysis revealed that synphilin-1 formed cytoplasmic inclusions (Fig. 4B) and that Siah-1 was distributed peripheral to the synphilin-1 inclusions. An overlay of Siah-1 and synphilin-1 staining demonstrated colocalization of both proteins.

**Siah-1 Mediates Synphilin-1 Degradation via the Ubiquitin-Proteasome Pathway**—Siah-1 has been demonstrated to specifically mediate degradation of its substrate proteins. Accordingly, the present study investigated whether Siah-1 mediates synphilin-1 degradation. Myc-Siah-1 was concomitantly expressed with HA-synphilin-1 by transient transfection of HEK 293 cells. Cell lysates were subjected to immunoblotting with anti-HA or anti-Myc antibodies. In cells expressing synphilin-1 alone, synphilin-1 was detected as a faint band (Fig. 5A). Nonetheless, in cells coexpressing synphilin-1 and Siah-1, synphilin-1 was detected as a faint band (Fig. 5A). Moreover, synphilin-1 expression recovered with addition of the potent proteasome inhibitor MG132 (20 μM) despite Siah-1 expression. On the other hand, with coexpression of E3-inactive SiahAN, synphilin-1 expression was not altered (Fig. 5A). Such results suggest that Siah-1 might play a role in synphilin-1 degradation by the ubiquitin-proteasome pathway. In addition, Siah-1 expression was also remarkably increased in the presence of MG132. To examine whether Siah-1 itself is also ubiquitinated, HEK 293 cells were transfected with ex-
In addition, to investigate whether Siah-1 would facilitate synphilin-1 degradation, a pulse-chase assay was performed. In cells transfected with pcDNA3-HA-synphilin-1 and pcDNA3-Myc-Siah-1, the synphilin-1 degradation rate was much higher than that of cells transfected with pcDNA3-HA-synphilin-1 and empty pcDNA3-Myc vectors. Moreover, the half-life of synphilin-1 was 8 h for cells cotransfected with expression vectors for Siah-1, whereas that for cells cotransfected with empty vectors was 44 h (Fig. 5C). Nonetheless, the synphilin-1 degradation rate in cells coexpressing Myc-SiahΔN was slower than that in cells cotransfected with empty vectors, suggesting that synphilin-1 degradation was inhibited by a dominant-negative effect of SiahΔN on endogenous Siah-1 (Fig. 5C). Such results are consistent with the notion that the RING finger domain of E3 is required for substrate ubiquitination that leads to degradation. As a negative control, β-TrCP/FWD1, an E3 for β-catenin, was utilized for the pulse-chase assay. Both Siah-1 and β-TrCP/FWD1 independently facilitate β-catenin degradation (35, 47, 48). In cells transfected with HA-synphilin-1 and Myc-β-TrCP/FWD1, the synphilin-1 degradation rate was almost the same as that of cells transfected with empty vectors (Fig. 5C), suggesting that Siah-1 specifically facilitated synphilin-1 degradation. Because Parkin has also been reported to regulate synphilin-1 degradation (20), the present study compared the effects of Siah-1 and Parkin on the synphilin-1 degradation rate. It was noted that the synphilin-1 degradation rate for cells coexpressing Myc-Siah-1 was higher than that for cells coexpressing Myc-Parkin. Moreover, the half-life of synphilin-1 was 8 h for cells expressing Siah-1 and 18 h for cells expressing Parkin (Fig. 5C). Siah-1 and Parkin cellular expression levels were similar (data not shown). Such results suggest that Siah-1 facilitates synphilin-1 degradation more efficiently than Parkin.

Siah-1 Ubiquitinates Synphilin-1 via Its RING Finger Domain—Because proteasome-dependent proteolysis involves ubiquitination of target proteins, the present study investigated whether Siah-1 would facilitate synphilin-1 degradation by promoting synphilin-1 ubiquitination. HEK 293 cells were transfected with expression vectors for HA-synphilin-1, FLAG-tagged ubiquitin (FLAG-Ub), and Myc-Siah-1 and subsequently cultured with MG132. Cells were lysed and immunoprecipitated with an anti-HA antibody, followed by immunoblotting with an anti-FLAG antibody to detect ubiquitination of target proteins. The present study demonstrated that Siah-1 ubiquitinates synphilin-1 via its RING finger domain. Studies have shown that the RING finger domain of E3 is required for substrate ubiquitination (58). Therefore, the RING finger domain of Siah-1 is also required for synphilin-1 ubiquitination. In addition, the present study investigated whether Siah-1 interacts with α-synuclein. Nonetheless, α-synuclein was not ubiquitinated by Siah-1 (Fig. 7A).
Anti-HA immunoblotting revealed that lysates prepared from HEK 293 cells cotransfected with pcDNA3-HA-synphilin-1, pcDNA3-FLAG-ubiquitin, pcDNA3-Myc-Siah-1, pcDNA3-Myc-SiahΔN, pcDNA3-Myc-SiahΔC, or pcDNA3.1-Myc-Parkin were immunoprecipitated with an anti-HA antibody (HA-IP). Immunoprecipitates were divided into two parts and analyzed by WB with anti-FLAG and anti-HA antibodies. Expression of Myc-Siah-1, Myc-SiahΔN, Myc-SiahΔC, or Myc-Parkin in each total cell lysate was detected by WB with an anti-Myc antibody. FLAG-Ub-conjugated proteins in each total cell lysate were detected by WB with an anti-FLAG antibody. Molecular mass markers are indicated on the right.

**Fig. 6.** Siah-1-mediated ubiquitination of synphilin-1. A, lysates prepared from HEK 293 cells cotransfected with expression vectors for HA-α-synuclein wild type, A53T, or A30P were lysed and precipitated with GST fusion Siah-1 protein. Anti-HA immunoblotting revealed that α-synuclein and Siah-1 did not bind to each other (Fig. 7B). It was also examined whether Siah-1 interacts with α-synuclein in mammalian cells. From lysates of HEK 293 cells cotransfected with expression vectors for HA-α-synuclein and Myc-Siah-1, an anti-HA antibody was found to not immunoprecipitate Myc-Siah-1 (data not shown). It was subsequently investigated whether Siah-1 mediates α-synuclein degradation. HEK 293 cells coexpressing HA-α-synuclein and Myc-Siah-1 were treated with MG132 for the indicated time. α-Synuclein expression levels were not altered by Siah-1 expression (Fig. 7C), suggesting that Siah-1 does not facilitate α-synuclein degradation.

**Effects of Siah-1 on Dopamine Release**—Siah-1 is known to be distributed in the central nervous system. In addition, recent papers have demonstrated that Siah-1 is involved in synaptic transmission (12, 38, 49). The present study examined whether Siah-1 affects dopamine release. Monoclonal cell lines, i.e., PC12-Tet-Siah (clones PS1–4) and PC12-Tet-ΔC (clones PDC1–4), that have Tet-repressible expression systems for Siah-1 and SiahΔC, respectively, were established. Immunoblotting with an anti-Myc antibody demonstrated that Tet in the culture medium could negatively regulate Siah-1 and SiahΔC protein cellular expression (PS1 and PDC1). Expressions were found to be very low at 500 ng/ml of Tet (Siah− or ΔC−), moderate at 50 ng/ml of Tet (Siah+), and high in the absence of Tet (Siah++ or ΔC+++) (Fig. 8A). Siah-1 reduced the total intracellular dopamine content without increasing dopamine metabolites such as DOPAC or HVA in PC12-Tet-Siah cells (PS1). In contrast, the total intracellular dopamine content in PC12-Tet-ΔC cells (PDC1) in the absence of Tet was similar to that in PC12-Tet cells, suggesting that Siah-1 might inhibit dopamine biosynthesis (Fig. 8B). Similar to PC12-Tet cells, high K+ (60 mM) stimulation induced dopamine release from PC12-Tet-Siah (PS1) and PC12-Tet-ΔC (PDC1) cells, whereas low K+ (4.7 mM) stimulation induced little dopamine release (Fig. 8B). High K+-induced dopamine release was rapid and reached a plateau at ~6 min. Nonetheless, maximum extracellular dopamine levels were decreased in relation to the decreased total intracellular dopamine contents resulting from Siah-1 expression (Fig. 8B). Siah-1 did not affect dopamine release kinetics, which was calculated as a percentage of the total intracellular dopamine content. Independent from Siah-1 expression, ~80–90% of the total intracellular dopamine content was released within 6 min (Fig. 8B). Similar results were obtained from other monoclonal cell lines (PS1–4, PDC1–4) (Fig. 8C).

**Synphilin-1 Inhibits High K+-induced Dopamine Release**—The role of synphilin-1 in dopamine release is poorly understood. The present study also examined whether synphilin-1 might be involved in dopamine release. PC12-Tet-Myc-Siah-1 cells were transfected with empty expression vectors or expression vectors for either synphilin-1 or α-synuclein, which were grown in the presence of Tet to inhibit Siah-1 expression (Siah−) and then stimulated with high K+ solution for 1 min. Synphilin-1 expression reduced the total intracellular dopamine content (Fig. 9A) without increasing dopamine metabo-
lites, such as DOPAC and HVA (data not shown). α-Synuclein expression also reduced the total intracellular dopamine content as described previously (10). Because the present work suggests that Siah-1 facilitates synphilin-1 degradation, it can also be said that Siah-1 might negatively regulate synphilin-1 function. Upon Siah-1 coexpression with synphilin-1 and α-synuclein, a state attained by removing Tet from the medium (Siah\(-\text{Tet}\)), the total intracellular dopamine content was further reduced (Fig. 9A). Expression of synphilin-1 and α-synuclein inhibited the high K\(^+\)-induced increase in extracellular dopamine levels, in proportion to the decreased total intracellular dopamine contents (Fig. 9B). With Siah-1 coexpression, the high K\(^+\)-induced increase in extracellular dopamine levels was further reduced. Nonetheless, when dopamine release kinetics were evaluated by percentage of dopamine release, high K\(^+\)-induced dopamine release from cells expressing synphilin-1 was depressed compared with controls, whereas release from cells expressing α-synuclein was almost equal to controls (Fig. 9C), suggesting that synphilin-1 might inhibit dopamine release. Furthermore, when Siah-1 expression in the same cells was induced by removing Tet from the medium (Siah\(+\)), dopamine release increased to control levels (Fig. 9C). Such results suggest that Siah-1 might abrogate the inhibitory effect of synphilin-1 on dopamine release by facilitating synphilin-1 degradation via the ubiquitin-proteasome pathway. Similar results were obtained from other monoclonal cell lines (Fig. 9D).

**DISCUSSION**

The present study identified Siah-1 as a binding partner for synphilin-1 that is implicated in the pathogenesis of Parkinson's disease. Siah/Sina family proteins are evolutionarily conserved E3 ubiquitin ligases that participate in regulating ubiquitination and proteasome-dependent degradation of multiple proteins. Siah-1 contains a RING finger domain at the N terminus that is required for interacting with E2s and a SBD at the C terminus that is required for substrate binding. Binding assays using GST fusion proteins indicated that Siah-1 also bound to synphilin-1 via the SBD. In particular, amino acid residues 180–240 in the SBD are necessary and sufficient for synphilin-1 binding. Recently, a binding motif for Siah, RPVA\(X\)PXXR, was identified (50). Furthermore, the core sequence PXAXXVP was found in the Siah interacting proteins SIP, OB1-F, DCC, and TIEG1, with more degenerate consensus sequences found in NUMB, Vav, Kid, and N-CoR (50). The most conserved residues in the motif appear to be VXP; mutagenesis of both of these residues reduced or abrogated Siah binding (50). The GST fusion protein binding assays of the present study indicate that the SBD of Siah-1 binds to the N-terminal region (aa 1–202) of synphilin-1, within which a consensus sequence, PXAXXP, is located at residues 74–80, suggesting the presence of a binding motif.

Pulse-chase and ubiquitination assays demonstrated that Siah-1 specifically facilitates synphilin-1 ubiquitination and
degradation. Although Parkin also exerts a similar function, synphilin-1 ubiquitination levels achieved with Siah-1 were higher than those attained with Parkin. In addition, synphilin-1 degradation rates achieved with Siah-1 were also faster than those attained with Parkin. Such results suggest that Siah-1 functions as a more efficient E3 for synphilin-1 than Parkin. Because Siah proteins interact with E2s, such as UbcH5, UbcH8, or UbcH9, and Parkin interacts with UbcH6, UbcH7, or Ubc12, interaction with different E2s might account for the distinct degradation rates. In addition to Siah-1 and Parkin, a recent study demonstrated that Dorfin also ubiquitinates synphilin-1 (21). Accordingly, three RING finger-type E3s, i.e., Siah-1, Parkin, and Dorfin, have been found to target synphilin-1 for ubiquitination and degradation.

The functional similarity among Siah-1, Parkin, and Dorfin raises important questions regarding redundancy and physiological significance of multiple pathways facilitating synphilin-1 degradation. Parkin and Dorfin bind the central portion of synphilin-1, which contains ANK repeats, a coiled-coil domain, and an ATP/GTP-binding site. Nonetheless, Siah-1 was not found to bind to that portion. As mentioned above, a specific peptide motif that mediates interaction of each E3 with a range of substrate proteins has been elucidated. Accordingly, it remains possible that if a single protein contains multiple peptide motifs for different E3s, such a protein could be ubiquitinated by multiple E3s, leading to degradation by multiple, synergistic mechanisms. It appears uncertain whether three proteins can simultaneously bind synphilin-1, but it remains possible that Siah-1 might synergistically ubiquitinate synphilin-1 with Parkin or Dorfin.

On the other hand, synphilin-1 is initially distributed in the cell bodies of immature neurons, subsequently becoming redistributed toward presynaptic nerve terminals during development after birth (51). It remains possible that Siah-1, Parkin, and Dorfin independently act as E3s for synphilin-1, depending on the distribution or developmental stage, despite concurrent
expression of all three E3s in the adult brain. Future studies will need to elucidate the reason underlying synphilin-1 ubiquitination by multiple E3s.

A previous study demonstrated that synphilin-1 strongly associated with synaptic vesicles; synphilin-1 was found to be located close to synaptic vesicles using electron microscopy (51). The present study demonstrated that Siah-1 is distributed in the central nervous system, and recent papers have shown that Siah-1 binds to group 1 metabotropic glutamate receptors (mGluR1 and mGluR5), which are involved in the regulation of synaptic transmission and regulates mGluR-mediated signaling (38). In addition, Siah-1 attenuates mGluR-mediated calcium current modulation at the synaptic terminal (49). Furthermore, it was demonstrated that Siah-1 binds to synaptophysin and that endogenous Siah-1 is localized on synaptic-like microvesicles in PC12 cells (12). Such findings suggest that Siah-1 might play a role in neurotransmitter release by facilitating ubiquitination of synaptic vesicle proteins, to include synphilin-1. In the present work, Siah-1 and synphilin-1 reduced intracellular dopamine content without increasing dopamine metabolites, suggesting that both proteins might inhibit dopamine biosynthesis. Siah-1 did not affect high K⁺/H₁₁₀₀₁-induced dopamine release from PC12 cells. Synphilin-1 was found to moderately inhibit high K⁺/H₁₁₀₀₁-induced dopamine release from cells, whereas coexpression of Siah-1 abrogated the inhibitory effect of synphilin-1 on dopamine release. Such a result suggests that association of Siah-1 with synphilin-1 might be involved in the regulation of dopamine release. It must be said.

**Fig. 9.** Synphilin-1-mediated inhibition of dopamine release was abrogated by Siah-1. A, PC12-Tet, PC12-Tet-Siah, or PC12-Tet-ΔC cells were transfected with empty expression vectors (Mock) or expression vectors for either HA-synphilin-1 (synph) or HA-α-synuclein (αS). Transfected cells were grown in either the presence or absence of 500 ng/ml of Tet to inhibit (Siah⁻ and SiahΔC⁻) or induce (Siah⁺ and SiahΔC⁺) Siah-1 expression, respectively. The total intracellular dopamine contents were measured by HPLC and normalized for total cell number. B and C, cells were treated with high K⁺ solution for 1 min. Extracellular dopamine levels were measured by HPLC and either normalized for total cell number (B) or calculated as a percentage of the total intracellular dopamine contents that were shown in A (C). Siah-1, SiahΔC, synphilin-1, and α-synuclein expression was confirmed by WB with anti-Myc and anti-HA antibodies (C). Data are presented as means ± S.D. from four independent experiments. Statistical analysis was performed by one-way analysis of variance. *, p < 0.05. D, dopamine release assays were performed using other monoclonal cell lines. The table presents the results of % dopamine release at 1 min from multiple monoclonal cell lines in the presence of 0 or 500 ng/ml of Tet. Data are presented as means from four independent experiments in each cell lines. Statistical analysis was performed by one-way analysis of variance. *, p < 0.05.
that the effects of Siah-1 and synphilin-1 on dopamine release is relatively small. Because multiple proteins close to synaptic vesicles are involved in neurotransmitter release, our observation might only represent one aspect of regulation of dopamine release. Further investigation should be performed to clarify the role of Siah-1 and synphilin-1 in neurotransmission.

Recently, Siah-1 has been shown to form an SCF-type complex with Skp1, Ebi, Sip, and APC, to facilitate β-catenin degradation in a p53-dependent manner (35, 48, 52). A recent report demonstrated that Parkin also facilitates synphilin-1 degradation in a p53-dependent manner (35, 48, 52). A recent box-type E3s containing Siah-1 or Parkin also facilitate syn-

The role of Siah-1 and synphilin-1 in neurotransmission. Further investigation should be performed to clarify the role of Siah-1 and synphilin-1 in neurotransmission.

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