ファージディスプレイ法を用いたシナプス部位蛋白相互作用の解析

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SUMMARY

We are interested in the dynamic change of protein composition and conformation at the mammalian central nervous system, which eventually leads to structural and functional change of the synapses. In this research project, we have tried to find novel synaptic protein-protein interactions using phage-display screening system.

The result reveals the usefulness of the phage display system. It can detect new protein interaction which cannot be detected by conventional two-hybrid assay theoretically. It is also evident that phage-display system has severe limitations which make this system unable to detect many known interactions. As a conclusion, phage display system is most useful when used in combination with conventional techniques like two-hybrid assay or affinity purification.

We first screened binding partners for NMDA-type glutamate receptor ectodomain or L1 adhesion molecule ectodomain using phage-display system. We found neuron-specific ubiquitin ligase fbx2 as a binding protein. Further analysis reveals that fbx2 recognizes N-linked glycosylation moiety on these ectodomain molecules and is thought to have some role in the quality control system of these membrane molecules.

This result opens up a new possibility: ubiquitin system plays an important role in the control of synaptic proteins. To further examine this possibility, we tested the biological activity of Siah1A (seven in absentia homolog 1A), another ubiquitin ligase identified as a binding protein to metabotropic glutamate receptor by two-hybrid screening. Siah1A, a member of the RING-finger-containing E3 ubiquitin ligases, was shown to bind to the specific sequence, termed Siah-interacting domain (SID), at the carboxy-terminal tails of the long splice variants of group1 metabotropic glutamate receptors (mGluR1a and mGluR5). We examined the function of Siah1A in ubiquitination and degradation of group1 mGluRs in heterologously expressing HEK293 cells. Coexpression of Siah1A markedly enhanced ubiquitination and downregulation of the SID-containing splice variants of group1 mGluRs among the mGluR family, and the enhanced downregulation of mGluR1a resulted from accelerated protein turnover, as revealed by pulse-chase experiments. The SID-lacking mGluR1b

splice variant and the SID-deleted mGluR1a mutant were both insusceptible to Siah1A-mediated degradation, indicating that the SID sequence is essential for Siah1A-mediated degradation of mGluRs. Effects on ubiquitination and degradation of group1 mGluRs were abrogated by mutations at the RING-finger domain of Siah1A. Replacements of lysine residues at the cytoplasmic regions of mGluR5 with arginine showed that Siah1A-mediated ubiquitination occurs at multiple lysine residues spanning both the 7 transmembrane region and carboxy-terminal tail. *In situ* hybridization histochemistry showed a widespread distribution of Siah1 mRNAs with high expression in the hippocampal pyramidal neurons and cerebellar Purkinje cells in the mouse brain. Group1 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 group1 mGluRs and would contribute to posttraslational downregulation of group1 mGluRs.

INTRODUCTION

Synaptic remodeling is a fundamental mechanism for information processing and storage in the developing and mature brain (1). In the process of synaptic remodeling, massive changes in the molecular composition and signaling properties occur in synapses. 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).

The ubiquitin system plays a central role in regulated protein degradation. Attachment of ubiquitin, a highly conserved 76-amino acid 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 upon 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), a conserved motif which is essential for E2 recruitment and subsequent ubiquitin conjugation (9). In Drosophila, Sina mediates degradation of tramtrack protein, which is essential for normal eye development (13). In mice, there are three highly homologous Siah proteins, Siah1A, Siah1B and Siah 2 (12). Siahs are known to recognize several target proteins including DCC (Deleted in Colorectal

Cancer), synaptophysin and Numb and promote the degradation of these proteins (14-16).

Glutamate receptors are major excitatory neurotransmitter receptors and play central roles in neuronal excitation in the mammalian central nervous system (17). Glutamate receptors are categorized into two classes, ionotropic and metabotropic glutamate receptors (17). Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors and consist of 8 different subtypes which are classified into three groups (17). Group1 mGluRs (mGluR1 and mGluR5) are coupled to inositol 1,4,5-trisphosphate (IP_3)-calcium signaling cascade (17) and play important roles in neural plasticity processes including long-term potentiation, long-term depression, synapse development and elimination (18-21). Both mGluR1 and mGluR5 comprise splice variants that differ in the cytoplasmic tails with long (mGluR1a and mGluR5) and short (mGluR1b and mGluR5d) carboxy-terminal domains (22,23). These group1 receptors are mainly localized postsynaptically and show regulated trafficking, internalization and desensitization in both neural cells and heterologously expressing cells (24-27). Recently, Siah1A has been shown to bind to the carboxy-terminal domain of long splice variants of mGluR1 and mGluR5 (28) and attenuate group1 mGluR-mediated calcium current inhibition in heterologously expressing superior cervical ganglion neurons (29). However, the biochemical events which are ensured by the direct interaction between Siah1A and group1 mGluRs remain elusive.

In this paper, we report that group1 mGluR-Siah1A interaction induces group1 mGluR-specific ubiquitination and subsequent degradation. The mRNA expressions of Siah1 and group1 mGluRs overlap in many brain regions including cerebellar Purkinje cells and hippocampal pyramidal neurons, where group1 mGluRs play essential roles in synaptic plasticity and synapse remodeling (18-21). These results imply that Siah regulates the mGluR turnover in the brain and could contribute to neural plasticity and remodeling in glutamatergic synapses.

EXPERIMENTAL PROCEDURES

Cell Culture and Plasmids – HEK293 cells (ATCC CRL-1573) were cultured in Dulbecco's modified Eagle's Medium (D-MEM) supplemented with 10% fetal bovine serum and antibiotics. All mGluR and NR1 cDNAs were tagged with Flag epitope at their amino-terminal ends just following 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 previously (30) 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 mutagenesis. DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Transfected cells were grown in the culture medium for 40 h before analysis. For inhibitor studies, cells were treated with MG-132 (30 μM), lactacystin (30 μM), E64 (100 μM), chloroquine (200 μM) or ammonium chloride (30 mM) at 24 h after transfection and incubated in the presence of respective inhibitors for 6 h before cell lysis. Inhibitors used were purchased from Calbiochem or Sigma.

Western Blotting and Immunoprecipitation – Transfected HEK293 cells were lysed in the RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40 and 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 RIPA buffer and the precipitated proteins were eluted with an excess Flag peptide (Sigma). Cell lysates or immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a Trans-Blot nitrocellulose membrane (Bio-Rad), followed by western blot analysis with anti-Flag M2 antibody (Sigma), anti-myc polyclonal antibody (Santa Cruz) or anti-actin polyclonal antibody (Sigma). Immunoreactive bands were detected with SuperSignal Femto ECL kit (Pierce).

Pulse-Chase Analysis – Flag-mGluR1a was transfected with or without Siah1A in HEK293 cells. Twenty-four h later, cells were washed and incubated for 45 min in a methionine/cysteine-free D-MEM medium. The medium was replaced with a methionine/cysteine-free D-MEM medium containing 200 μCi/ml of a mixture of [^{35}S]-methionine and [^{35}S]-cysteine (1000 Ci/mmol, Amersham). After 1 h incubation, the radioactive medium was removed and cells were extensively washed with non-radioactive D-MEM. Cells were cultured in the D-MEM medium containing 10% fetal bovine serum and an excess amount of methionine and cysteine. Cells were taken after appropriate chase time, lysed with the RIPA 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 proteins were quantified with BAS-5000 image analyzer (Fuji Film).

Northern Blot and In Situ Hybridization Analysis – Total RNA was isolated from transfected cells with Trizol reagent (Invitrogen). The isolated total RNA (10 μg) was electrophoresed on a 1.5% agarose-formaldehyde gel and transferred onto a GeneScreen Plus membrane (NEN) in 20 X SSC (1 X SSC is 0.15 M NaCl plus 0.015 M sodium citrate, adjusted to pH 7.0). The *PmaC*I-*Bgl*II fragment of mGluR1a cDNA (nucleotide residues 1489-2076 of the protein-coding region) was used as a probe. [^{32}P]-labeled probe was synthesized using BcaBEST Labeling kit (Takara) and hybridization was performed in PerfectHyb Plus hybridization buffer (Sigma). 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 previously (31). 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 in HEK293 cells by immunoblotting of 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, which corresponded to a monomeric and a dimeric form of mGluR1a (Fig. 1A) (32). As the appearance of the dimeric form of mGluRs was more prominent and consistent in our experimental condition (Fig. 1A), 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. 1A). In contrast, levels of mGluR1a mRNA remained unchanged by increasing the transfected Siah1A cDNA (Fig. 1A), indicating that Siah1A influences levels of mGluR1a protein at the posttranscriptional level. Coexpression of Siah1B or Siah2 similarly decreased mGluR1a protein levels in HEK293 cells (data not shown), indicating that all three members of the Siah family have the ability to decrease mGluR1a protein levels. In control, Siah 1A had no effect on levels of actin protein measured as an internal marker (Fig. 1A).

Siah1A binds to a specific amino acid stretch (Siah-interacting domain: SID) at the carboxy-terminal cytoplasmic tail of mGluR1a and mGluR5 and does not interact with the SID-lacking group2 and group3 mGluRs (28). We examined the specificity of Siah1A-mediated downregulation of receptor proteins by cotransfection of Siah1A with representative subtypes of group1, group2 (mGluR3) and group3 (mGluR7) subfamily as well as the NR1 subunit of N-methyl-D-aspartate-type ionotropic glutamate receptors. Of these receptors, only group1 mGluRs (mGluR1a and mGluR5) decreased and no other mGluR subtypes or the NR1 subunit were affected by coexpression with Siah1A (Fig. 1B). These results indicate that Siah1A specifically downregulates group1 mGluRs in the mGluR family, depending on its interaction with group1 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 extensively washed with a non-radioactive medium, and radioactivity of [^{35}S]-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 approximately 4 h and 20 h, respectively (Fig. 2B). These results indicate that Siah1A promotes the degradation of mGluR1a in HEK293 cells.

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 carboxy-terminal sequence (Fig. 3A). Protein levels of mGluR1b were unaffected by coexpression of Siah1A (Fig. 3B). More directly, an mGluR1a mutant (mGluR1a-Δ1) in which the SID sequence (amino acid residues 905-932) was deleted from the carboxy-terminal tail of mGluR1a was constructed and tested (Fig. 3A and B). This deletion mutant showed no decrease in protein levels by coexpression with Siah1A, indicating 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 (12,33-35). The zinc-containing RING-finger structure is essential for interaction with ubiquitin-conjugating enzyme (E2) and subsequent ubiquitination and degradation of target proteins (34,35). 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), while Siah1A-H59Y was a single amino acid mutant in which the critical histidine at position 59 within the RING-finger domain was

substituted for tyrosine (Fig. 4A) (34). 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. 4B). In spite of the elevation of mutant Siah1A proteins, both Siah1A-ΔR and Siah1A-H59Y failed to decrease cotransfected mGluR1a in HEK293 cells (Fig. 4B), indicating that Siah1A serves as a specific E3 ubiquitin ligase for degradation of mGluR1a.

Siah1A Induces Group1 mGluRs Ubiquitination - To address whether Siah1A promotes ubiquitination of group1 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). Extents of ubiquitination of both mGluR1a and mGluR5 were markedly enhanced by coexpression with Siah 1A (Fig. 5). Ubiquitinated mGluR1a and mGluR5 were detected as broad bands at the upper part of the gel (Fig. 5), 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), indicating 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 group1 mGluRs, lysine residues at the cytoplasmic regions of mGluR5 were substituted for arginine, and the effects of lysine substitutions on ubiquitination and degradation of mGluR5 were analyzed. There are 10 residues of lysine in the cytoplasmic loops and linings of the 7 transmembrane segments and 12 lysine residues at the carboxy-terminal tail of mGluR5

(36). All 22 lysine residues at the cytoplasmic regions of mGluR5 were replaced with arginine in mGluR5-Δall (K) mutant. Neither enhanced ubiquitination nor accelerated degradation was observed for mGluR5-Δall (K) by cotransfection with Siah1A (Fig. 6), indicating that 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-substituted mutants; mGluR5-ΔTM (K) and mGluR5-ΔC (K) in which 10 lysine residues at the cytoplasmic region of the 7 transmembrane segements (positions 610, 664, 676, 677, 678, 682, 683, 759, 771, 827) and 12 lysine residues at the carboxy-terminal tail (positions 850, 866, 880, 889, 906, 907, 917, 921, 962, 1065, 1113, 1155) of mGluR5 were substituted for arginine, respectively. Unexpectedly, both mGluR5-ΔTM (K) and mGluR5-ΔC (K) mutants were susceptible to Siah1A- mediated ubiquitination and degradation (Fig. 6). These results indicate that ubiquitination occurs at multiple lysine residues spanning from the transmembrane-connecting cytoplasmic loops to the carboxy-terminal tail of mGluR5 and multiple ubiquitination sites contribute to degradation of mGluR5.

Effects of Proteasome and Lysosome Inhibitors – In the classical view, polyubiquitinated protein substrates are recognized and degraded to small peptides by the proteasome complex (9). Recent evidence has also shown that in the case of membrane proteins such as receptors, transporters and channels, ubiquitination is used as a signal for endocytosis and intracellular trafficking and ubiquitinated proteins are transported to vacuole/lysosome for degradation (9-11). To examine which system predominantly serves for Siah1A-mediated degradation of mGluR1a, we tested several inhibitors, which selectively abrogate the proteasome function (MG-132 or lactacystin) or the lysosome function (E64, chloroquine or ammonium chloride). None of these compounds, however, were effective in blocking Siah1A-mediated mGluR1a degradation (Fig. 7). Both MG-132 and lactacystin inhibited Siah1A degradation (Fig. 7), confirming that these inhibitors were effective in blocking the proteasome-dependent

Siah1A degradation. At present, the pathway that links the mGluR1a ubiquitination to its degradation remains elucive.

Overlapping Expression of Siah1 and Group1 mGluRs in the Mouse Brain – Siah1 mRNA is expressed in various tissues, 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 non-overlapping probes (residues 1-456 and 457-849) were prepared from the mouse Siah1A clone, 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 8, 12, 24 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 postnatal day 12 (P12), is presented in Fig. 8. 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. 8A). This expression pattern of Siah1 mRNAs was confirmed by the two non-overlapping probes of Siah1A mRNA (data not shown). Cell populations expressing Siah1 mRNAs in the hippocampus and cerebellum were further analyzed by *in situ* hybridization of emulsion-dipped sections (Fig. 8C-8F). A high expression of Siah1 mRNAs was detected in pyramidal neurons of the hippocampus and Purkinje cells of the cerebellum (Fig. 8C-8F). Pyramidal neurons in the hippocampus and Purkinje cells in the cerebellum express a high amount of mGluR5 and mGluR1a, respectively (31,36-38). Thus, Siah1 and group1 mGluR mRNAs are coexpressed in the same cell populations at least in the hippocampus and cerebellum.

DISCUSSION

This investigation has indicated that Siah1A, initially identified as a binding protein to group1 mGluRs by yeast two-hybrid screening (28), acts as a specific E3 ubiquitin ligase that leads to ubiquitination and degradation of group1 mGluRs. Analysis of

mGluR subtype specificity and deletion mutation revealed that the SID sequence of group1 mGluRs is indispensable for the specific Siah1A-mediated ubiquitination and degradation of group1 mGluRs. Mutational analysis of Siah1A also disclosed that the RING-finger domain of Siah1A, like other E3 ubiquitin ligases, is essential for both ubiquitination and degradation of group1 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 carboxy-terminal tail of group1 mGluRs and facilitates the selective ubiquitination and degradation of this subclass of mGluRs. mGluR1 consists of two major splice variants, a long mGluR1a splice variant and a short mGluR1b splice variant (22). Both splice variants are highly expressed in a variety of neurons but distinctly distributed, depending on different neuronal cell types (37). They are also substantially different in the subcellular localization, activation kinetics of intracellular effectors and agonist-independent activity (39-42). Importantly, mGluR1b lacks the SID sequence at the carboxy-terminal region and is insusceptible to Siah1A-mediated ubiquitination and degradation. It is likely that Siah1A is important not only for controlling protein levels of mGluR1a at the posttranslational level but also distinctly regulating two different splice variants of mGluR1, depending on their susceptibility to Siah1A-mediated degradation.

Siah is involved in the ubiquitin-mediated degradation of DCC, synaptophysin and Numb in mammalian cells (14-16). The degradation of these proteins is mediated by the ubiquitin-proteasome pathway. In the case of group1 mGluRs, inhibitors of both proteasome and lysosome functions were ineffective in blocking the Siah1A-mediated degradation of group1 mGluRs. The similar proteasome- and lysosome-independent degradation has been reported for downregulation of β_2-adrenergic receptors (β_2AR) in heterologously β_2AR-expressing L cells and endoneously β_2AR-expressing A431 cell lines (43), although β-arrestin-facilitated β_2AR degradation has been shown to be sensitive to proteasomal inhibitors in other report (44). The proteasome- and lysosome-independent β_2AR degradation has been interpreted by the possible

mechanism in which some proteases at the plasma membrane may be involved in degradation of membrane proteins (43). Additional studies are required to uncover the precise mechanisms of Siah1A-mediated degradation of group1 mGluRs.

Siah1 mRNAs are, though widely expressed in various brain regions, enriched in hippocampal pyramidal neurons and cerebellar Purkinje cells where mGluR5 and mGluR1a mRNAs are highly expressed, respectively. Gene targeting and other studies indicated that mGluR1a and mGluR5 play a key role in inducing long-term depression in Purkinje cells and long-term potentiation in hippocampal neurons, respectively (19-21). Neural plasticity is now thought to be evoked by dynamic changes in receptor and signaling molecules in synapses (1,6). Furthermore, increasing evidence indicated 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 is therefore 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 group1 mGluRs. Because little is known about the posttranslational regulation of group1 mGluRs, our findings will aid the possibility to examine the regulatory mechanisms of downregulation of group1 mGluRs in glutamatergic synapses.

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FOOTNOTES

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[1]The abbreviations used are: Siah, seven in absentia homologues; mGluR, metabotropic glutamate receptor; Sina, seven in absentia; SID, Siah-interacting domain; DCC, deleted in colorectal cancer; D-MEM, Dulbecco's modified Eagle's medium; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; IB, immunoblot; IP, immunoprecipitation; WT, wild-type; myc-Ub, myc-ubiquitin;

FIGURE LEGENDS

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. Amounts of mGluR1a, but not actin, decreased in a dose-dependent manner by increasing levels of expression of Siah1A. Levels of mGluR1a mRNA were determined by Northern blotting of the corresponding transfected cells, indicating that no change occurred by increasing expression of Siah1A. *B*. 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.

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 extensively washed with an excess of methionine/cysteine and further cultured 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 a 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 Siah-interacting domain of mGluR1a for its**

degradation. *A*. The schematic structures of mGluR1a, mGluR1b and mGluR1a-Δ1 are indicated; SID, Siah-interacting domain; the caboxy-terminal sequence characteristic of the mGluR1b splice variant (residues 887-906) is indicated by a slash box. *B*. Flag-mGluR1a, Flag-mGluR1b and Flag-mGluR1a-Δ1 were transfected with or without myc-Siah1A and cell lysates were immunoblotted with anti-Flag, anti-myc and anti-actin antibodies. Levels of mGluR1b and mGluR1a-Δ1 were not affected by coexpression with Siah1A. Levels of actin measured as an internal control marker remained unchanged by Siah1A coexpression.

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 with anti-Flag, anti-myc and anti-actin antibodies. Siah1A–ΔR and Siah1A-H59Y failed to degrade mGluR1a and showed a marked and a moderate increase of its own protein as compared with wild-type (WT) Siah1A, respectively.

FIG. 5. **Siah1A induces mGluR1a polyubiquitination.** 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 antibody to confirm that Siah1A, but not Siah1A-ΔR, enhanced degradation of mGluR1a and mGluR5. Flag-mGluR1a and Flag-mGluR5 were immunoprecipitated from the corresponding cell lysates with anti-Flag antibody, followed by immunoblotting with anti-myc antibody to detect mGluR ubiquitination (mGluR-[Ub]n); IP, immunoprecipitation . Anti-actin blotting was conducted as an internal control. mGluR1a or mGluR5 was highly ubiquitinated by Siah1A but not by Siah1A-ΔR and this enhanced polyubiquitination was correlated with accelarated degradation of mGluR1a/mGluR5 by Siah1A.

FIG. 6. **Effects of lysine replacements at the cytoplasmic regions of mGluR5 on its degradation and polyubiquitination.** Flag-mGluR5 and lysine-replaced Flag-mGluR5 mutants together with myc-ubiquitin (myc-Ub) 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 . Neither enhancement of degradation nor polyubiquitination of mGluR5 was detected in mGluR5-Δall (K) mutant. In contrast, significant polyubiquitination and degradation were detected in mGluR5-ΔTM(K) and mGluR5-ΔC(K).

FIG. 7. **Effects of proteasome or lysosome inhibitors.** Cells were transfected with Flag-mGluR1a and myc-Siah1A and incubated in the presence of proteasome inhibitors (30 μM MG-132 or 30 μM lactacystin) or lysosome inhibitors (100 μM E64, 200 μM chloroquine or 30 mM ammonium chloride). Levels of mGluR1a and Siah1A were measured by immunoblotting with anti-Flag and anti-myc antibodies, respectively. Anti-actin blotting was conducted as an internal control. None of these inhibitors were effective in blocking Siah1A-mediated mGluR1a degradation. In contrast, levels of Siah1A were higher in cells treated with proteasome inhibitors than those untreated or treated with lysosome inhibitors.

FIG. 8. **Siah1 mRNA distribution in the mouse brain.** *A, 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, E,* Dark-field images of emulsion-dipped sections of the cerebellum *(C)* and hippocampus (*E*) of the P12 mouse. *D, F,* bright-field images of emulsion-dipped sections of the cerebellar cortex (*D),* and the pyramidal cell layer of 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, 2 mm for *A* and *B*, 0.4 mm for *C* and *E* and 50 μm for *D* and *F*.

Figure 1

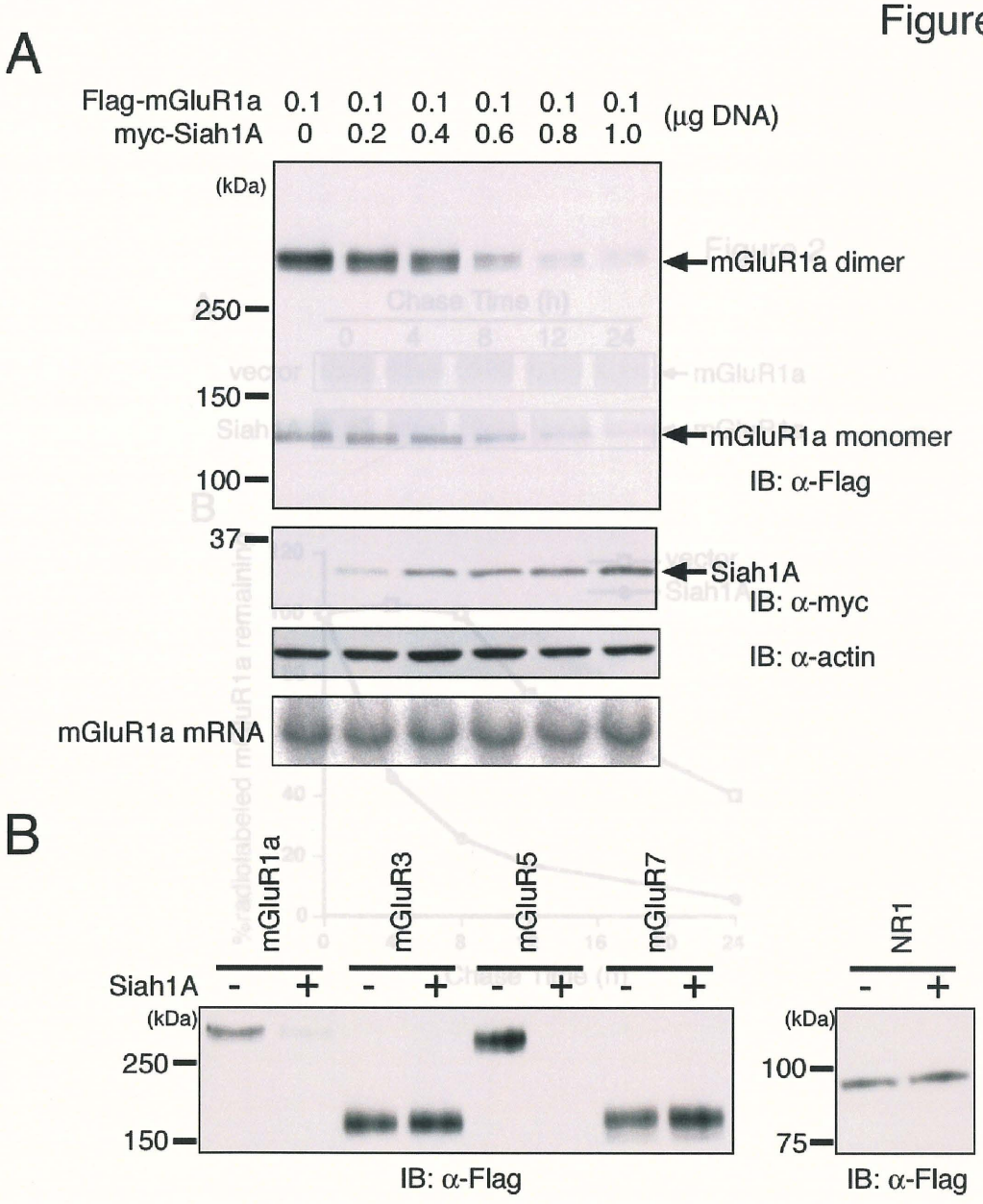
A
Flag-mGluR1a 0.1 0.1 0.1 0.1 0.1 0.1 (μg DNA)
myc-Siah1A 0 0.2 0.4 0.6 0.8 1.0
(kDa)
250
150
100
37
mGluR1a dimer
mGluR1a monomer
IB: α-Flag
Siah1A
IB: α-myc
IB: α-actin
mGluR1a mRNA
B
mGluR1a
mGluR3
mGluR5
mGluR7
NR1
Siah1A - + - + - + - + - +
(kDa)
250
150
100
75
IB: α-Flag
IB: α-Flag

Figure 2

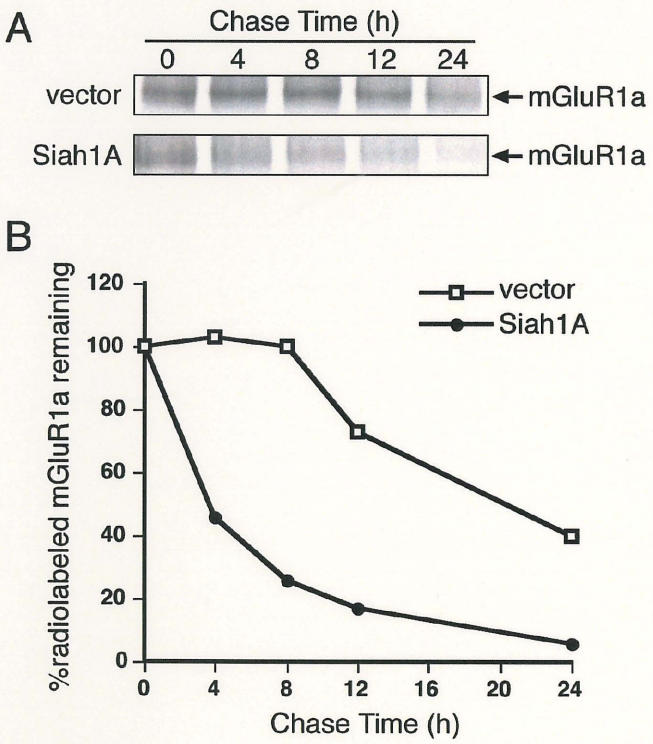
A
Chase Time (h)
0
4
8
12
24
vector
mGluR1a
Siah1A
mGluR1a
B
%radiolabeled mGluR1a remaining
120
100
80
60
40
20
0
0
4
8
12
16
20
24
Chase Time (h)
vector
Siah1A

Figure 3

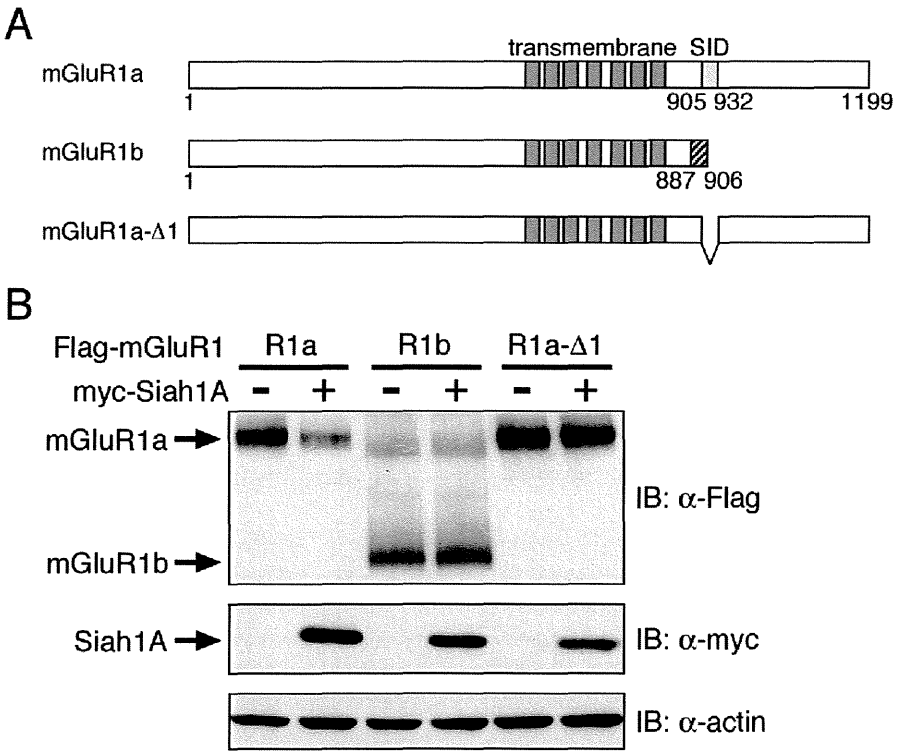
A
transmembrane SID
mGluR1a
1
905 932
1199
mGluR1b
1
887 906
mGluR1a-Δ1
B
Flag-mGluR1
R1a
R1b
R1a-Δ1
myc-Siah1A
- + - + - +
mGluR1a
mGluR1b
IB: α-Flag
Siah1A
IB: α-myc
IB: α-actin

Figure 4

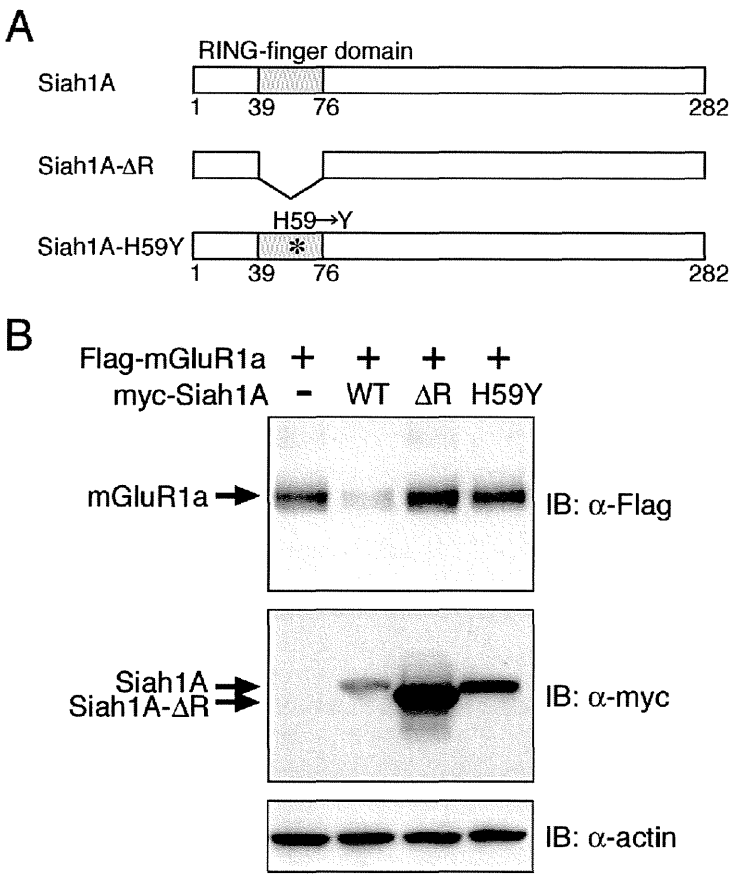
A
RING-finger domain
Siah1A
1
39
76
282
Siah1A-ΔR
H59→Y
Siah1A-H59Y
1
39
76
282
B
Flag-mGluR1a + + + +
myc-Siah1A - WT ΔR H59Y
mGluR1a
IB: α-Flag
Siah1A
Siah1A-ΔR
IB: α-myc
IB: α-actin

Figure 5

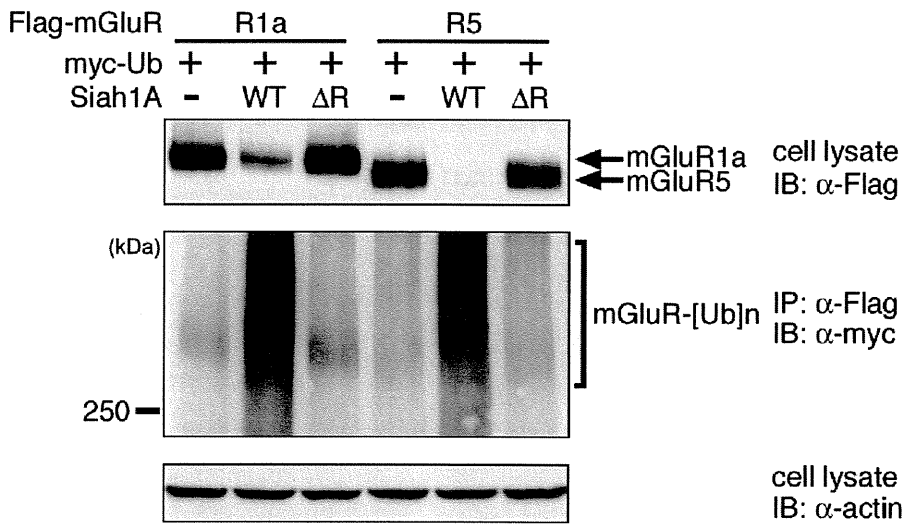
Flag-mGluR
R1a
R5
myc-Ub
Siah1A
WT
ΔR
mGluR1a
mGluR5
cell lysate
IB: α-Flag
(kDa)
250
mGluR-[Ub]n
IP: α-Flag
IB: α-myc
cell lysate
IB: α-actin

Figure 6

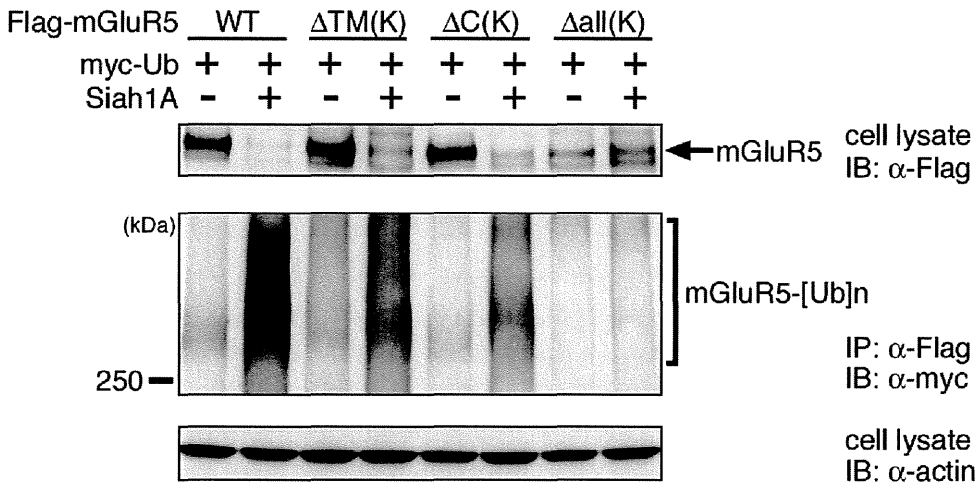
Flag-mGluR5
WT
ΔTM(K)
ΔC(K)
Δall(K)
myc-Ub + + + + + + + +
Siah1A - + - + - + - +
mGluR5
cell lysate
IB: α-Flag
(kDa)
250
mGluR5-[Ub]n
IP: α-Flag
IB: α-myc
cell lysate
IB: α-actin

Figure 7

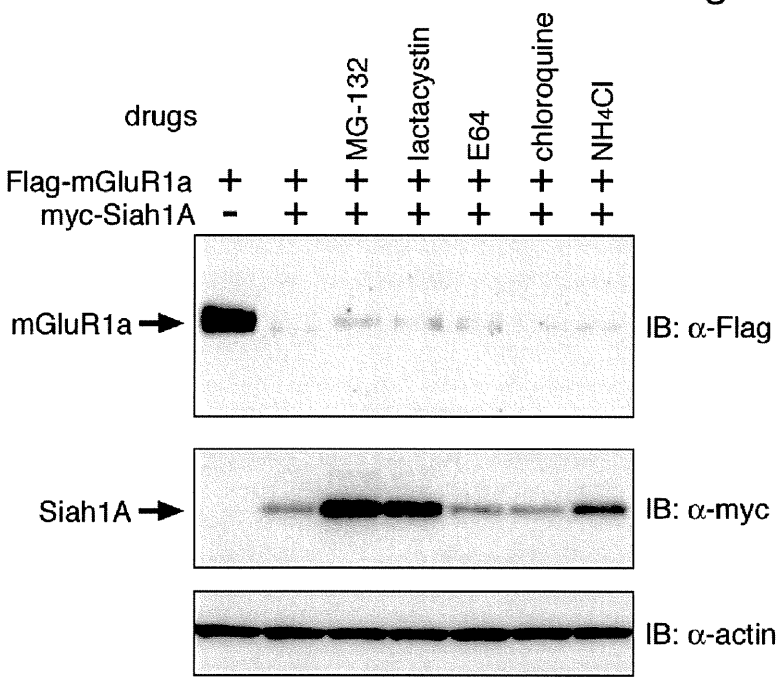
drugs
MG-132
lactacystin
E64
chloroquine
NH4Cl
Flag-mGluR1a + + + + + + +
myc-Siah1A - + + + + + +
mGluR1a
IB: α-Flag
Siah1A
IB: α-myc
IB: α-actin

Figure 8