Seven in absentia homolog 1A mediates ubiquitination and degradation of group 1 metabotropic glutamate receptors

Koki Moriyoshi*, Kouichirou Iijima*, Hajime Fujii†, Hiroshi Ito†, Yoshimi Cho†, and Shigetada Nakanishi*†‡

Departments of *Molecular and System Biology, Graduate School of Biostudies, and †Biological Sciences, Faculty of Medicine, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan

Contributed by Shigetada Nakanishi, April 30, 2004

Seven in absentia homolog 1A (Siah1A) is a member of the RING-finger-containing E3 ubiquitin ligases and has been shown to bind to the Siah-interacting domain (SID) at the carboxyl-terminal tails of the long splice forms of group 1 metabotropic glutamate receptors (mGluR1a and mGluR5). We examined the function of Siah1A in ubiquitination and degradation of group 1 mGluRs in heterologously expressing cell lines. Coexpression of Siah1A markedly decreased the SID-containing splice forms of group 1 mGluRs but not the SID-lacking mGluR1b splice form or the SID-deleted mGluR1a mutant. The decrease of mGluR1a resulted from accelerated protein turnover, as revealed by pulse–chase experiments. The Siah1A-mediated degradation of group 1 mGluRs was abrogated by not only mutations at the RING-finger domain of Siah1A but also treatment with a proteasome inhibitor. Siah1A coexpression induced strong ubiquitination of group 1 mGluRs. Replacements of lysine residues with arginine showed that Siah1A-mediated ubiquitination occurs at multiple lysine residues spanning both the seven-transmembrane region and carboxyl-terminal tail of mGluR5. *In situ* hybridization histochemistry showed a widespread distribution of Siah1 mRNAs, with high expression in the hippocampal pyramidal neurons and cerebellar Purkinje cells. Group 1 mGluRs play critical roles in the neural plasticity in both the hippocampal neurons and Purkinje cells. This investigation indicates that Siah1A serves as a selective ubiquitin ligase that mediates ubiquitination-dependent degradation of long splice variants of group 1 mGluRs and would contribute to posttranslational down-regulation of group 1 mGluRs.

Synaptic remodeling is a fundamental mechanism for information processing and storage in the developing and mature brain (1). In the process of synaptic remodeling, many postsynaptic proteins including neurotransmitter receptors, scaffolding proteins, and signaling molecules increase or decrease in response to stimulation or inhibition of synaptic activities (2). These molecular events are controlled by multiple mechanisms including transcriptional control (3), regulated dendritic protein translation (4), dynamic changes in protein localization to or from synapses (5, 6), and selective protein degradation through the ubiquitin system (2). Among them, little attention has been paid to involvement of the ubiquitin system until recently (7, 8). In the ubiquitin system, attachment of ubiquitin, a highly conserved 76-aa polypeptide, to lysine residues of target proteins is mediated by the sequential actions of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases (E3) (9). There is a variety of E3 ligases, and each of them recognizes specific target proteins and brings them to the degradation pathway (9). Ubiquitinated proteins are usually degraded by proteasome (9), but a number of membrane proteins including receptors and channels are endocytosed and transported to vacuole/lysosome after ubiquitination in a proteasome-independent manner (10, 11). *Drosophila* Sina (seven in absentia) and its mammalian homolog Siah (seven in absentia homolog) are members of the E3 ubiquitin ligase family with the RING-finger protein motif (12). In mice, there are three highly homologous Siah proteins, Siah1A, Siah1B, and Siah 2 (12). Siahs are known to recognize several target proteins including Deleted in Colorectal Cancer (DCC), synaptophysin, and Numb and promote the degradation of these proteins (13–15).

Glutamate receptors play central roles in neuronal excitation in the mammalian central nervous system (16). Glutamate receptors are categorized into two classes, ionotropic glutamate receptors and metabotropic glutamate receptors (mGluRs) (16). mGluRs are G protein-coupled receptors and consist of eight different subtypes, which are classified into three groups (16). Group 1 mGluRs (mGluR1 and mGluR5) are coupled to inositol 1,4,5-trisphosphate-calcium signaling cascade (16) and play important roles in neural plasticity processes including long-term potentiation, long-term depression, and synapse development and elimination (17–20). Both mGluR1 and mGluR5 comprise splice variants that differ in the cytoplasmic tails with long (mGluR1a and mGluR5) and short (mGluR1b and mGluR5d) carboxyl-terminal domains (21, 22). Recently, Siah1A has been shown to bind to the carboxyl-terminal domain of long splice variants of mGluR1 and mGluR5 (23) and attenuate group 1 mGluR-mediated calcium current inhibition in heterologously expressing superior cervical ganglion neurons (24). However, the biochemical events that are ensured by the direct interaction between Siah1A and group 1 mGluRs remain elusive.

In this article we report that group 1 mGluR–Siah1A interaction induces group 1 mGluR-specific ubiquitination and subsequent degradation. The mRNA expressions of Siah1 and group 1 mGluRs overlap in many brain regions including cerebellar Purkinje cells and hippocampal pyramidal neurons, in which group 1 mGluRs play essential roles in synaptic plasticity and synapse remodeling (17–20). These results imply that Siah regulates the mGluR turnover in the brain and could contribute to neural plasticity and remodeling in glutamatergic synapses.

Materials and Methods

Cell Culture and Plasmids. HEK293 (ATCC CRL-1573), COS-7 (ATCC CRL-1651), and BHK-21 (ATCC CCL-10) cells were cultured in DMEM supplemented with 10% FBS and antibiotics. All mGluR and NR1 cDNAs were tagged with Flag epitope at their amino-terminal ends just after signal sequences and subcloned into the expression vector pCMV-Tag3 (Stratagene). Mouse Siah1A cDNA, either tagged with myc epitope at its amino-terminal end or untagged, was subcloned into the expression vector pCI-neo (Promega). myc-tagged mouse ubiquitin was constructed as described in ref. 25 and subcloned into the expression vector pCI-neo (Promega). Deletion mutants of mGluR1a and Siah1A were made by PCR. Mutants containing amino acid replacements were generated by site-directed mu-

Abbreviations: Siah, seven in absentia homolog; Sina, seven in absentia; mGluR, metabotropic glutamate receptor; SID, Siah-interacting domain; P*n*, postnatal day *n*.

‡To whom correspondence should be addressed. E-mail: snakanis@phy.med.kyoto-u.ac.jp.

www.pnas.org/cgi/doi/10.1073/pnas.0403042101

tagenesis. DNA transfection was performed by using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions; 1.0 μg of myc-Siah1A DNA and 0.1 μg of receptor DNA were transfected, unless otherwise stated. Transfected cells were grown in the culture medium for 40 h before analysis. For inhibitor study, cells were treated with MG-132 (30 μM; Calbiochem) 24 h after transfection and incubated for 6 h before cell lysis.

Western Blotting and Immunoprecipitation. Transfected cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl/0.5% sodium deoxycholate/1% Nonidet P-40/0.1% SDS) supplemented with protease inhibitor cocktail (Sigma) and 50 μM MG-132. Immunoprecipitation of Flag-mGluR was carried out with anti-Flag M2 agarose beads (Sigma) in the radioimmunoprecipitation assay buffer, and the precipitated proteins were eluted with an excess Flag peptide (Sigma). Cell lysates or immunoprecipitates were subjected to Western blot analysis with anti-Flag M2 (Sigma), anti-myc (Santa Cruz Biotechnology), or anti-actin (Sigma) antibodies as described (26). Immunoreactive bands were detected with a SuperSignal Femto ECL kit (Pierce).

Pulse–Chase Analysis. Flag-mGluR1a was transfected with or without Siah1A in HEK293 cells. Twenty-four hours later, cells were washed and incubated for 45 min in a methionine/cysteine-free DMEM. The medium was replaced with a methionine/cysteine-free DMEM containing 200 μCi/ml (1 Ci = 37 GBq) of a mixture of [^{35}S]methionine and [^{35}S]cysteine (1,000 Ci/mmol; Amersham Pharmacia). After 1 h of incubation, the radioactive medium was removed, and cells were washed extensively with nonradioactive DMEM. Cells were cultured in the DMEM containing 10% FBS and an excess amount of methionine and cysteine. Cells were taken after appropriate chase time and lysed with the radioimmunoprecipitation assay buffer, and radiolabeled mGluR1a was immunoprecipitated with anti-Flag M2 agarose beads from an equivalent amount of lysates at each chase time. Immunoprecipitates were subjected to SDS/PAGE followed by autoradiography. Relative amounts of ^{35}S-labeled mGluR1a protein were quantified with BAS-5000 image analyzer (Fuji Film).

Northern Blot and *in Situ* Hybridization Analysis. Northern blot analysis was performed with total RNA (10 μg) isolated from transfected cells as described in ref. 27. The *Pma*CI–*Bgl*II fragment of mGluR1a cDNA (nucleotide residues 1489–2076 of the protein-coding region) was used as a probe. For *in situ* hybridization, freshly frozen brain sections of C57/BL6 mice were hybridized with ^{35}S-labeled antisense riboprobe corresponding to nucleotide residues 1–456 or 457–849 of the mouse Siah1A protein-coding region as described in ref. 26. Control hybridization was carried out in adjacent sections with use of sense riboprobes corresponding to the same region.

Results

Siah1A Induces mGluR1a Degradation. We examined the effect of Siah1A coexpression on protein levels of mGluR1a by immunoblotting cell lysates of HEK293 cells cotransfected with myc-Siah1A and Flag-mGluR1a for 40 h. Immunoblotting with anti-Flag antibody gave rise to two bands that corresponded to a monomeric and a dimeric form of mGluR1a (Fig. 1*A*) (28). Because the appearance of the dimeric form of mGluRs was more prominent and consistent in our experimental condition (Fig. 1*A*), we pursued changes in levels of the dimeric mGluRs in the subsequent experiments. When the expression of Siah1A was increased by increasing amounts of the transfected Siah1A cDNA in HEK293 cells, levels of mGluR1a protein in cell lysates decreased in a dose-dependent manner (Fig. 1*A*). In control,

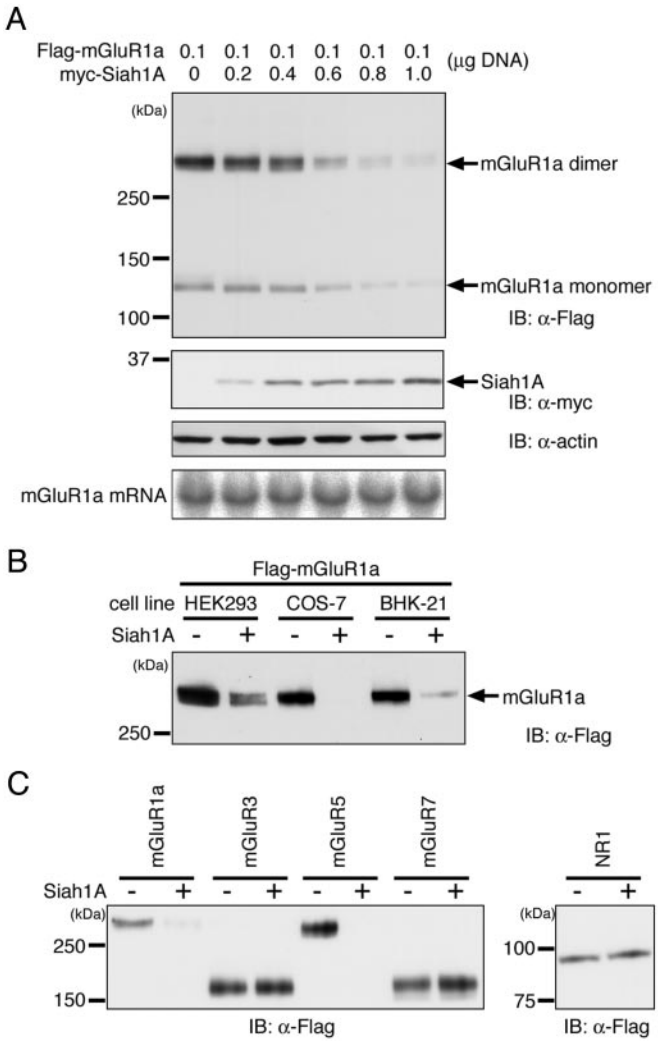

Fig. 1. Specific degradation of mGluR1a and mGluR5 by Siah1A. (*A*) HEK293 cells were transfected with a fixed amount of the Flag-mGluR1a DNA together with increasing amounts of the myc-Siah1A DNA. A vector DNA was added to make the total amounts of transfected DNAs equivalent in all experiments. Amounts of Flag-mGluR1a, myc-Siah1A, and actin in cell lysates were determined by immunoblotting with anti-Flag, anti-myc, and anti-actin antibodies, respectively. Immunoblotting of actin was conducted as an internal control. Levels of mGluR1a mRNA were determined by Northern blotting of the corresponding transfected cells. (*B*) HEK293, COS-7, or BHK-21 cells were transfected with Flag-mGluR1a DNA with or without myc-Siah1A DNA. Amounts of mGluR1a were determined by immunoblotting of cell lysates with anti-Flag antibody. (*C*) Various subtypes of mGluRs and the NR1 subunit of ionotropic glutamate receptors were Flag-tagged, and each of them was expressed with or without Siah1A in HEK293 cells. The specificity of Siah1A-mediated decrease of the receptor proteins was analyzed by immunoblotting with anti-Flag antibody. Molecular sizes (kilodaltons) of marker proteins are indicated on the left side of immunoblot analysis. IB, immunoblot; α-Flag, α-myc, and α-actin, anti-Flag, anti-myc, and anti-actin antibodies, respectively.

Siah 1A had no effect on levels of actin protein measured as an internal marker (Fig. 1*A*). In contrast to mGluR1a protein, levels of mGluR1a mRNA remained unchanged by increasing the transfected Siah1A cDNA (Fig. 1*A*). These results indicate that Siah1A influences levels of mGluR1a protein at the post-transcriptional level. Siah1A-dependent decrease of mGluR1a protein was also observed in COS-7 cells and BHK-21 cells (Fig. 1*B*), indicating that this process utilizes a cellular machinery common in many cell types. Coexpression of Siah1B or Siah2 similarly decreased mGluR1a protein levels in HEK293 cells

BIOCHEMISTRY

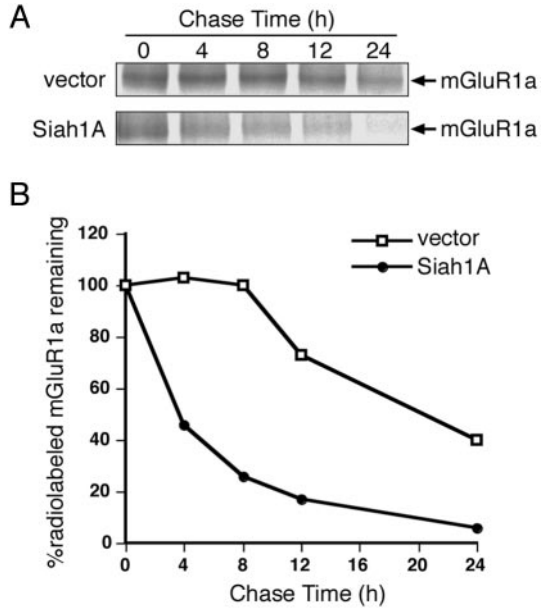

Fig. 2. Accelerated turnover of mGluR1a by Siah1A. (*A*) HEK293 cells transfected with Flag-mGluR1a, together with or without myc-Siah1A, were radiolabeled in the culture medium containing a mixture of [^{35}S]methionine/cysteine for 1 h. Cells were washed extensively with an excess of methionine/cysteine and cultured further in the methionine/cysteine-containing standard medium. Cells were collected and lysed at the indicated time of cell culture, and radiolabeled mGluR1a was immunoprecipitated with anti-Flag antibody. mGluR1a immunoprecipitates were subjected to SDS/PAGE and autoradiography. (*B*) Extents of ^{35}S-labeled mGluR1a radioactivity were quantified by the BAS-5000 image analyzer, and turnover of ^{35}S-labeled mGluR1a was determined by plotting radioactivity of ^{35}S-labeled mGluR1a against the time of cell lysis.

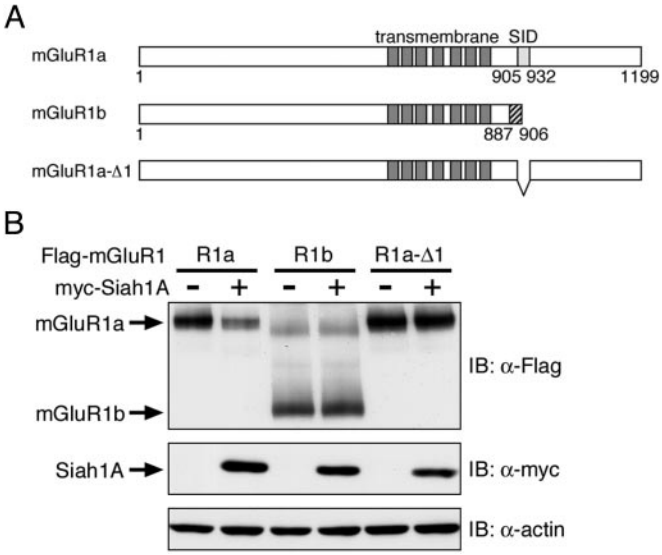

Fig. 3. Requirement of SID of mGluR1a for its degradation. (*A*) The schematic structures of mGluR1a, mGluR1b, and mGluR1a-Δ1 are indicated; the carboxyl-terminal sequence characteristic of the mGluR1b splice variant (residues 887–906) is indicated the cross-hatched box. (*B*) Flag-mGluR1a, Flag-mGluR1b, and Flag-mGluR1a-Δ1 were transfected with or without myc-Siah1A, and cell lysates were immunoblotted (IB) with anti-Flag (α-Flag), anti-myc (α-myc), and anti-actin (α-actin) antibodies.

(data not shown). All three members of the Siah family thus have the ability to decrease mGluR1a protein levels.

Siah1A binds to a specific amino acid stretch [Siah-interacting domain (SID)] at the carboxyl-terminal cytoplasmic tails of mGluR1a and mGluR5 and does not interact with the SID-lacking group 2 and 3 mGluRs (23). We examined the specificity of Siah1A-mediated down-regulation of receptor proteins by cotransfection of Siah1A with representative subtypes of group 1, 2 (mGluR3), and 3 (mGluR7) subfamilies as well as the NR1 subunit of *N*-methyl-D-aspartate-type ionotropic glutamate receptors. Of these receptors, only group 1 mGluRs (mGluR1a and mGluR5) decreased, and no other mGluR subtypes or the NR1 subunit were affected by coexpression with Siah1A (Fig. 1*C*). These results indicate that Siah1A specifically down-regulates group 1 mGluRs in the mGluR family, depending on its interaction with group 1 mGluR subtypes.

To further assess the Siah1A-mediated mGluR1a degradation, the turnover of mGluR1a protein was determined in the presence and absence of Siah1A by pulse–chase experiments (Fig. 2). One day after transfection of mGluR1a with or without Siah1A, HEK293 cells were incubated with a mixture of [^{35}S]methionine/cysteine in the methionine/cysteine-free medium for 1 h. Cells were washed extensively with a nonradioactive medium, and radioactivity of ^{35}S-labeled amino acids incorporated into mGluR1a was chased by quantitative autoradiography of immunoprecipitated ^{35}S-labeled mGluR1a in cell lysates. This analysis showed that the turnover of mGluR1a was accelerated by coexpression of Siah1A, a half-life of mGluR1a protein in the presence and absence of Siah1A being ≈4 and 20 h, respectively (Fig. 2*B*). These results indicate that Siah1A promotes the degradation of mGluR1a.

Siah1A Interaction for Siah1A-Mediated mGluR1a Degradation. To substantiate the importance of the interaction of Siah1A with the SID sequence of mGluR1a for Siah1A-mediated mGluR1a degradation, we examined whether coexpression of Siah1A has any effect on degradation of mGluR1b, a short splice variant of mGluR1 lacking the SID sequence at the carboxyl-terminal sequence (Fig. 3*A*). Protein levels of mGluR1b were unaffected by coexpression of Siah1A (Fig. 3*B*). More directly, an mGluR1a mutant (mGluR1a-Δ1) in which the SID sequence (amino acid residues 905–932) was deleted from the carboxyl-terminal tail of mGluR1a was constructed and tested (Fig. 3). This deletion mutant showed no decrease in protein levels by coexpression with Siah1A. The results indicate that the direct interaction between Siah1A and mGluR1a is responsible for Siah1A-mediated degradation of mGluR1a.

The RING-Finger Domain of Siah1A Is Necessary for mGluR1a Degradation. Siah possesses the RING-finger structure characteristic of E3 ligases (12, 29–31). The zinc-containing RING-finger structure is essential for interaction with ubiquitin-conjugating enzyme (E2) and subsequent ubiquitination and degradation of target proteins (30, 31). To test whether the RING-finger domain of Siah1A is necessary for mGluR1a degradation, two Siah1A mutants were constructed and analyzed for mGluR1a degradation. Siah1A-ΔR was a deletion mutant at the entire RING-finger domain (amino acid residues 39–76), whereas Siah1A-H59Y was a single amino acid mutant in which the critical histidine at position 59 within the RING-finger domain was replaced with tyrosine (Fig. 4*A*) (30). Both Siah1A-ΔR and Siah1A-H59Y mutants showed an increase in levels of mutant Siah1A proteins as compared with wild-type Siah1A, reflecting the reduced ability of these Siah1A mutants to degrade their own proteins (Fig. 4*B*). Despite the elevation of mutant Siah1A proteins, both Siah1A-ΔR and Siah1A-H59Y failed to decrease cotransfected mGluR1a (Fig. 4*B*). The results indicate that Siah1A serves as a specific E3 ubiquitin ligase for degradation of mGluR1a.

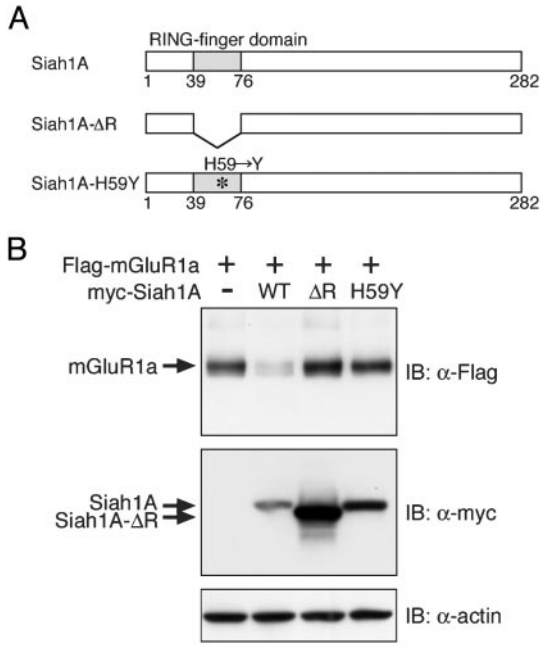

Fig. 4. The RING-finger domain of Siah1A for mGluR1a degradation. (*A*) The mutant structures of Siah1A-ΔR and Siah1A-H59Y are depicted on the basis of a schematic structure of Siah1A. (*B*) myc-Siah1A, myc-Siah1A-ΔR, and myc-Siah1A-H59Y were transfected with Flag-mGluR1a, and cell lysates were immunoblotted (IB) with anti-Flag (α-Flag), anti-myc (α-myc), and anti-actin (α-actin) antibodies.

Siah1A Induces Group 1 mGluRs Ubiquitination. To address whether Siah1A promotes ubiquitination of group 1 mGluRs, Flag-mGluR1a or Flag-mGluR5 was cotransfected with myc-ubiquitin with or without coexpression of Siah1A in HEK293 cells. Flag-mGluR1a and Flag-mGluR5 were immunoprecipitated with anti-Flag antibody, and extents of ubiquitination of mGluR1a and mGluR5 were analyzed by immunoblotting of mGluR immunoprecipitates with anti-myc antibody. A small amount of ubiquitinated mGluR1a or mGluR5 was detected in cell lysates untransfected with Siah1A (Fig. 5*A*). Extents of ubiquitination of both mGluR1a and mGluR5 were markedly enhanced by coexpression with Siah 1A (Fig. 5*A*). Ubiquitinated mGluR1a and mGluR5 were detected as broad bands at the upper part of the gel (Fig. 5*A*), suggesting that the mGluR proteins were polyubiquitinated by Siah1A. As expected, no such enhancement of ubiquitination of both mGluR1a and mGluR5 was conferred by coexpression with the Siah1A-ΔR mutant (Fig. 5*A*). The results indicate that the ubiquitin ligase activity of Siah1A is necessary for the enhanced ubiquitination and accelerated degradation of both mGluR1a and mGluR5.

Siah1A Ubiquitinates Lysine Residues at the Cytoplasmic Region of mGluR5. E3 ubiquitin ligases ubiquitinate lysine residues of target proteins (9). To further address whether Siah1A ubiquitination is essential for degradation of group 1 mGluRs, lysine residues at the cytoplasmic regions of mGluR5 were replaced with arginine, and the effects of lysine replacements on ubiquitination and degradation of mGluR5 were analyzed. There are 10 residues of lysine in the cytoplasmic loops and linings of the seven transmembrane segments and 12 lysine residues at the carboxyl-terminal tail of mGluR5 (27). All 22 lysine residues at the cytoplasmic regions of mGluR5 were replaced with arginine in the mGluR5-Δall (K) mutant. Neither enhanced ubiquitination nor accelerated degradation were observed for mGluR5-Δall (K) by cotransfection with Siah1A (Fig. 5*B*), indicating that

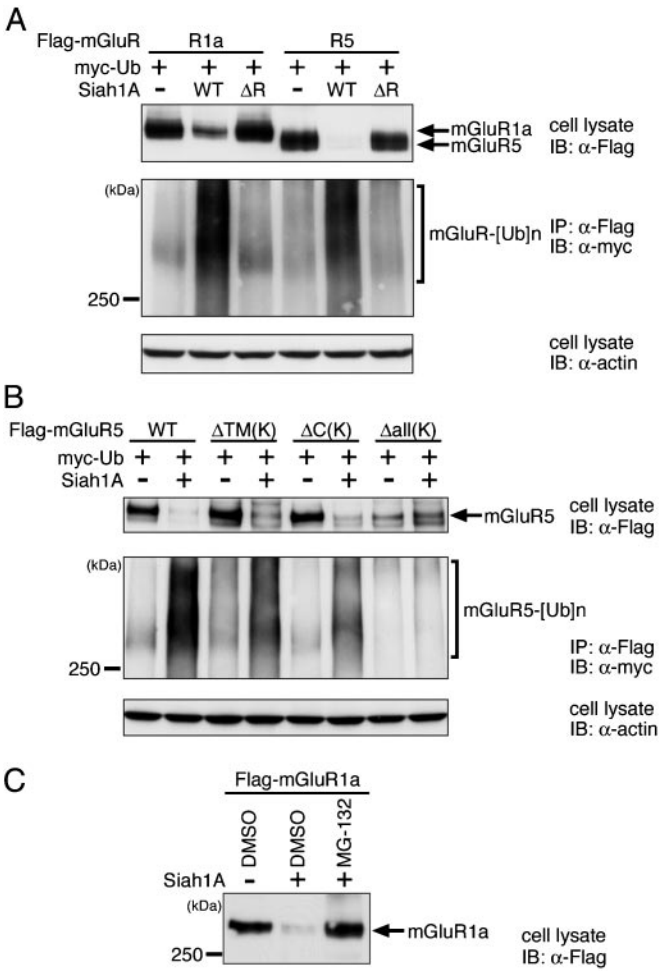

Fig. 5. Siah1A-mediated ubiquitination at multiple lysine residues of group 1 mGluRs and inhibition of Siah1A-mediated degradation of mGluR1a by a proteasome inhibitor. (*A*) Flag-mGluR1a or Flag-mGluR5 together with myc-ubiquitin (myc-Ub) was expressed with or without wild-type (WT) Siah1A or Siah1A-ΔR. Cell lysates were immunoblotted with anti-Flag (α-Flag) antibody to confirm that Siah1A but not Siah1A-ΔR enhanced degradation of mGluR1a and mGluR5. Flag-mGluR1a and Flag-mGluR5 were immunoprecipitated (IP) from the corresponding cell lysates with anti-Flag antibody followed by immunoblotting (IB) with anti-myc (α-myc) antibody to detect mGluR ubiquitination (mGluR-[Ub]n). α-actin, anti-actin. (*B*) Flag-mGluR5 and lysine-replaced Flag-mGluR5 mutants together with myc-ubiquitin were transfected with or without Siah1A. Flag-mGluR5 degradation was examined by immunoblotting of cell lysates with anti-Flag antibody. Extents of ubiquitination of mGluR5 (mGluR5-[Ub]n) were measured by immunoprecipitation of mGluR5 with anti-Flag antibody followed by immunoblotting with anti-myc antibody. (*C*) COS-7 cells were transfected with Flag-mGluR1a and myc-Siah1A. Twenty-four hours after transfection, cells were treated with either 30 μM MG-132 or a vehicle (DMSO) and incubated for 6 h before cell lysis. Amounts of mGluR1a were determined by immunoblotting with anti-Flag antibody.

ubiquitination of lysine residues of mGluR5 is indispensable for degradation of this receptor protein. To identify the ubiquitination site of mGluR5, we subdivided the cytoplasmic regions of mGluR5 into two parts by constructing two lysine-replaced mutants, mGluR5-ΔTM (K) and mGluR5-ΔC (K), in which 10 lysine residues at the cytoplasmic region of the seven transmembrane segments (positions 610, 664, 676, 677, 678, 682, 683, 759, 771, and 827) and 12 lysine residues at the carboxyl-terminal tail (positions 850, 866, 880, 889, 906, 907, 917, 921, 962, 1065, 1113, and 1155) of mGluR5 were replaced with arginine. Unexpectedly, both mGluR5-ΔTM (K) and mGluR5-ΔC (K) mutants

were susceptible to Siah1A-mediated ubiquitination and degradation (Fig. 5*B*). These results indicate that ubiquitination occurs at multiple lysine residues spanning from the transmembrane-connecting cytoplasmic loops to the carboxyl-terminal tail of mGluR5 and that multiple ubiquitination sites contribute to degradation of mGluR5.

Inhibition of Siah1A-Mediated mGluR Degradation by a Proteasome Inhibitor. In previous reports, Siah has been shown to degrade several substrates [for example, Deleted in Colorectal Cancer (13), synaptophysin (15), and Numb (14)] in a proteasome-dependent manner. We tested the effectiveness of the proteasome inhibitor MG-132 on the Siah1A-dependent degradation of mGluR1a protein. When Siah1A-cotransfected cells were treated with 30 μM MG-132 for 6 h, Siah1A-dependent decrease of mGluR1a protein was abrogated (Fig. 5*C*). The results suggest that the proteasome-dependent mechanism underlies Siah1A-mediated degradation of mGluR1a.

Overlapping Expression of Siah1 and Group 1 mGluRs in the Mouse Brain. Siah1 mRNA is expressed in various tissues, with expression being the highest in the brain and moderate in the lung, testis, and thymus (12). We examined a detailed distribution of Siah1 mRNA in the mouse brain with *in situ* hybridization histochemistry. In these experiments, two nonoverlapping probes (residues 1–456 and 457–849) were prepared from the mouse Siah1A cDNA, but the high homology between Siah1A and Siah1B mRNAs (97% nucleotide identity in their protein-coding regions) could not allow distinguishing the distribution of the two Siah1 mRNA species. *In situ* hybridization analysis of brain sections of postnatal day (P)8, P12, P24, and adult mice showed a wide distribution of Siah1 mRNAs (Siah1A plus Siah1B mRNA) at all stages analyzed, and an expression pattern of Siah1 mRNAs, analyzed at P12, is presented in Fig. 6. A high expression of Siah1 mRNAs was observed in the hippocampus and the cerebellum, and other brain regions including the olfactory bulb, olfactory nucleus, striatum, cerebral cortex, and thalamus also expressed moderate levels of Siah1 mRNAs (Fig. 6*A*). This expression pattern of Siah1 mRNAs was confirmed by the two nonoverlapping probes of Siah1A mRNA (data not shown). Cell populations expressing Siah1 mRNAs in the hippocampus and cerebellum were analyzed further by *in situ* hybridization of emulsion-dipped sections (Fig. 6 *C–F*). A high expression of Siah1 mRNAs was detected in pyramidal neurons of the hippocampus and Purkinje cells of the cerebellum (Fig. 6 *C–F*). Pyramidal neurons in the hippocampus and Purkinje cells in the cerebellum express a high amount of mGluR5 and mGluR1a, respectively (26, 27, 32, 33). Thus, Siah1 and group 1 mGluR mRNAs are coexpressed in the same cell populations, at least in the hippocampus and cerebellum.

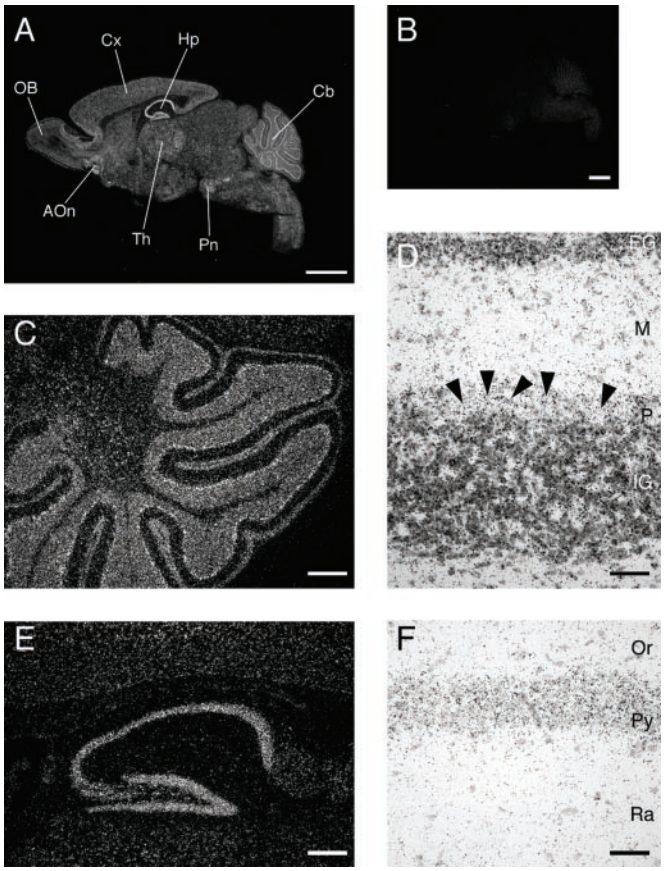

Fig. 6. Siah1 mRNA distribution in the mouse brain. (*A* and *B*) Negative images of sagittal sections of the P12 mouse brain with an antisense Siah1A riboprobe (nucleotide residues 1–456) (*A*) and the corresponding sense riboprobe (*B*). OB, main olfactory bulb; AOn, anterior olfactory nucleus; Cx, cerebral cortex; Hp, hippocampus; Th, thalamus; Pn, pontine nucleus; Cb, cerebellum. (*C* and *E*) Dark-field images of emulsion-dipped sections of the cerebellum (*C*) and hippocampus (*E*) of the P12 mouse. (*D* and *F*) Bright-field images of emulsion-dipped sections of the cerebellar cortex (*D*) and the pyramidal cell layer of the CA1 area of hippocampus (*F*) of the P12 mouse. Arrowheads indicate the locations of Purkinje cells. EG, external granular layer; M, molecular layer; P, Purkinje cell layer; IG, internal granular layer; Or, stratum oriens; Py, pyramidal cell layer; Ra, stratum radiatum. (Scale bars: *A* and *B*, 2 mm; *C* and *E*, 0.4 mm; *D* and *F*, 50 μm.)

Discussion

This investigation indicates that Siah1A, initially identified as a binding protein to group 1 mGluRs by yeast two-hybrid screening (23), acts as a specific E3 ubiquitin ligase that leads to ubiquitination and degradation of group 1 mGluRs. Analysis of mGluR subtype specificity and deletion mutation revealed that the SID sequence of group 1 mGluRs is indispensable for the specific Siah1A-mediated ubiquitination and degradation of group 1 mGluRs. Mutational analysis of Siah1A also disclosed that the RING-finger domain of Siah1A, similar to other E3 ubiquitin ligases, is essential for both ubiquitination and degradation of group 1 mGluRs. Furthermore, replacements of lysine residues with arginine at the cytoplasmic region of mGluR5 abolished not only the Siah1A-mediated ubiquitination but also the degradation of mGluR5. These results demonstrate that Siah1A interacts with the SID sequence at the carboxyl-terminal tail of group 1 mGluRs and facilitates the selective ubiquitination and degradation of this subclass of mGluRs. The Siah1A-mediated mGluR1a degradation is revealed to be blocked by a proteasome inhibitor MG-132, suggesting that the proteasome-dependent mechanism is involved. However, the involvement of the lysosome pathway, as in the cases of other membrane proteins (10, 11), remains possible, and the precise mechanism of degradation of the ubiquitinated mGluRs may be elucidated by additional investigation.

mGluR1 consists of two major splice variants, a long mGluR1a splice variant and a short mGluR1b splice variant (21). Both splice variants are highly expressed in a variety of neurons but are distinctly distributed depending on different neuronal cell types (32). They also are substantially different in the subcellular localization, activation kinetics of intracellular effectors, and agonist-independent activity (34–37). Importantly, mGluR1b lacks the SID sequence at the carboxyl-terminal region and is insusceptible to Siah1A-mediated ubiquitination and degradation. It therefore is likely that Siah1A is important not only for controlling protein levels of mGluR1a at the posttranslational level but also for distinctly regulating two different splice

variants of mGluR1, depending on their susceptibility to Siah1A-mediated degradation.

Siah1 mRNAs, although widely expressed in various brain regions, are enriched in hippocampal pyramidal neurons and cerebellar Purkinje cells, in which mGluR5 and mGluR1a mRNAs, respectively, are highly expressed. Gene targeting and other studies indicate that mGluR1a and mGluR5 play a key role in inducing long-term depression in Purkinje cells and long-term potentiation in hippocampal neurons, respectively (18–20). Neural plasticity is now thought to be evoked by dynamic changes in receptor and signaling molecules in synapses (1, 6). Furthermore, increasing evidence indicates that neural activity controls the activity of the ubiquitination complex and leads to dynamic changes in the molecular composition of postsynaptic structures including receptors and signaling molecules (2). Importantly, E3 ubiquitin ligases consist of diverse members of the protein family, and each recognizes a specific protein substrate (9). It therefore is important to identify a ubiquitin ligase specific for a key receptor molecule. In this investigation, Siah1A is revealed to act as a functional ubiquitin ligase specific for ubiquitination and degradation of group 1 mGluRs. Because little is known about the posttranslational regulation of group 1 mGluRs, our findings may aid further investigation of the molecular mechanisms underlying regulation of group 1 mGluRs in glutamatergic synapses.

This work was supported in part by research grants from the Ministry of Education, Science, and Culture of Japan and the Suntory Institute for Bioorganic Research.

1. Luscher, C., Nicoll, R. A., Malenka, R. C. & Muller, D. (2000) *Nat. Neurosci.* **3,** 545–550.
2. Ehlers, M. D. (2003) *Nat. Neurosci.* **6,** 231–242.
3. West, A. E., Chen, W. G., Dalva, M. B., Dolmetsch, R. E., Kornhauser, J. M., Shaywitz, A. J., Takasu, M. A., Tao, X. & Greenberg, M. E. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 11024–11031.
4. Steward, O. & Schuman, E. M. (2001) *Annu. Rev. Neurosci.* **24,** 299–325.
5. Ehlers, M. D. (2000) *Neuron* **28,** 511–525.
6. Malinow, R. & Malenka, R. C. (2002) *Annu. Rev. Neurosci.* **25,** 103–126.
7. Murphey, R. K. & Godenschwege, T. A. (2002) *Neuron* **36,** 5–8.
8. Hegde, A. N. & DiAntonio, A. (2002) *Nat. Rev. Neurosci.* **3,** 854–861.
9. Glickman, M. H. & Ciechanover, A. (2002) *Physiol. Rev.* **82,** 373–428.
10. Hicke, L. (1999) *Trends Cell Biol.* **9,** 107–112.
11. Hicke, L. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 195–201.
12. Della, N. G., Senior, P. V. & Bowtell, D. D. (1993) *Development (Cambridge, U.K.)* **117,** 1333–1343.
13. Hu, G., Zhang, S., Vidal, M., Baer, J. L., Xu, T. & Fearon, E. R. (1997) *Genes Dev.* **11,** 2701–2714.
14. Susini, L., Passer, B. J., Amzallag-Elbaz, N., Juven-Gershon, T., Prieur, S., Privat, N., Tuynder, M., Gendron, M. C., Israel, A., Amson, R., *et al.* (2001) *Proc. Natl. Acad. Sci. USA* **98,** 15067–15072.
15. Wheeler, T. C., Chin, L. S., Li, Y., Roudabush, F. L. & Li, L. (2002) *J. Biol. Chem.* **277,** 10273–10282.
16. Nakanishi, S., Nakajima, Y., Masu, M., Ueda, Y., Nakahara, K., Watanabe, D., Yamaguchi, S., Kawabata, S. & Okada, M. (1998) *Brain Res. Brain Res. Rev.* **26,** 230–235.
17. Aiba, A., Chen, C., Herrup, K., Rosenmund, C., Stevens, C. F. & Tonegawa, S. (1994) *Cell* **79,** 365–375.
18. Aiba, A., Kano, M., Chen, C., Stanton, M. E., Fox, G. D., Herrup, K., Zwingman, T. A. & Tonegawa, S. (1994) *Cell* **79,** 377–388.
19. Lu, Y. M., Jia, Z., Janus, C., Henderson, J. T., Gerlai, R., Wojtowicz, J. M. & Roder, J. C. (1997) *J. Neurosci.* **17,** 5196–5205.
20. Ichise, T., Kano, M., Hashimoto, K., Yanagihara, D., Nakao, K., Shigemoto, R., Katsuki, M. & Aiba, A. (2000) *Science* **288,** 1832–1835.
21. Tanabe, Y., Masu, M., Ishii, T., Shigemoto, R. & Nakanishi, S. (1992) *Neuron* **8,** 169–179.
22. Malherbe, P., Kew, J. N., Richards, J. G., Knoflach, F., Kratzeisen, C., Zenner, M. T., Faull, R. L., Kemp, J. A. & Mutel, V. (2002) *Brain Res. Mol. Brain Res.* **109,** 168–178.
23. Ishikawa, K., Nash, S. R., Nishimune, A., Neki, A., Kaneko, S. & Nakanishi, S. (1999) *Genes Cells* **4,** 381–390.
24. Kammermeier, P. J. & Ikeda, S. R. (2001) *BMC Neurosci.*, www.biomedcentral.com/1471-2202/2/15.
25. Ellison, M. J. & Hochstrasser, M. (1991) *J. Biol. Chem.* **266,** 21150–21157.
26. Masu, M., Tanabe, Y., Tsuchida, K., Shigemoto, R. & Nakanishi, S. (1991) *Nature* **349,** 760–765.
27. Abe, T., Sugihara, H., Nawa, H., Shigemoto, R., Mizuno, N. & Nakanishi, S. (1992) *J. Biol. Chem.* **267,** 13361–13368.
28. Romano, C., Yang, W. L. & O'Malley, K. L. (1996) *J. Biol. Chem.* **271,** 28612–28616.
29. Hu, G., Chung, Y. L., Glover, T., Valentine, V., Look, A. T. & Fearon, E. R. (1997) *Genomics* **46,** 103–111.
30. Hu, G. & Fearon, E. R. (1999) *Mol. Cell. Biol.* **19,** 724–732.
31. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S. & Weissman, A. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 11364–11369.
32. Grandes, P., Mateos, J. M., Ruegg, D., Kuhn, R. & Knopfel, T. (1994) *NeuroReport* **5,** 2249–2252.
33. Blumcke, I., Behle, K., Malitschek, B., Kuhn, R., Knopfel, T., Wolf, H. K. & Wiestler, O. D. (1996) *Brain Res.* **736,** 217–226.
34. Flor, P. J., Gomeza, J., Tones, M. A., Kuhn, R., Pin, J. P. & Knopfel, T. (1996) *J. Neurochem.* **67,** 58–63.
35. Prezeau, L., Gomeza, J., Ahern, S., Mary, S., Galvez, T., Bockaert, J. & Pin, J. P. (1996) *Mol. Pharmacol.* **49,** 422–429.
36. Mary, S., Gomeza, J., Prezeau, L., Bockaert, J. & Pin, J. P. (1998) *J. Biol. Chem.* **273,** 425–432.
37. Francesconi, A. & Duvoisin, R. M. (2002) *J. Neurosci.* **22,** 2196–2205.