Glycogen Synthase Kinase 3β-mediated Phosphorylation in the Most C-terminal Region of Protein Interacting with C Kinase 1 (PICK1) Regulates the Binding of PICK1 to Glutamate Receptor Subunit GluA2

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Background: Despite extensive research, the mechanisms regulating the interaction between protein interacting with C kinase 1 (PICK1) and GluA2 are still unclear.

Results: Glycogen synthase kinase-3β (GSK-3β) phosphorylates PICK1. Phosphorylated PICK1 binds to GluA2.

Conclusion: GSK-3β regulates the GluA2-PICK1 interaction.

Significance: This study contributes to our understanding of the mechanisms of long-term depression, an Alzheimer disease-related event.

Protein interacting with C kinase 1 (PICK1) is a synaptic protein interacting with the AMPA receptor subunits GluA2/3. The interaction between GluA2 and PICK1 is required for the removal of GluA2 from the synaptic plasma membrane during long-term depression (LTD). It has been suggested that glycogen synthase kinase 3β (GSK-3β) is activated during LTD, but the relationships between GluA2, PICK1, and GSK-3β are not well understood. In particular, the substrate(s) of GSK-3β have not yet been determined. Here we showed that PICK1 is a substrate of GSK-3β. We found that Ser⁴¹⁶, Ser⁴³⁴, Ser⁴¹², and Ser⁴⁰⁰ of PICK1 were putative GSK-3β-mediated phosphorylation sites. Among these sites, Ser⁴¹⁶ played a crucial role in regulating the interaction between GluA2 and PICK1. We showed that replacing Ser⁴¹⁶ with Ala disrupted the GluA2-PICK1 interaction, whereas substituting Ser⁴¹⁶ with Glu or Asp retained this interaction. However, deletion of Ser⁴¹⁶ did not affect the GluA2-PICK1 interaction, and substitution of Ser⁴¹⁶ with Ala did not alter the PICK1-PICK1 interaction. Using image analysis in COS-7 cells with AcGFP1-fused PICK1, we showed that substitution of Ser⁴¹⁶ with Ala increased the formation of AcGFP1-positive clusters, suggesting an increase in the association of PICK1 with the membrane. This may have resulted in the dissociation of the GluA2-PICK1 complexes. Our results indicated that GSK-3β-mediated phosphorylation of PICK1 at Ser⁴¹⁶ was required for its association with the AMPA receptor subunit. Therefore, the GSK-3β-mediated phosphorylation of PICK1 may be a regulating factor during LTD induction.

The synaptic protein interacting with C kinase 1 (PICK1) (1) interacts with the AMPA receptor subunits GluA2/3 (2, 3). The interaction between GluA2 and PICK1 is required for the removal of GluA2 from the synaptic plasma membrane during long-term depression (LTD) in hippocampal and cerebellar neurons (4–10). The contribution of PICK1 to LTD has been confirmed by the reduction of LTD in PICK1 knockout mice (11).

PICK1 has a PDZ (PSD-95/Dlg/ZO-1) domain and a BAR (Bin/amphiphysin/Rvs) domain (12). GluA2 and PICK1 interact with each other via the C-terminal region of GluA2 and the PDZ domain of PICK1 (3). However, the precise mechanisms regulating this interaction remain to be determined.

Recently, LTD has received much attention with regard to neurodegenerative diseases, including Alzheimer disease (AD). The hallmark of AD is the deposition of amyloid β protein (Aβ) (senile plaques) and hyperphosphorylated tau (neurofibrillary tangles) (13), both of which have been shown to be related to LTD. Aβ dimers inhibit long-term potentiation and induce LTD (14), and the presence of tau is required for LTD induction (15).

The abbreviations used are: LTD, long-term depression; AD, Alzheimer disease; IP, immunoprecipitation.
Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine kinase that phosphorylates various substrates and is involved in cellular and synaptic functions (16, 17). GSK-3β has also been studied in relation to AD (18). For example, Aβ facilitates GSK-3β signaling (19, 20), and tau is a well known substrate of GSK-3β (21, 22). GSK-3β is regarded as a critical molecular link between Aβ and tau (23). Interestingly, GSK-3β is required for LTD (24). Therefore, identification of the mechanisms of LTD with regard to these AD-related proteins will provide new insights into the molecular mechanisms of AD and other neurodegenerative diseases.

Here we aimed to elucidate the relationships between GluA2, PICK1, and GSK-3β. We showed that GSK-3β phosphorylated PICK1 and that this phosphorylation promoted the GluA2-PICK1 interaction. We identified GSK-3β-mediated phosphorylation sites of PICK1, among which phosphorylation at the most C-terminal Ser416 residue was essential for the GluA2-PICK1 interaction. Analysis with AcGFP1-fused PICK1 revealed that dephosphorylation at Ser416 caused PICK1 to have a tendency to associate with the membrane. This study proposes a new model for regulating the GluA2-PICK1 interaction and provides new insights into the molecular mechanisms of LTD and AD, extending to learning and memory.

**Experimental Procedures**

**Antibodies**—For Western blotting and immunoprecipitation, the following antibodies were used: mouse monoclonal anti-GluA2 (catalog no. MAB397, Merck Millipore, Billerica, MA) recognizing amino acids residues 175–430 with no cross-reactivity with other AMPA/Kainate glutamate receptor subunits; rabbit polyclonal anti-PICK1 (catalog no. PA1-073, Thermo Fisher Scientific, Waltham, MA) recognizing amino acids residues 1–18; rabbit polyclonal anti-tau, JM (25); rabbit polyclonal anti-phospho-Tau (Ser(P)400, catalog no. T1700-1VL, Sigma); anti-DYKDDDDK tag monoclonal antibodies (Wako, Osaka, Japan); anti-c-Myc monoclonal antibodies (Wako); anti-glutathione S-transferase monoclonal antibodies (Wako); and anti-His6 monoclonal antibodies (Wako).

**DNA Construction**—Expression constructs for PICK1 were cloned into the EcoRI-MluI site of pCI-neo (Promega, Madison, WI). Expression constructs for AcGFP1-PICK1 (AcGFP1 fused to the N-terminal of PICK1) were cloned into the EcoRI-BamHI site of pAcGFP1-C2 (Clontech, Mountain View, CA). AcGFP1 is a GFP derived from *Aequorea coeruleusens*. The construct for GluA2 expressed rat GluA2 (NM_001083811), a short/flop form of the protein with an Arg at the Gln/Arg site. The construct for GST-ct-GluA2 expressed the last C-terminal 50 amino acid residues of GluA2, which were fused with GST (2). Expression constructs for His6-PICK1 (a His6 tag fused to the N-terminal of PICK1) were cloned into the HindIII-BamHI site of pmCherry (Clontech).

**Cell Culture and DNA Transfection**—COS-7 cells were maintained in Dulbecc’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. DNA was transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. After transfection, the cells were subjected to biochemical analysis or microscopic analysis.

**In Vitro GSK-3β-mediated GluA2/PICK1 Phosphorylation Assay**—COS-7 cells transiently expressing GluA2, wild-type PICK1, or various PICK1 mutants were harvested and stored at −80°C until use. One day before the *in vitro* assay, the cells were homogenized in TBS containing 1% Triton X-100 with various protease and phosphatase inhibitors. Homogenates were ultracentrifuged at 180,000 × g for 20 min at 4°C (MLA55, Beckman Coulter, Brea, CA). The supernatants were subjected to immunoprecipitation (IP) with control IgG, anti-GluA2 antibodies, or anti-PICK1 antibodies in the presence of magnetic beads (Dynabeads M-280, Invitrogen) at 4°C for 16 h. Bead-
Regulation of the GluA2-PICK1 Interaction Mediated by GSK-3β

A

1

PDZ

BAR

FVSTMSKY YNDCYAVLQDA
339 342

PTDKGSWCD S
412 416

B

1

PDZ

BAR

WT

FVSTMSKY

PTDKGSWCD S

S339A/S342A

FVATMAKY

PTDKGSWCD S

S412A/S416A

FVSTMSKY

PTDKGGAWCD A

4SA

FVATMAKY

PTDKGGAWCD A

S339A

FVSTMSKY

PTDKGSWCD S

S342A

FVATMAKY

PTDKGSWCD S

S412A

FVSTMSKY

PTDKGGAWCD A

S416A

FVSTMSKY

PTDKGGSWCD A

C

D

Incubation

WT S339A S342A S412A S416A 4SA

Phospho-Level vs. WT

58K

46K

**

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E

F

Incubation

WT S339A S342A S412A S416A

Phospho-Level vs. WT

60K

47K

*
bound immunoprecipitated proteins were washed with TBS containing 0.5% Tween 20 (TBS-T) twice, followed by a wash with TwG buffer (92 mM MES, 1.3 mM Mg(AcO)₂, 1 mM EGTA (pH 6.5), 10% glycerol, 10% Tween 20, and 0.03% 2-mercaptoethanol), and then mixed with recombinant active GST-fused GSK-3β (final concentration, 0.082 μM; Active Motif, Carlsbad, CA), ATP (final concentration, 0.2 mM), and [γ-32P]ATP (0.0185 MBq/assay). Mixtures were incubated at 30 °C, followed by four washes with TwG buffer. Proteins were eluted from the beads, separated on precast SDS gels (SuperSep Ace, BAS2500 or FTA-7000 (Fuji Film, Tokyo, Japan). Homogenates were ultracentrifuged at 125,000 × g for 1–2 h. Harvested COS-7 cells were homogenized in TBS containing 0.5% Tween 20 (TBS-T) twice, followed by a wash with TBS-T for 1–2 h, washed twice with TBS-T, and added to COS-7 cell lysates that were prepared as follows. For co-IP assays, to investigate the interaction between myc-tagged PICK1 and FLAG-tagged PICK1, we used anti-DYKDDDDK tag antibody magnetic beads (Wako) that were blocked with 2.5% bovine serum albumin in TBS-T for 1–2 h. Harvested COS-7 cells were homogenized in TBS containing 1% Triton X-100 with various protease and phosphatase inhibitors by using 29-gauge needles (Terumo, Tokyo, Japan). Homogenates were ultracentrifuged at 125,000 × g for 20 min (rotor TLA45, Beckman Coulter). Protein concentrations of supernatants were determined using a BCA protein assay kit (Nacalai Tesque, Kyoto, Japan) and adjusted to 1.5–2 μg/μl. An equal volume of sample buffer was then added. These samples were used as “input.” Proteins (300–400 μg) of the remaining supernatants were mixed with anti-PICK1 antibody-bead complexes prepared as described above. Mixtures were rotated at 4 °C for 16 h and washed four times with TBS-T. Proteins were eluted from the beads by incubating in sample buffer at 96 °C for 10 min. Samples were subjected to Western blotting using anti-GluA2 antibodies.

GST Pulldown Assay—We performed GST pulldown assays. GST or GST-ct-GluA2 proteins were obtained from Escherichia coli strain DH5α. Lysates of bacterial cultures (A₆₀₀ of 0.4–0.5) were prepared by sonication and solubilization in ice-cold PTxE buffer (PBS, 1% Triton X-100, and 0.1 mM EDTA (pH 7.4)), and lysates were stored at −80 °C until use. Glutathione-Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) was rotated with bacterial lysates at 4 °C overnight, followed by blocking with 2.5% bovine serum albumin. His₆-PICK1 proteins were obtained from E. coli strain DH5α by purification using His GraviTrap™ (GE Healthcare). Purified His₆-PICK1 was subjected to GSK-3β-mediated phosphorylation as described above and mixed with Glutathione-Sepharose 4B, followed by rotation at 4 °C overnight. After washing four times, proteins were eluted from the beads by incubating in sample buffer at 96 °C for 10 min. Samples were subjected to Western blotting using anti-GST or anti-His₆ tag antibodies.

Clusterig Assay by Imaging of Live Cells—COS-7 cells (0.3–0.4 × 10⁵) were cultured in 35-mm-diameter glass-bottom dishes (catalog no. D110400, Matsunami Glass, Osaka, Japan) for 2 days before transfection. Two days after transfection, the cellular distribution of AcGFP1-PICK1 was analyzed by fluorescence microscopy (Birevo Keyence BZ-9000, Keyence, Osaka, Japan). Cells containing more than five clusters were counted as reported previously (26), and the ratio of cluster-positive cells to AcGFP1 signal-positive cells was calculated. Approximately 550–800 cells were counted per sample.

Western Blotting—Proteins were separated on precast SDS gels (SuperSep Ace 5–20%, Wako), and transferred to nitrocellulose membranes. After blocking with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T), the membranes were incubated with the primary antibodies overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and washing with PBS-T three times. The membranes were treated with reagent for exposure (SuperSignal West Pico chemiluminescent substrate or SuperSignal West Femto maximum sensitivity substrate, Thermo Fisher Scientific; ImmunoStar LD, Wako). Images of the membranes were captured using an LAS-3000, LAS-4000 (Fuji Film), ChemiDoc XRS+ system (Bio-Rad), or C-DiGit blot scanner (LI-COR, Lincoln, NE), and subjected to ImageJ analysis.

Statistical Analysis—Prism 6 (GraphPad Software Inc., San Diego, CA) and one-way analysis of variance, followed by Dunnnett test or Tukey-Kramer test, were used for statistical analysis. For two-group comparisons, Student’s t test was applied. Differences with p < 0.05 were considered significant.

Results

GSK-3β Phosphorylated PICK1 in Vitro—To identify the substrate(s) of GSK-3β, we focused on GluA2 and PICK1 as candidates. Lysates of COS-7 cells overexpressing GluA2 or PICK1 WT (Fig. 1A) was subjected to IP with anti-GluA2 or anti-PICK1 antibodies, respectively, followed by in vitro phosphorylation assays using GSK-3β. Signals were detected with PICK1 but not with GluA2 (Fig. 1B), indicating that GSK-3β phosphorylated PICK1 but not GluA2. We detected nonspecific signals, indicated by asterisks in Figs. 1 and 2, that appeared between the 80- and 58-kDa markers. Because we used recombinant GST-fused GSK-3β for which the molecular weight was ~73 kDa, the nonspecific bands may reflect autophosphorylation of GSK-3β.

To confirm the phosphorylation of PICK1 by GSK-3β, we used two negative controls: IP of untransfected cell lysates with anti-PICK1 antibodies (Fig. 1C, lanes 4 and 5) and IP of cell
**FIGURE 3. Effects of PICK1 phosphorylation on the GluA2-PICK1 interaction.** A, co-IP assays using anti-PICK1 antibodies were performed with no cell lysates (lane 1), lysates of untransfected cells (lane 2), lysates of cells expressing either GluA2 or PICK1 alone (lanes 3 and 4, respectively), and lysates of cells co-expressing GluA2 and PICK1 (lane 5). Representative blots are shown. IB, immunoblot. B and C, co-IP assays were performed with PICK1 WT, PICK1 4SA, and PICK1 4SE. Representative blots (B) and quantification data (C) are shown. Quantification data represent the mean ± S.E. (n = 5). ***, p < 0.001 in comparisons as indicated. D, COS-7 cells expressing tau, treated with 0.3 μM of the GSK-3β inhibitor VIII, were subjected to Western blot analysis with antibodies against total tau and Ser(P)400. Representative blots and quantification data are shown. Quantification data represent the mean ± S.E. (n = 5). **, p < 0.01 versus 0 μM. E, co-IP assay of the GluA2-PICK1 WT interaction in the presence of 0 or 0.3 μM GSK-3β inhibitor. Representative blots are shown. Quantification data represent the mean ± S.E. (n = 10). **, p < 0.01 versus 0 μM. F, co-IP assay of the GluA2-PICK1 4SE interaction in the presence of 0 μM or 0.3 μM GSK-3β inhibitor. Representative blots are shown. Asterisks indicate nonspecific bands (also detected in a lane with untransfected cells). Arrowheads indicate co-immunoprecipitated GluA2.
lysates expressing PICK1 WT with control rabbit IgG (Fig. 1C, lanes 6 and 7). Accordingly, signals were detected with IP of lysates from cells expressing PICK1 WT with anti-PICK1 antibodies (Fig. 1C, lanes 1–3) but not with the negative controls (Fig. 1C, lanes 4–7), confirming that GSK-3β/H9252 phosphorylated PICK1 in vitro.

Identification of Phosphorylation Sites—To identify the phosphorylation sites within PICK1, we analyzed the sequence of PICK1 (12). Because GSK-3β/H9252 phosphorylates serine/threonine residues, particularly recognizing the motif -SXXXS(P)- (17), we decided to analyze Ser339, Ser342, Ser412, and Ser416 (Fig. 2A), among which the sequence from Ser412 to Ser416 corresponded to this motif. We generated PICK1-encoding DNA constructs with one, two, or all putatively phosphorylated Ser residues substituted by Ala (Fig. 2B), and COS-7 transfectants were then used in the in vitro phosphorylation assay.

First, we compared the phosphorylation levels of PICK1 S339A/S342A, PICK1 S412A/S416A, and PICK1 4SA (all four Ser residues were mutated to Ala) with that of PICK1 WT. Phosphorylation levels of PICK1 mutants were reduced to ~50% (S339A/S342A and S412A/S416A) or to ~30% (4SA) (Fig. 2, C and D). This indicated that some of the four Ser residues were involved in GSK-3β-mediated phosphorylation.

Second, we investigated the role of each single Ser residue in phosphorylation using PICK1 with single Ser residues substituted by Ala, i.e., S339A, S342A, S412A, and S416A. PICK1 S339A and PICK1 S412A showed almost the same phosphorylation levels as PICK1 WT, whereas PICK1 S342A and PICK1 S416A showed 50% lower phosphorylation levels compared with PICK1 WT (Fig. 2, E and F). These results indicated that Ser342 and Ser416 played an important role in GSK-3β-mediated phosphorylation.

GSK-3β-mediated Phosphorylation of PICK1 Regulated the GluA2-PICK1 Interaction—GluA2 is the best-known ligand of PICK1, and interaction between GluA2 and PICK1 is the crucial event for LTD induction (5). To elucidate whether PICK1 phosphorylation affected the GluA2-PICK1 interaction, we performed co-IP assays with various PICK1 mutants and GluA2. We transiently expressed GluA2 and PICK1 in COS-7 cells, and estimated the levels of PICK1-associated GluA2 by IP with anti-PICK1 antibodies.

At first, as shown Fig. 3A, we performed co-IP assays using anti-PICK1 antibodies with no cell lysates (lane 1), lysates of untransfected cells (lane 2), lysates of cells expressing either GluA2 or PICK1 alone (lanes 3 and 4, respectively), and lysates of cells co-expressing GluA2 and PICK1 (lane 5). We specifically detected co-immunoprecipitated GluA2 in lane 5 (Fig. 3A, arrowhead). We also detected additional upper bands (Fig. 3A, asterisk) that could be detected in lanes 2–5 but not in lane 1 (just anti-PICK1 antibodies in the absence of cell lysates). This
result suggested that the additional bands might be derived from a certain protein included in COS7 lysates.

Next we performed a co-IP assay using unphosphorylated PICK1 or pseudophosphorylated PICK1 in which all four putatively phosphorylated Ser residues were substituted by Ala (4SA) or Glu (4SE) (27), respectively. Again, we confirmed the specificity of IP by showing that no GluA2 was immunoprecipitated using untransfected cells (Fig. 3B, lane 1) or cells expressing either GluA2 or PICK1 alone (Fig. 3B, lanes 2 and 3). When COS-7 cells were transfected with GluA2 and each PICK1 (WT, 4SA, or 4SE), the level of PICK1-associated GluA2 was reduced significantly in the case of PICK1 4SA (Fig. 3, B, lanes 4 and 5, and C), whereas PICK1 4SE-associated GluA2 displayed the same level as PICK1 WT-associated GluA2 (Fig. 3, B, lanes 4 and 6, and C). As discussed later, in this experiment it was assumed that PICK1 WT was phosphorylated by GSK-3β. These results indicated that phosphorylated PICK1 interacted with GluA2, whereas dephosphorylated PICK1 impeded this interaction.

To investigate whether GSK-3β activity affected the GluA2-PICK1 interaction, we performed the co-IP assay in the presence of the GSK-3β inhibitor VIII (Calbiochem, La Jolla, CA). First we confirmed the effects of the inhibitor by using COS-7 cells overexpressing tau. Because GSK-3β phosphorylates tau at Ser400 (28), we estimated GSK-3β activity according to the level of phosphorylated Ser400 detected by a Ser(P)400-specific antibody. The inhibitor (0.3 μM) suppressed GSK-3β activity to ~60% that of the untreated control (Fig. 3D). When we performed the co-IP assay in the presence of the inhibitor at 0.3 μM, we found that PICK1 WT-associated GluA2 significantly decreased to ~70% that of the untreated control (Fig. 3E), indicating that GSK-3β-mediated phosphorylation facilitated the GluA2-PICK1 interaction.

In addition to the four Ser residues (Ser339, Ser342, Ser412, and Ser416), PICK1 may be phosphorylated at other sites. To investigate whether phosphorylation at other sites, if present, affected the GluA2-PICK1 interaction, we performed the co-IP assay with PICK1 4SE in the presence of the inhibitor. If the phosphorylation at other sites also facilitated the GluA2-PICK1 interaction, the inhibitor would be expected to reduce the level of PICK1 4SE-associated GluA2. However, we found that the inhibitor increased the level of PICK1 4SE-associated GluA2, suggesting that, under certain circumstances, GSK-3β phosphorylation at other sites could inhibit the GluA2-PICK1 interaction (Fig. 3F). We also confirmed that the inhibitor did not alter the level of PICK1 4SA-associated GluA2 (Fig. 3G). These results suggested that the other phosphorylation sites were less likely to contribute to the enhancement of GluA2-PICK1 interaction. Therefore, we concluded that the phosphorylation event mediated by the four Ser residues facilitated the binding of PICK1 to GluA2.

The Most C-terminal Ser416 Residue Was Essential for Binding to GluA2—To determine the sites responsible for the interaction of PICK1 with GluA2, we performed co-IP assays using PICK1 S339A/S342A or PICK1 S412A/S416A and found that the GluA2-PICK1 S412A/S416A interaction was weaker than that of GluA2-PICK1 S339A/S342A (Fig. 4, A, lanes 3 and 4, and C). The interaction of GluA2-PICK1 S339A/S342A was comparable with that of GluA2-PICK1 WT (Fig. 4, A, lanes 2 and 3, and C). Therefore, the C-terminal region of PICK1, including Ser412 and Ser416, seemed to regulate the GluA2-PICK1 interaction.

We finally investigated which Ser residue (Ser412 or Ser416) was responsible for the interaction with GluA2 by performing co-IP assays using PICK1 S412A and PICK S416A. Although PICK1 S412A interacted with GluA2 as tightly as PICK1 WT (Fig. 4, B, lanes 2 and 5, and C), the level of PICK1 S416A-associated GluA2 was reduced significantly (Fig. 4, B, lanes 2 and 6, and C). We also confirmed that the level of PICK1 S416E-associated GluA2 was comparable with the level of PICK1 WT-associated GluA2 (Fig. 4D). We next investigated whether substitution of the phosphorylation site(s) with Asp affected the GluA2-PICK1 interaction. COS-7 cells were transfected with PICK1 4SD, in which all four putatively phosphorylated Ser residues (Ser339, Ser342, Ser412, and Ser416) were substituted by Asp, and PICK1 S416D. After standardization according to the expression levels of PICK1, the levels of PICK1 4SD-associated GluA2

FIGURE 5. GluA2-PICK1 interaction in the presence or absence of tau. Shown are co-IP assays with PICK1 WT or PICK1 S416A in the presence or absence of tau. Representative blots (A) and quantification data (B) are shown. The arrowhead indicates PICK1-associated GluA2. Quantification data represent the mean ± S.E. (n = 3), *, p < 0.05 in comparison as indicated. IB, immunoblot.
The substitution of Ser416 with Ala had a severe effect on the GluA2-PICK1 interaction, despite the presence of tau (Fig. 5, A). The interaction of GluA2 with PICK1 S416A was not strengthened, whereas dephosphorylation at Ser416 impeded the interaction.

**Tau Enhanced the GluA2-PICK1 WT Interaction but not the GluA2-PICK1 S416A Interaction**—We previously indicated that the phosphorylation state of Ser416 regulated the GluA2-PICK1 interaction. That is, phosphorylation at Ser416 promoted the interaction, whereas dephosphorylation at Ser416 impeded the interaction.

**Deletion of the C-terminal Region of PICK1 did not affect the GluA2-PICK1 interaction.** A, left panel, deletion mutants of PICK1 used in this study. Right panel, expression of PICK1 C-terminal deletion mutants. We could detect substantial expressions of PICK1 WT, ΔC1, and ΔC5 but only weak expression of PICK1 ΔC30 and ΔCT (arrows). IB, immunoblot. B and C, co-IP assay with GluA2 and PICK1 deletion mutants. Representative blots (B) and quantification data (C) are shown. The arrowhead indicates PICK1-associated GluA2. Quantification data represent the mean ± S.E. (n = 7).

and PICK1 S416D-associated GluA2 were comparable with the level of PICK1 WT-associated GluA2 (Fig. 4E). These results indicated that the phosphorylation state of Ser416 regulated the GluA2-PICK1 interaction. That is, phosphorylation at Ser416 promoted the interaction, whereas dephosphorylation at Ser416 impeded the interaction.

The S416A Mutation in PICK1 Increased the Association of GluA2 with PICK1—To elucidate the mechanism through which Ser416 regulated the GluA2-PICK1 interaction, we generated PICK1 mutants in which the C-terminal region was deleted, i.e. PICK1 ΔC1 (one amino acid was deleted from the C-terminal) and ΔC5 (five amino acids were deleted from the C-terminal) as shown in Fig. 6A. Although we also constructed PICK1 ΔC30 (30 amino acids were deleted from the C-terminal) and ΔCT (60 amino acids were deleted from the C-terminal up to the C-terminal end of the BAR domain), the expression levels of these mutants were too low for analysis (Fig. 6A, right panel). Therefore, we performed experiments using PICK1 ΔC1 and ΔC5.

COS-7 cells were transfected with GluA2 and each PICK1 (WT, ΔC1, or ΔC5) and subjected to the co-IP assay. PICK1-associated GluA2 levels were comparable between PICK1 WT, PICK1 ΔC1, and PICK1 ΔC5 (Fig. 6, B and C), indicating that the C-terminal region was not required for the association between GluA2 and PICK1. However, as shown in Figs. 4 and 5, the substitution of Ser416 with Ala dramatically disrupted the GluA2-PICK1 interaction. These results suggested that the S416A mutation altered the properties of PICK1. Subsequent experiments were performed to further examine these altered characteristics of PICK1.

**The S416A Mutation in PICK1 Did Not Affect the PICK1-PICK1 Interaction**—We investigated whether the S416A mutation altered the PICK1-PICK1 interaction using myc-tagged PICK1 and FLAG-tagged PICK1. The co-IP assay showed that PICK1 S416A interacted with itself as did PICK1 WT (Fig. 7A). Therefore, disruption of the GluA2-PICK1 S416A could not be explained by the increase in the PICK1-PICK1 interaction.

**The S416A Mutation in PICK1 Altered the Association Property**—To investigate the direct interaction between GluA2 and PICK1, we performed a pulldown assay to using GST-ct-GluA2 (2) and His6-PICK1. Purified His6-PICK1 proteins were reacted with recombinant GSK-3β and subjected to pulldown by GST or GST-ct-GluA2. We confirmed GluA2-specific binding of His6-PICK1 WT (Fig. 7B, bottom panel, lanes 1 and 3). In contrast, His6-PICK1 S416A showed association with GST (Fig. 7B, bottom panel, lanes 2 and 4). This result suggested that the S416A mutation in PICK1 caused an alteration in its association property. Therefore, there was a possibility that the S416A amino acid substitution per se, rather than the blockade of phosphorylation, caused changes in PICK1 protein interactions and that these protein interactions might be nonspecific.

**The S416A Mutation in PICK1 Increased the Association between PICK1 and the Membrane**—Finally, to investigate the effects of substitution at Ser416 on the properties of PICK1, we
assessed subcellular localization using AcGFP1-fused PICK1 in which AcGFP1 was linked to the N-terminal of PICK1. We performed in vitro live cell imaging using COS-7 cells transfected with AcGFP1-PICK1 WT, AcGFP1-PICK1 S416A, AcGFP1-PICK1 S416E, AcGFP1-PICK1 ΔC1, or AcGFP1-PICK1 ΔC5.

Most cells showed diffuse signals (Fig. 8A). However, we could detect cells harboring many small AcGFP1-positive clusters (Fig. 8B). Therefore, we calculated the ratio of cells with clusters per AcGFP1-positive cells, as reported previously (26). For PICK1 WT, PICK1 S416E, PICK1 ΔC1, and PICK1 ΔC5, few cells harbored AcGFP1-positive clusters. In contrast, PICK1 S416A formed AcGFP1-positive clusters within cells (Fig. 8C), reflecting an increased association with the membrane (26).

These results (Figs. 6–8) indicated that the disruption of the GluA2-PICK1 S416A interaction (Figs. 4 and 5) could be explained by increases in the association between PICK1 and the membrane. We also confirmed that PICK1 S416E, ΔC1, and ΔC5 showed similar properties as PICK1 WT in live-cell imaging, corresponding to the results of the co-IP assay (Figs. 4 and 6). In summary, the phosphorylation state of the most C-terminal region of PICK1 determined the preference for its association with either GluA2 or the membrane.

Discussion

The presence of LTD has been shown to be associated with the interaction between GluA2 and PICK1 (5) as well as the activation of GSK-3β (24). However, the relationship between the GluA2-PICK1 interaction and GSK-3β activity remains unknown.

Here we showed that GSK-3β phosphorylated PICK1 but not GluA2 (Fig. 1) and determined the putative phosphorylation sites within PICK1 (Fig. 2). Substitution of the four Ser residues

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**FIGURE 7.** Effect of the S416A mutation in PICK1 on the PICK1-PICK1 interaction and on the direct interaction with GluA2. A, co-IP assay with myc-tagged PICK1 and FLAG-tagged PICK1. Myc-tagged PICK1 was shown to associate with FLAG-tagged PICK1. Representative blots, labeled with antibodies for each tag, and quantification data are shown. Quantification data represent the mean ± S.E. (n = 6). B, pulldown assay using GST-ct-GluA2 and His6-tagged PICK1 (WT or S416A). Representative blots, labeled with antibodies for each tag, are shown.
Regulation of the GluA2-PICK1 Interaction Mediated by GSK-3β

Our co-IP assay results with unphosphorylated and pseudophosphorylated PICK1 revealed that phosphorylation at the four Ser residues facilitated the binding of PICK1 to GluA2 (Figs. 3, B and C, and 4E). We also showed that treatment with a GSK-3β inhibitor significantly decreased the level of PICK1 WT-associated GluA2 (Fig. 3E) but not that of pseudophosphorylated PICK1 (PICK1 4SE)-associated GluA2 (Fig. 3F). These results indicated that GSK-3β-mediated phosphorylation of the four Ser residues was responsible for facilitating the GluA2-PICK1 interaction.

Consequently, we confirmed the crucial role of Ser416 for the GluA2-PICK1 interaction (Fig. 4). Substitution of Ser416 with Ala disrupted the GluA2-PICK1 interaction, whereas substitution with Glu or Asp retained the interaction. The GluA2-PICK1 WT interaction was enhanced in the presence of tau, supporting the hypothesis that tau is required for LTD (15). In contrast, the GluA2-PICK1 S416A interaction was not affected by the presence of tau (Fig. 5). If phosphorylation at Ser416 enables PICK1 to interact with GluA2, then it seems likely that deleting the C-terminal disrupts the GluA2-PICK1 interaction. However, we showed that PICK1 ΔC1 and PICK1 ΔC5 could associate with GluA2 (Fig. 6). We also confirmed that PICK1 S416A could interact with PICK1 itself, as did PICK1 WT (Fig. 7A). The pulldown assay, however, showed that the S416A mutation in PICK1 altered its association property (Fig. 7B). Finally, we found that, in contrast to PICK1 WT and the other PICK1 mutants, AcGFP1-PICK1 S416A had a tendency to form AcGFP1-positive clusters (Fig. 8). These results suggested that the S416A mutation increased the association between PICK1 and the membrane, leading to the disruption of the GluA2-PICK1 interaction in COS-7 cells.

From these data, we concluded that GSK-3β-mediated phosphorylation of PICK1 regulated the GluA2-PICK1 interaction. In particular, phosphorylation at Ser416 facilitated the association of PICK1 with GluA2, whereas dephosphorylation of Ser416 facilitated the association between PICK1 and the membrane. Our results suggested that activated GSK-3β phosphorylates PICK1, resulting in its association with GluA2. A model summarizing these results is depicted in Fig. 9.

To the best of our knowledge, our study is the first report indicating that PICK1 is a substrate of GSK-3β and that PICK1 phosphorylation regulates the GluA2-PICK1 interaction. Because previous reports have suggested that LTD involves GluA2, PICK1, GSK-3β, and tau (4–10, 15, 24), this study provides new insights into the molecular mechanisms of LTD.

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FIGURE 8. Effect of the S416A mutation in PICK1 on intracellular cluster formation. Shown are COS-7 cells expressing various PICK1 mutants (WT, S416A, S416E, ΔC1, and ΔC5) fused to the C-terminal of AcGFP1. A, representative figure of cells with diffuse AcGFP1 signals. B, representative figure of cells harboring AcGFP1-positive clusters. C, quantification data of the ratio of cluster-positive cells to AcGFP1-positive cells. Quantification data represent the mean ± S.E. (WT, n = 48; S416A, n = 54; S416E, n = 52; ΔC1, n = 60; ΔC5, n = 59). *, p < 0.05 versus WT.

FIGURE 9. Model explaining the regulation of the GluA2-PICK1 interaction. A rectangle or an ellipse indicates a molecule of GluA2 or PICK1, respectively. A star indicates phosphorylation. Previous reports have suggested that an LTD stimulus activates GSK-3β (indicated by a broken line with an arrowhead). When GSK-3β-mediated phosphorylation occurs, phosphorylated PICK1 associates with GluA2 (right). When PICK1 is dephosphorylated, PICK1 has a tendency to associate with the membrane, resulting in disruption of the GluA2-PICK1 interaction (left).
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However, further studies of PICK1 phosphorylation states are required.

Author Contributions—S. Y. and A. T. designed the research and wrote the manuscript. S. Y. and M. M. performed the biochemical experiments and analyses. S. Y. and T. E. performed the cluster assay using COS-7 cells, and S. Y., T. E., and K. M. analyzed the data.

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