Cellular/Molecular

Phosholipase C-Related Inactive Protein Is Involved in Trafficking of γ2 Subunit-Containing $GABA_A$ Receptors to the Cell Surface

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The subunit composition of $GABA_A$ receptors is known to be associated with distinct physiological and pharmacological properties. Previous studies that used phospholipase C-related inactive protein type 1 knock-out (PRIP-1 KO) mice revealed that PRIP-1 is involved in the assembly and/or the trafficking of γ2 subunit-containing $GABA_A$ receptors. There are two PRIP genes in mammals; thus the roles of PRIP-1 might be compensated partly by those of PRIP-2 in PRIP-1 KO mice. Here we used PRIP-1 and PRIP-2 double knock-out (PRIP-DKO) mice and examined the roles for PRIP in regulating the trafficking of $GABA_A$ receptors. Consistent with previous results, sensitivity to diazepam was reduced in electrophysiological and behavioral analyses of PRIP-DKO mice, suggesting an alteration of γ2 subunit-containing $GABA_A$ receptors. The surface numbers of diazepam binding sites (α/γ2 subunits) assessed by [^{3}H]flumazenil binding were reduced in the PRIP-DKO mice as compared with those of wild-type mice, whereas the cell surface GABA binding sites (α/β subunits, assessed by [^{3}H]muscimol binding) were increased in PRIP-DKO mice. The association between $GABA_A$ receptors and $GABA_A$ receptor-associated protein (GABARAP) was reduced significantly in PRIP-DKO neurons. Disruption of the direct interaction between PRIP and $GABA_A$ receptor β subunits via the use of a peptide corresponding to the PRIP-1 binding site reduced the cell surface expression of γ2 subunit-containing $GABA_A$ receptors in cultured cell lines and neurons. These results suggest that PRIP is implicated in the trafficking of γ2 subunit-containing $GABA_A$ receptors to the cell surface, probably by acting as a bridging molecule between GABARAP and the receptors.

Key words: **benzodiazepine; $GABA_A$ receptor; GABARAP; knock-out mice; PRIP; trafficking**

Introduction

$GABA_A$ receptors are the major target of the endogenous inhibitory neurotransmitter GABA and mediate the bulk of fast inhibitory neurotransmission in the mammalian brain. They are heteropentamers composed of subunits including α1–6, β1–3, γ1–3, δ, ε, θ, and π, and their characteristics depend mainly on the subunit composition of the individual receptor (Korpi et al., 2002). Benzodiazepine-type drugs are widely used for anxiolytic, hypnotic, anticonvulsant, and muscle-relaxing actions, and benzodiazepine sites absolutely require the presence of the γ2 subunit in $GABA_A$ receptors (Günther et al., 1995). Both quantitative and qualitative changes in the number of benzodiazepine-sensitive $GABA_A$ receptors have been implicated in several CNS disorders, including anxiety, depression, epileptogenic activity, muscle tension, and memory (Crestani et al., 1999).

The $GABA_A$ receptor-associated protein (GABARAP) has been identified as a protein that interacts specifically with the γ2 subunit (Wang et al., 1999) and has been implicated in the clustering of $GABA_A$ receptors (Chen et al., 2000) and the trafficking of $GABA_A$ receptors to the cell surface because of its ability to

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interact with microtubules (Wang et al., 1999) and *N*-ethylmaleimide-sensitive factor (Kittler et al., 2001). Recently, GABARAP has been reported to be important for the trafficking of the receptors, especially the γ2 subunit-containing type, to the surface membrane (Leil et al., 2004; Chen et al., 2005). However, the precise molecular mechanisms that underlie GABARAP-dependent transport of γ2 subunit-containing receptors remain unclear.

Phospholipase C-related catalytically inactive protein type 1 (PRIP-1), a novel D-*myo*-inositol 1,4,5-trisphosphate binding protein, has a number of binding partners, including GABARAP (Kanematsu et al., 2002), the catalytic subunit of protein phosphatase-1α (Yoshimura et al., 2001) and protein phosphatase-2A (Kanematsu et al., 2006), and $GABA_A$ receptor β subunits (Terunuma et al., 2004; Kanematsu et al., 2006); thus it regulates $GABA_A$ receptor functions as analyzed by PRIP-1 knock-out (PRIP-1 KO) mice (Kanematsu et al., 2002; Terunuma et al., 2004). In addition to PRIP-1, PRIP-2, a second isoform of PRIP, also is expressed in the brain and is able to bind with the molecules described above (Uji et al., 2002). This suggests that compensation of PRIP-1 activity by PRIP-2 might occur in PRIP-1 KO mice.

In this study we further elucidated the function of PRIP proteins in the regulation of $GABA_A$ receptor activity by analyzing PRIP-1 and PRIP-2 double knock-out (PRIP-DKO) mice (Kanematsu et al., 2006). PRIP-DKO mice exhibited a decreased number of cell surface-expressed γ2 subunit-containing $GABA_A$ receptors. Interestingly, this correlated with a significant reduction in the amount of GABARAP bound to $GABA_A$ receptors in the brains of PRIP-DKO mice. Furthermore, we found that disruption of the interaction between PRIP and the β subunits by using a peptide corresponding to the β subunit binding site in PRIP (Kanematsu et al., 2006) decreased the cell surface expression of γ2 subunits in the rat pituitary cell line (GH3), human embryonic kidney 293 (HEK293), and cultured hippocampal cells. Collectively, these results suggest that PRIP plays an important role in the membrane trafficking of γ2 subunit-containing $GABA_A$ receptors, presumably by facilitating the delivery of GABARAP to the γ2 subunit via association with the β subunit.

Materials and Methods

PRIP-DKO mice. The PRIP-1 KO mice (Kanematsu et al., 2002) and PRIP-2 KO mice (Takenaka et al., 2003), both of which were backcrossed against the C57BL/6J background ($n = 7$ and $n = 2$, respectively), were crossed to generate a PRIP-DKO mouse strain and corresponding wild-type (WT) as previously published (Kanematsu et al., 2006). Genotyping for both of the loci was performed by PCR [design of PCR primers has been described in Kanematsu et al. (2002) and Takenaka et al. (2003)], using mouse tail genomic DNA as a template. Homozygous PRIP-DKO and WT mice were mated *inter se* to obtain the required number of mice, and only F1 and F2 generations of both genotypes were used for experiments. The handling of mice and all of the procedures that were performed were approved by the Animal Care Committee of Kyushu University, following the guidelines of the Japanese Council on Animal Care.

Electrophysiological analysis. Hippocampal CA1 cells from 10- to 14-d-old mice were freshly dissociated without the use of enzymes, as described previously (Mizoguchi et al., 2003). Electrical measurements were performed by using the nystatin perforated patch recording method (Nabekura et al., 1996). All recordings were performed by using voltage clamp at a holding potential of −50 mV and using a patch-clamp amplifier (EPC-7, List Biologic, Campbell, CA). All experiments were performed at a temperature of 30 ± 1°C. Drug solutions were applied by using the Y-tube perfusion system, allowing for rapid exchange of the solution surrounding a cell (Kakazu et al., 1999; Nabekura et al., 2002). All data are expressed as the mean ± SEM.

Behavioral analysis. Male mice (WT and PRIP-DKO) were reared in a specific pathogen-free facility with a 12 h light/dark cycle at Kyushu University, Japan, and then moved to a conventional facility for the experiments at 9–12 weeks of age. Food and water were available *ad libitum*. For locomotor activity measurement the mice were placed in an ambulation chamber equipped with an infrared beam to count the number of crosses for every 6 min over a period of 60 min. The elevated plus maze test was performed to provide measures of anxiolytic activity. The test was performed 30 min after the intraperitoneal administration of diazepam (0.1 mg/kg), as described previously (Rudolph et al., 1999).

Ligand-binding assay. After decapitation the brains were removed immediately and placed in ice-cold saline from which hippocampi were dissected rapidly on ice. Hippocampi were homogenized by 10 strokes of a Teflon glass homogenizer in ice-cold homogenization buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and a mixture of protease inhibitors [containing the following (in μg/ml): 5 pepstatin A, 5 leupeptin, 2 aprotinin, 5 phenylmethylsulfonyl fluoride]. The homogenates were centrifuged at 50,000 × *g* for 60 min at 4°C. The pellets were washed twice by being resuspended in the same buffer, followed by centrifugation. After the final wash the pellets were resuspended in an assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% bovine serum albumin) and used at a final protein concentration of 1 mg/ml. The membrane suspension was incubated with [^{3}H]muscimol or [^{3}H]flumazenil (Ro15-1788; specific radioactivity, 1350.5 or 2619.6 GBq/mmol, respectively; PerkinElmer, Boston, MA) at various concentrations approaching saturation for 60 min on ice. Nonspecific binding also was determined in the presence of 1 μM unlabeled muscimol (Sigma–Aldrich, St. Louis, MO) or 1 μM flumazenil (gift from Astellas Pharma, Tokyo, Japan), respectively. Mixtures were filtered under negative pressure on GF/C filters (Whatman, Maidstone, UK) that twice were rinsed rapidly with 8 ml of ice-cold wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). Radioactivity on a filter was counted with a liquid scintillation counter (LSC-5100, Aloka, Tokyo, Japan).

Reverse transcription-PCR. Total RNA was prepared from cultured GH3 and HEK293 cells, using an RNeasy mini kit (Qiagen, Valencia, CA). The total RNA (2 μg) from each cultured cell was used for reverse transcription (RT). PCR was performed in a volume of 25 μl, using the following primers: 5′-CTCGAGGATCCATGAAGTTCGTGTACAAAG-3′ (GABARAP forward) and 5′-TAAGTGCAGGTCCTGAAG-3′ (GABARAP reverse). As a control glycerol-3-phosphate dehydrogenase (G3PDH) primers were used.

Whole-cell ELISA. GH3 cells were transfected by electroporation (250 V, 975 μF; Gene Electropulser II, Bio-Rad, Hercules, CA) with a total of 10 μg of DNA [$\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits, $\alpha1^{Flag}$, $\beta2^{Flag}$, and $\gamma2^{Myc}$ subunits, or $\alpha1^{Flag}$, $\beta3^{Flag}$, and δ^{Myc} subunits (Connolly et al., 1996) with PRIP-binding peptide (amino acid residues 553–565 of rat PRIP-1) (Kanematsu et al., 2006) or its scrambled peptide (synthesized sense oligonucleotide fragment, 5′-GATCTATGGAGGAAACTG-AGATGGATGCTGGAGATGAAGTGTCTGAGGAATAACTAG-3′) in phosphorylated internal ribosomal entry site-enhanced green fluorescent protein (pIRES2-EGFP) vector; 2.5 μg each] and incubated in DMEM with 10% fetal bovine serum. To examine an influence of exogenous expression of PRIP-1 and GABARAP on the surface expression of γ2-containig receptors, we transfected $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Myc}$ subunits with genes for PRIP-1 and/or GABARAP in African green monkey kidney cells (COS-7) cells. After 24 h of incubation at 37°C the cells were fixed in 4% paraformaldehyde for 10 min and then blocked in PBS containing 10% fetal bovine serum and 0.5% bovine serum albumin (blocking buffer) for 15 min. Staining was performed under nonpermeabilized conditions with anti-Myc (9E10) monoclonal antibodies (0.5 μg/ml) diluted in blocking buffer for 1 h. Secondary detection was performed by using HRP-sheep anti-mouse IgG (Amersham Biosciences, Arlington Heights, IL) in blocking buffer (1:5000) for 1 h, washed with PBS four times, and incubated with 1 ml of 3,3′,5,5′-tetramethylbenzidine. The supernatant was transferred to a cuvette, and absorbance was determined at 655 nm.

Immunofluorescence analysis. HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were transfected by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) with a

total of 10 μg of DNA ($\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits or $\alpha1^{Flag}$, $\beta2^{Flag}$, and $\gamma2^{Myc}$ subunits with PRIP-binding peptide or scrambled peptide; 2.5 μg each), following the manufacturer's protocols. After 24 h of incubation at 37°C the cells were fixed with 4% paraformaldehyde for 15 min and blocked with the blocking solution described above for 15 min. Myc-tagged subunits of the surface receptors were stained under nonpermeabilized conditions with rabbit anti-Myc polyclonal antibody (1:1000; Sigma–Aldrich) for 1 h, followed by secondary staining with anti-rabbit cyanine 3 (Jackson ImmunoResearch, West Grove, PA) at 1:1000. Cells then were permeabilized with 0.2% Triton X-100 in blocking solution for 10 min and incubated with mouse anti-Myc monoclonal antibody (9E10) to stain Myc-tagged subunits of the internal receptors. Secondary detection was performed with cascade blue-conjugated anti-mouse secondary antibody (1:1000; Invitrogen, Eugene, OR). All of the antibody dilutions were performed in blocking solution, and washes were done in PBS. Coverslips were examined with a confocal microscope (Radience 2100, Bio-Rad).

Glutathione S-transferase protein pull-down and immunoprecipitation assays. Glutathione *S*-transferase (GST) protein pull-down assay was performed as described previously (Goto et al., 2005). Briefly, GST-β3 or GST alone immobilized on glutathione Sepharose 4B beads (Amersham Biosciences) was incubated with His_6-PRIP-1 and GABARAP in a binding buffer (300 μl) containing (in mM) 25 Tris-HCl, pH 7.5, 150 NaCl, 5 EDTA, and 1% Triton X-100 for 1 h at 4°C, followed by centrifugation at 3500 × *g* for 1 min. Bound material was washed four times with the same buffer and then separated by SDS-PAGE, followed by Western blot analysis. For immunoprecipitation assay the cerebra of WT and PRIP-DKO mice (9–12 weeks, male) were homogenized in buffer A (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2% bovine serum albumin, and 0.5% Triton X-100) containing a mixture of protease inhibitors (described above). After homogenization the cross-linker solution [2.5 mM DTSSP (3,3′-dithiobis[sulfosuccinimidylproppionate]) and 2.5 mM DSP (dithiobis[succinimidylpropionate]) (Pierce, Rockford, IL)] was added, and the membrane proteins were extracted in 1% Na-deoxycholate for 30 min at 4°C. After the cross-linking reaction was performed for 30 min at room temperature, it was stopped by adding 50 mM Tris-HCl, pH 7.5. Protein (5 mg) from the extract obtained by centrifugation was subjected to immunoprecipitation with 6.5 μg of mouse anti-β2/3 antibody (Upstate Biotechnology, Lake Placid, NY), followed by the addition of 20 μl of 50% slurry of protein G-Sepharose beads (Amersham Biosciences). The beads were washed once with buffer A and three times with buffer A without bovine serum albumin. After the final wash the beads were resuspended in 20 μl of sample buffer for SDS-PAGE, followed by Western blot analysis. The antibodies used for immunoblotting were as follows: anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β2/3 antibody, anti-GABARAP antibody (Tanida et al., 2004), anti-PRIP-1 antibody (Kanematsu et al., 2002), anti-PRIP-2 antibody (Takenaka et al., 2003), and HRP-conjugated secondary antibodies. Signals were detected by SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) or Enhanced Chemiluminescence kit (ECL; Amersham Biosciences), using the LAS-1000 plus gel documentation system (Fujifilm, Tokyo, Japan) and analyzed by densitometric measurements (NIH Image software 1.55). For the inhibition of the association between PRIP-1 and β subunit by PRIP-binding peptide, GH3 cells were transfected with $\alpha1^{Flag}$, $\beta2^{Myc}$, $\gamma2^{Flag}$ subunit plasmids (2.5 μg each) and 5 μg of PRIP-binding or scrambled peptide plasmid, followed by homogenization in buffer A containing 1% SDS and a mixture of protease inhibitors. The lysates were diluted with buffer A to obtain a 0.2% SDS solution. Extract (3 mg of protein) obtained by centrifugation was subjected to immunoprecipitation with 8 μg of mouse anti-Myc antibody (9E10), followed by the addition of 20 μl of a 50% slurry of protein G-Sepharose beads. Beads were washed three times, followed by an SDS-PAGE/immunoblotting with rabbit anti-Myc polyclonal antibody (Sigma–Aldrich), and were detected by an ECL Plus kit. For immunoprecipitation assays using cells labeled with [^{35}S/^{35}S]cysteine/methionine, HEK293 cells were transfected with $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ along with PRIP-binding peptide or scrambled peptide and then radiolabeled with [^{35}S/^{35}S]cysteine/methionine (50 μCi/ml; specific radioactivity, 11.0 mCi/ml; PerkinElmer) for 2 h. After being washed twice with ice-cold PBS, the cells were lysed in buffer A containing 1% SDS and a mixture of protease inhibitors. The lysates were diluted fivefold with buffer A and treated with 10 μg of anti-Flag antibody (M2 monoclonal antibody, Sigma–Aldrich) for immunoprecipitation, followed by SDS-PAGE and autoradiography. Experiments also were performed by using cells transfected with $\alpha1^{Flag}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ and anti-Myc antibody for immunoprecipitation. Identification of the radioactive band corresponding to each subunit was determined by advanced experiments in which cells were transfected with $\alpha1^{Myc}\beta2\gamma2$, $\alpha1\beta2^{Myc}\gamma2$, or $\alpha1\beta2\gamma2^{Myc}$; the subsequent procedures were the same as described above.

Transfection of peptide to rat hippocampal neurons. PRIP-binding peptide and scrambled peptide plasmids were purified by using EndoFree Plasmid Kits (Qiagen) and were resuspended in Tris-HCl/EDTA buffer. Hippocampus was removed from embryonic day 16–18 rat brain, trypsinized, and then dissociated by trituration. Cells were transfected with 5 μg of DNA (PRIP-binding peptide or scrambled peptide plasmids) with a nucleofection system (Amaxa, Cologne, Germany) according to the manufacturer's instructions and were plated onto poly-D-lysine-coated glass coverslips ($2–4 \times 10^5$ cells/cm^2). After 4 d the coverslips were used for electrophysiological analysis. All recordings were performed with a whole-cell patch recording at a holding potential of 0 mV.

Results

Pharmacological and physiological characterization of PRIP-DKO mice

Figure 1, *A* and *B*, shows genotyping of mutant mice assessed by PCR, which exhibited successful targeting of PRIP-1 and PRIP-2 genes, and Western blotting of cortex and hippocampus extracts, which exhibited little presence of PRIP molecules. PRIP-DKO mice appeared to grow normally and became fertile.

We first analyzed GABA-induced Cl^- current (I_{GABA}) by using hippocampal CA1 cells freshly isolated from young mice (10–14 d) of both genotypes. The GABA dose–response curve in PRIP-DKO cells showed a pattern similar to that observed in control cells (Fig. 1*C*). Sensitivity to diazepam, a typical benzodiazepine drug for which the targets are the interface of α/γ2 subunits, was examined next. As shown in Figure 1*D*, PRIP-DKO hippocampal neurons showed less response to diazepam, indicating that the number of benzodiazepine receptors was decreased in PRIP-DKO hippocampal neurons. These results were essentially similar to those observed with PRIP-1 KO mice [Kanematsu et al. (2002), their Fig. 4*D*,*E*].

Behavioral phenotype of PRIP-DKO mice

To examine additionally the deficiencies in the PRIP-1 and PRIP-2 genes, we next performed behavioral analyses. We compared the ambulation counts between PRIP-DKO and WT mice at the age of 10–12 weeks to determine the alteration of spontaneous locomotor activity. The ambulation was counted for every 6 min over a period of 60 min, and it was found that the locomotor activity of PRIP-DKO mice as compared with that of WT decreased throughout the time examined (Fig. 1*E*), a result opposite to that obtained with PRIP-1 KO mice [Kanematsu et al. (2002), their Fig. 5*B*]. We next examined the anxiolytic effect of diazepam in the elevated plus maze test. As shown in Figure 1*F*, WT and PRIP-DKO mice spent the majority of their time in the closed arms of the maze. However, an injection of diazepam at 0.1 mg/kg to WT mice resulted in an increase of the time spent in the open arm, indicating the anxiolytic activity of this drug. In contrast, PRIP-DKO mice did not show such an increase, indicating little sensitivity to diazepam. The results were the same as those previously observed with the PRIP-1 KO mice [Kanematsu et al. (2002), their Fig. 5*C*].

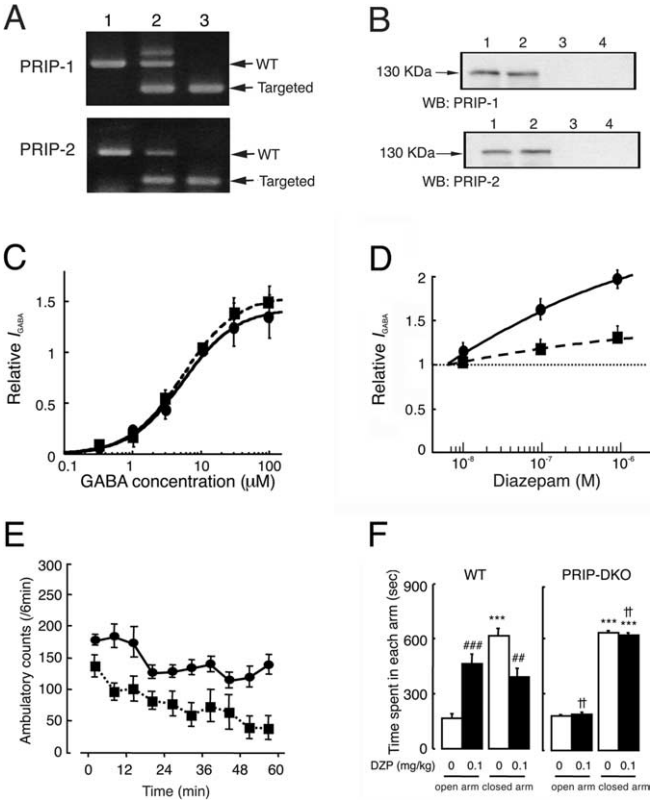

Figure 1. Generation and characterization of PRIP-DKO mice. ***A***, Genotype of the mice. Shown is genotyping for both of the loci by PCR from the tail genomic DNA. Lane 1, *PRIP-1* $^{+/+}$, *PRIP-2* $^{+/+}$ (WT); lane 2, *PRIP-1* $^{+/-}$, *PRIP-2* $^{+/-}$; lane 3, *PRIP-1* $^{-/-}$, *PRIP-2* $^{-/-}$ (PRIP-DKO). Sizes of the fragments include PRIP-1 WT 830 bp, targeted 450 bp; PRIP-2 WT 420 bp, targeted 120 bp. ***B***, Western blot analysis of WT and PRIP-DKO mice with rabbit anti-PRIP-1 (top) and PRIP-2 polyclonal antibody (bottom). Shown are WT and PRIP-DKO hippocampus (lanes 1, 3) and cortex (lanes 2, 4), respectively (15 μg/lane). ***C***, ***D***, Electrophysiological analysis. Shown is the concentration–response relationship of GABA-elicited currents in the WT (solid line with filled circles) and PRIP-DKO (dotted line with filled squares) hippocampal CA1 neurons (***C***; $n = 3$ for each genotype). Also shown is the dose–response relationship (WT, solid line with filled circles; PRIP-DKO, dotted line with filled squares) for the effect of diazepam on I_{GABA} induced by 3 μM GABA (***D***; $n = 3$ for each genotype). ***E***, ***F***, Behavioral analysis of PRIP-DKO mice. ***E***, Spontaneous locomotor activity was measured in a chamber equipped with an infrared beam to count the number of crosses for every 6 min over a 60 min period [WT, solid line with filled circles ($n = 3$); PRIP-DKO, dotted line with filled squares ($n = 5$)]. Results are represented as the mean ± SEM (***C–E***). ***F***, Elevated plus maze test on WT (left) and PRIP-DKO (right) mice was performed 30 min after intraperitoneal administration of diazepam (DZP; 0.1 mg/kg; filled bars) or vehicle (open bars). Data are represented as the mean ± SEM of the time spent in the open arms of the maze. Mann–Whitney *U* test; $^{***}p < 0.001$ versus open arm; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus vehicle (control); $^{\dagger\dagger}p < 0.01$ versus WT.

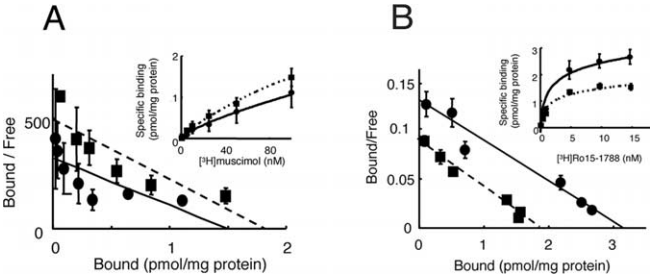

Figure 2. Ligand binding in hippocampal neurons from WT and PRIP-DKO. [^{3}H]muscimol (***A***) and [^{3}H]flumazenil binding assays (***B***) were performed by using the membrane fraction of hippocampus from WT (solid line with filled circles) and PRIP-DKO (dotted line with filled squares) mice (10–14 d old of both genotypes). Saturation isotherm (inset) and Scatchard analysis are shown. B_{max} and K_D values include the following: ***A***, 1.5 ± 0.2 pmol/mg protein and 34.7 ± 3.6 nM for WT and 1.8 ± 0.4 pmol/mg and 31.9 ± 3.4 nM for PRIP-DKO ($n = 10$ mice; 5 independent experiments for each genotype); ***B***, 3.2 ± 0.4 pmol/mg protein and 2.5 ± 0.2 nM for WT and 1.9 ± 0.2 pmol/mg protein and 2.2 ± 0.3 nM for PRIP-DKO ($n = 8$ mice; 4 independent experiments for each genotype).

Alteration of subunit expression of GABA$_A$ receptors in PRIP-DKO mice

A modulation of GABA$_A$ receptors in number and subunit composition is implicated in behavioral states such as the regulation of anxiety, sedation, epileptogenic activity, motor coordination, and drug sensitivity (Korpi et al., 2002). We assessed the subunit expression levels of GABA$_A$ receptors in PRIP-DKO mice by quantitative real-time RT-PCR. In the hippocampus of PRIP-DKO mice at the age of 9–12 weeks, mRNA expression levels of α1, α2, β1, and β2 subunits increased to ~160–250% of the control (WT), whereas the levels of α4, β3, γ2, and δ subunits were increased only slightly, but with no statistical significance (data not shown). To examine the alteration of the surface-expressed receptor number and composition, we performed ligand-binding assays, using [^{3}H]muscimol, a GABA agonist, and [^{3}H]flumazenil, a benzodiazepine antagonist, on a membrane fraction of hippocampus from both genotypes (10–14 d). [^{3}H]muscimol saturation binding studies revealed ~20% elevation in binding sites (B_{max}), with unaltered affinity (K_D value) in PRIP-DKO mice as compared with WT mice (Fig. 2*A*). The benzodiazepine binding using [^{3}H]flumazenil revealed that the maximal binding (B_{max}) decreased by ~40% without alteration of the affinity (Fig. 2*B*). The interface of α/β subunits or α/γ subunits provides for [^{3}H]muscimol or [^{3}H]flumazenil binding sites, respectively (Möhler et al., 2001; Korpi et al., 2002), suggesting that PRIP-DKO hippocampal neurons express more GABA$_A$ receptors with fewer γ2 subunits on their cell surface.

Most of the physiological pentameric structures for GABA$_A$ receptors in brain are those made of three subunits (Fritschy and Möhler, 1995; Whiting et al., 1999). Therefore, we tested the possibility that substitution of the γ2 subunit occurs with other subunits, including the δ subunit in PRIP-DKO neurons. For this purpose several chemicals specific to δ subunit were used. 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP; Sigma–Aldrich) is a GABA agonist that generates maximal current greater than that by GABA itself and displays markedly higher efficacy at the δ-containing receptor (Ebert et al., 1997; Brown et al., 2002). Furthermore, the trivalent cation lanthanum (La^{3+}) inhibits the THIP-gated current in α4/6βδ GABA$_A$ receptors (Saxena et al., 1997; Zhu et al., 1998; Brown et al., 2002). Therefore, the maximal THIP-gated current, the EC_{50} for THIP, and La^{3+} sensitivity to THIP-evoked current would be an estimate of the δ subunit substitution. The responses to THIP at various concentrations were recorded by using acutely isolated hippocampal neurons from WT and PRIP-DKO and compared with that evoked by GABA at 100 μM, which provoked the maximal current (Fig. 1*C*). As shown in Figure 3*A*, THIP at a maximal concentration provided a current equivalent to that evoked by 100 μM GABA in neurons from both WT and PRIP-DKO mice, and the EC_{50} values to THIP were similar. The La^{3+} effect was rather positive and unchanged between the genotypes; the current evoked by 100 μM THIP was increased to 126 ± 5.9% ($n = 4$) or 130 ± 5.9% ($n = 4$) in the presence of 100 μM La^{3+} in WT and PRIP-DKO neurons, respectively. Furthermore, the neurosteroid tetrahydrodeoxycorticosterone (THDOC; Sigma–Aldrich) consistently augmented the currents evoked by GABA in receptors containing the δ subunit, but not the γ2 subunit, in the recombinant cell expression system (Wohlfarth et al., 2002; Bianchi and Macdonald,

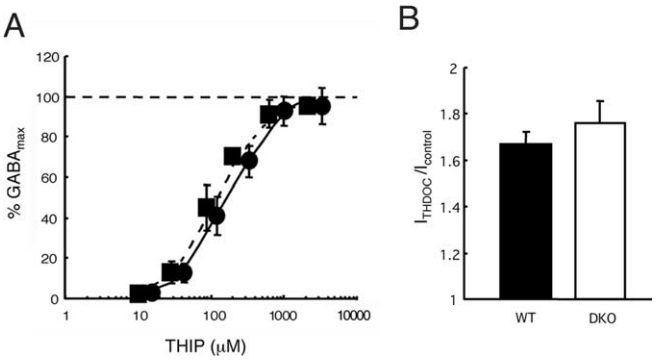

Figure 3. Effect of THIP, La^{3+}, and THDOC in PRIP-DKO hippocampal neurons. ***A***, THIP-evoked current was recorded with whole-cell patch-clamp techniques, using acutely isolated CA1 cells. Peak responses to THIP were expressed as a percentage to the maximal GABA-gated (100 μM) current ($GABA_{max}$). Dotted line indicates 100% of response (GABA-gated current at 100 μM). The EC_{50} for THIP was 123.2 $\pm$ 11.2 μM (for WT, $n = 5$) and 133.7 $\pm$ 40.6 μM (for DKO, $n = 3$), statistically insignificant. ***B***, THDOC (0.1 μM) enhancement of peak current amplitudes evoked by 3 μM GABA for acutely isolated hippocampal CA1 neuron (WT, filled bar; PRIP-DKO, open bar; $n = 4$ for each genotype). Data are represented as the mean $\pm$ SEM.

2003). We then examined the enhancement of GABA-evoked (at 3 μM) current by 100 nM THDOC. PRIP-DKO neurons exhibited a slightly larger enhancement by THDOC, but with no statistical significance (Fig. 3*B*). These results indicate no significant increase of the δ subunit-containing $GABA_A$ receptor in PRIP-DKO hippocampal neurons, excluding the possibility that the δ subunit is a substitute for the γ2 subunit in PRIP-DKO mice.

Together with electrophysiological and behavioral analyses, the double knock-out experiment on PRIP genes suggests that PRIP molecules are involved in subunit-dependent cell surface $GABA_A$ receptor expression, probably by regulating the trafficking of γ2-containing $GABA_A$ receptors.

Interaction between PRIP and β subunits modulates cell surface $GABA_A$ receptor expression

It has been reported that PRIP molecules and receptor γ2 subunits bind GABARAP in a competitive manner (Kanematsu et al., 2002; Uji et al., 2002) and that GABARAP facilitates the transport of γ2 subunit-containing receptors to the cell surface (Leil et al., 2004; Chen et al., 2005). On the basis of these findings we predicted that gene knock-out of the PRIP molecules might increase the cell surface expression of γ2 subunit-containing $GABA_A$ receptors. However, as described above, deletion of PRIP appeared to decrease γ2 subunit surface trafficking. In an attempt to understand this observation better, we focused additionally on characterizing the recent observation of a direct interaction of PRIP with $GABA_A$ receptor β subunits (Terunuma et al., 2004). We recently mapped the region (amino acid residues 544–568 of rat PRIP-1) responsible for the interaction with receptor β subunits (Kanematsu et al., 2006, 2007). This region is located remotely from that for GABARAP (Kanematsu et al., 2002). Therefore, we tested whether PRIP-1 forms a trimeric complex with the receptor β3 subunit and GABARAP. As shown in Figure 4*A*, the GST-β3 subunit, but not GST alone (lanes 4, 5), directly bound to PRIP-1 (lane 3), but not to GABARAP (lane 2). Importantly, GABARAP was coprecipitated with the GST-β3 subunit in the presence of PRIP-1 (lane 1). Furthermore, PRIP-1 and GABARAP were coimmunoprecipitated with β2/3 subunits when mouse brain lysates were used (Fig. 4*B*). Hence we hypothesized that the association between PRIP and the β subunits is implicated in facilitating GABARAP-dependent surface trafficking of γ2 subunit-containing $GABA_A$ receptors.

To examine this possibility, we used an expression vector to express a peptide corresponding to the β subunit-binding region in PRIP-1 and then determined whether the dissociation of the interaction by the peptide altered $GABA_A$ receptor cell surface expression and subunit composition. We used GH3 and HEK293 cells, which contain endogenous PRIP-1 and PRIP-2 and GABARAP (Fig. 4*C*). The PRIP-binding peptide (amino acid residues 553–565 of rat PRIP-1) was inserted in the pIRES2-EGFP vector (Kanematsu et al., 2006), which was transfected into GH3 cells along with $\alpha1^{Flag}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits. A scrambled version of this peptide also was used as a control (see Materials and Methods). Disruption of the interaction between PRIP and $\beta2^{Myc}$ subunits by the PRIP-binding peptide was confirmed by using a coimmunoprecipitation assay to precipitate $GABA_A$ receptors from cell lysates with an anti-Myc antibody (9E10), followed by immunoblot analysis with anti-PRIP-1 antibody. The amount of PRIP-1 immunoprecipitated with $\beta2^{Myc}$ subunits was decreased sufficiently in the presence of PRIP-binding peptide as compared with that by control scrambled peptide, as shown in Figure 4*D*. The $\beta2^{Myc}$ subunits also were immunoblotted with rabbit anti-Myc polyclonal antibody, but unfortunately the chemiluminescence signals on the blot were difficult to define because of their hiding behind the nonspecific signals of mouse IgG heavy chain (data not shown). However, because immunoprecipitation was performed by using the same amount of cell extract and the same amount of mouse anti-Myc monoclonal antibody (see Materials and Methods), it will be shown that the PRIP-binding peptide disrupts the association.

To test an influence of the disruption for surface trafficking of γ2 subunit-containing $GABA_A$ receptors, we transfected GH3 cells with combinations of $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits or $\alpha1^{Flag}$, $\beta2^{Flag}$, and $\gamma2^{Myc}$ subunits with the PRIP-binding peptide or the scrambled peptide. The cell surface expression of $\alpha1^{Myc}$ $\beta2^{Myc}$, or $\gamma2^{Myc}$ subunit-containing receptors was quantified by a whole-cell ELISA under nonpermeabilized conditions (see Materials and Methods). As shown in the left panel of Figure 4*E*, either scrambled or PRIP-binding peptide had little effect on the expression of α and β subunits, suggesting little influence on β subunit-dependent receptor trafficking. When the expression of the γ2 subunit was assessed, the presence of the PRIP-binding peptide slightly but perceptibly reduced the cell surface expression of $\gamma2^{Myc}$ subunit-containing $GABA_A$ receptors, as shown in the middle panel. We also examined whether the peptide alters the trafficking of the δ-containing $GABA_A$ receptor by a similar experimental procedure, but in this case the β3 subunit in place of the β2 subunit was used (Korpi et al., 2002). As shown in the right panel of Figure 4*E*, the PRIP-binding peptide exhibited little effect on the cell surface expression of δ subunit-containing receptors, indicating that the effect of PRIP-binding peptide is specific to the case of the γ2 subunit.

To confirm additionally the effects of PRIP-binding peptide on the trafficking of γ2 subunit-containing receptors, we transfected HEK293 cells with combinations of $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits or $\alpha1^{Flag}$, $\beta2^{Flag}$, and $\gamma2^{Myc}$ subunits with or without the PRIP-binding peptide plasmid, and we visualized the localizations of cell surface and internal $GABA_A$ receptors by using anti-Myc antibody under nonpermeabilized and permeabilized conditions, respectively (Fig. 4*F*). Under nonpermeabilized and permeabilized conditions positive signals were seen mainly on the cell surface and in the cytoplasmic area, as expected. The localization of α1 and β2 subunits seemed to be unaffected by the

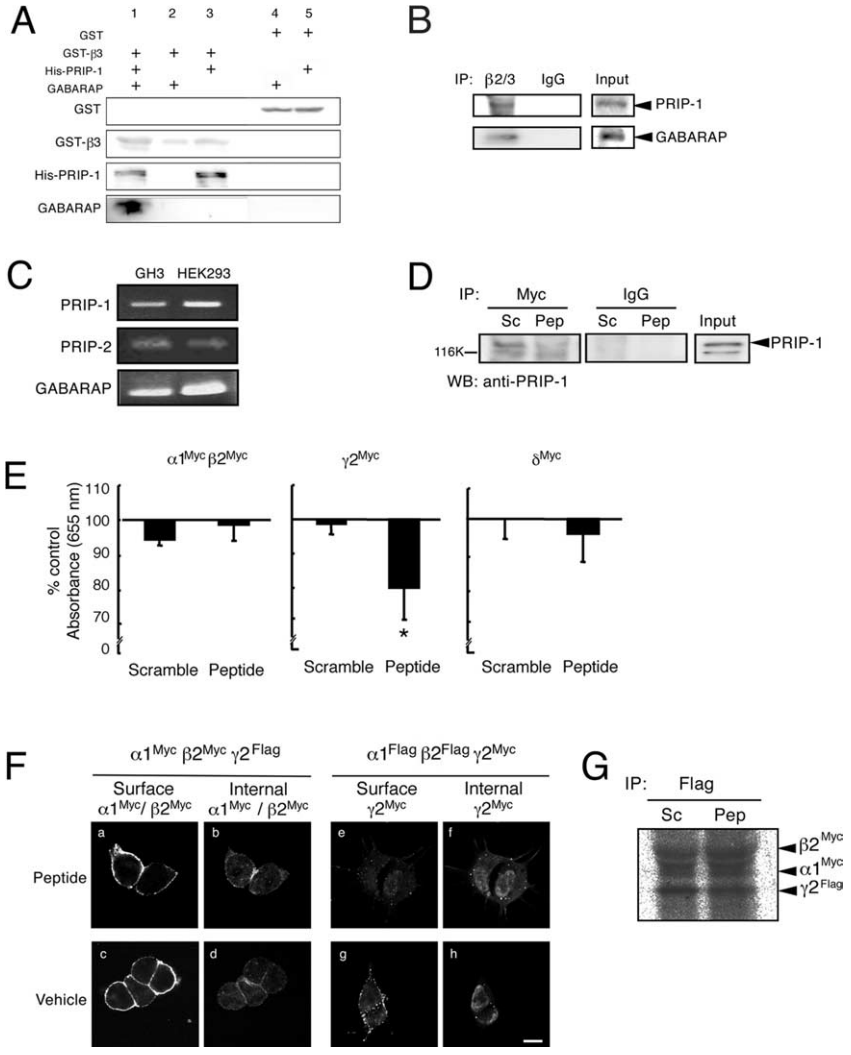

Figure 4. Importance of the association between PRIP and β subunits of GABA$_A$ receptor in trafficking of γ2 subunit-containing receptors. ***A***, Complex formation among PRIP-1, GABARAP, and GST-β3. A pull-down assay was performed by using recombinant PRIP-1 and/or GABARAP to the GST-β3 subunit of GABA$_A$ receptor and control GST protein. The combination of these proteins in the assay is shown as (+) on the top side. Similar results were seen in two other experiments. ***B***, Interaction of PRIP-1 and GABARAP with GABA$_A$ receptors in brain extracts. Brain extracts (5 mg of protein) from WT mice were immunoprecipitated by an antibody against the GABA$_A$ receptor β2/3 subunit, followed by SDS-PAGE and Western blotting with an anti-PRIP-1 antibody or anti-GABARAP antibody. Similar results were seen in three independent experiments. ***C***, Intrinsic expression of PRIP-1, PRIP-2, and GABARAP mRNA in HEK293 and GH3 cells. ***D***, Inhibition of the association between PRIP-1 and β2 subunits of GABA$_A$ receptor by PRIP-binding peptide. Lysates from GH3 cells transfected with $\alpha1^{Flag}$, $\beta2^{Myc}$, $\gamma2^{Flag}$ subunits, PRIP-binding peptide (Pep), or control scrambled peptide (Sc) were immunoprecipitated by anti-Myc antibody or control mouse IgG, followed by Western blotting with anti-PRIP-1 antibody. An arrowhead indicates PRIP-1, and a band seen at bottom in both lanes with a similar amount appears to be nonspecific. Standard protein size marker (116,000) is indicated at the left. Two other experiments provided similar results. Similar results also were obtained from the experiments in which HEK293 cells were used (data not shown). ***E***, Reduction of γ2 subunit cell surface expression by PRIP-binding peptide in GH3 cells as assessed by whole-cell ELISA. GH3 cells were transfected with $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits (left) or $\alpha1^{Flag}$, $\beta2^{Flag}$, and $\gamma2^{Myc}$ subunits (middle) in the presence of PRIP-binding peptide plasmid (Peptide), scrambled peptide plasmid (Scramble), or vector plasmid. The combination of $\alpha1^{Flag}$, $\beta2^{Flag}$, and δ^{Myc} subunits (right) also was transfected with each peptide in GH3 cells. Then a nonpermeabilized whole-cell ELISA was performed. The graph shows the summary of the results ($n = 4$) representing the percentage of the control (vector). Data are represented as the mean ± SEM. Student's *t* test; $^*p < 0.05$. ***F***, Reduction of γ2 subunit cell surface expression by PRIP-binding peptide in HEK293 cells as assessed by fluorescent image analysis. HEK293 cells were transfected with $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits (left) or $\alpha1^{Flag}$, $\beta2^{Flag}$, and $\gamma2^{Myc}$ subunits (right) in the presence of PRIP-binding peptide (Peptide) or in the absence of the peptide (Vehicle). Then immunocytochemical analysis was performed. Representative confocal microscopic images (peptide, ***a***, ***b***, ***e***, ***f***; vehicle, ***c***, ***d***, ***g***, ***h***) are shown. Signals were visualized under nonpermeabilized (surface, ***a***, ***c***, ***e***, ***g***) and permeabilized (internal, ***b***, ***d***, ***f***, ***h***) conditions (see also Materials and Methods). More than 20 fields in three independent experiments produced similar images. Scale bar, 10 μm. ***G***, Little effect of the PRIP-binding peptide on assembly of $\alpha/\beta/\gamma$ subunits. HEK293 cells were transfected with $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ along with PRIP-binding peptide (Pep) or scrambled peptide (Sc) and then radiolabeled with [^{35}S/^{35}S]cysteine/methionine, followed by an immunoprecipitation of the lysates with anti-Flag antibody and autoradiography. Two other experiments gave similar results, and reverse experiments that used anti-Myc antibody in place of anti-FLAG antibody for immunoprecipitation (see Materials and Methods) also provided similar results.

presence of the peptide (Fig. 4*F*, compare *a*, *b* with *c*, *d*). However, dissociation of the interaction between β subunits and PRIP molecules by the PRIP-binding peptide reduced the cell surface expression of γ2 subunits (Fig. 4*F*, compare *e*, *g*) and resulted in an accumulation of γ2 subunits in an internal compartment (Fig. 4*F*, compare *f*, *h*).

Moreover, to examine the possibility that the interference peptide affects the GABA$_A$ receptor assembly, we performed an immunoprecipitation assay that used HEK293 cells transfected with $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Flag}$ subunits in the presence of PRIP-binding or scrambled peptide. Each subunit is difficult to define by Western blot analysis because of the nonspecific signals of mouse IgG heavy chain. Therefore, here the cells were radiolabeled with [^{35}S/^{35}S]cysteine/methionine; then immunoprecipitation of the lysate was performed with anti-Flag antibody, followed by autoradiography. Similar radioactivities corresponding to the $\beta2^{Myc}$ and $\alpha1^{Myc}$ as well as $\gamma2^{Flag}$ were observed in PRIP-binding peptide like those in scrambled peptide (Fig. 4*G*). Reverse experiments that used anti-Myc antibody also showed similar radioactivities corresponding to the $\gamma2^{Flag}$ (data not shown), indicating little effect of the peptide on assembly of $\alpha/\beta/\gamma$ subunits.

To address whether exogenous expression of GABARAP and/or PRIP-1 promotes surface expression of $\alpha1/\beta2/\gamma2$ receptors, we next performed a whole-cell ELISA by using COS-7 cells, which have endogenous GABARAP, but not PRIP (Takeuchi et al., 2000). The cells were transfected with combinations of $\alpha1^{Myc}$, $\beta2^{Myc}$, and $\gamma2^{Myc}$ subunits with the GABARAP, PRIP-1, or control vector. The exogenous expression of PRIP-1 or GABARAP increased the surface expression levels of the GABA$_A$ receptors to 128.2 ± 5.9% ($n = 6$) or 131.6 ± 9.0% ($n = 6$) of the control, respectively. The effect was more evident when both PRIP-1 and GABARAP were transfected; the upregulation was 164.1 ± 18.9% ($n = 6$) of the control. The upregulation by PRIP appeared to be dependent mainly on the interaction with $\beta2$ subunit, because it was inhibited by the PRIP-binding peptide, but not by the scrambled peptide.

Interaction of PRIP with β subunits is implicated in the cell surface expression of $\gamma2$ subunit-containing GABA$_A$ receptors in neurons

Finally, to elucidate the physiological relevance of PRIP molecules in the

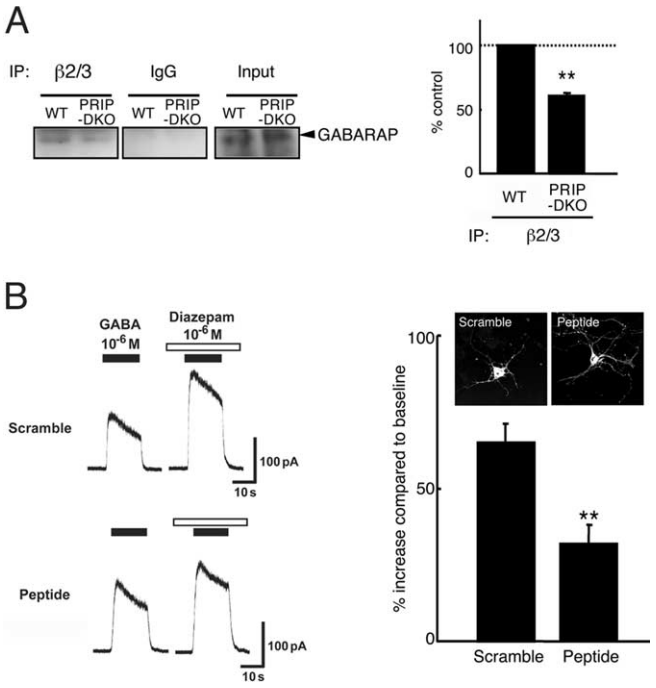

Figure 5. Involvement of PRIP in the γ2 subunit trafficking machinery. ***A***, Reduction of GABARAP bound to the $GABA_A$ receptors in brains from PRIP-DKO mice. Brain lysates from WT or PRIP-DKO mice were immunoprecipitated by anti-β2/3 antibody or mouse IgG as a control. Then Western blotting was performed with anti-GABARAP antibody. A representative image is shown (left). Graph at right shows the summary of the results ($n = 3$) representing the percentage of WT. Data are represented as the mean ± SEM. Student's *t* test; $^{**}p < 0.01$. ***B***, Surface expression of γ2 subunits is downregulated by the PRIP-binding peptide. Rat cultured hippocampal neurons expressing scrambled peptide (left inset) or PRIP-binding peptide (right inset) were visualized by GFP via fluorescence microscopy and were used for I_{GABA} recording. Left panel shows a representative trace of I_{GABA} induced by 1 μM GABA in cultured hippocampal cells with scrambled (top) and PRIP-binding (bottom) peptide in the presence of 1 μM diazepam. Solid and open bars indicate the periods of GABA and diazepam application, respectively. Right bar graph shows the percentage of increase in I_{GABA} compared with the current in the absence of diazepam ($n = 11$ for each peptide). Data are represented as the mean ± SEM. Student's *t* test; $^{**}p < 0.01$.

GABARAP-mediated γ2 subunit trafficking machinery, we performed coimmunoprecipitation experiments to analyze the formation of $GABA_A$ receptor–GABARAP protein complexes in the brains of PRIP-DKO mice. Brain extracts (5 μg) from PRIP-DKO and WT mice were immunoprecipitated with anti-$GABA_A$ receptor β2/3 antibody and control mouse IgG (6.5 μg each), and the amount of GABARAP coimmunoprecipitated with the receptors was assessed. The bands of immunoprecipitated β2/3 subunits (53/57 kDa) were hidden behind the strong nonspecific chemiluminescent signal of the IgG heavy chain (data not shown). As shown in Figure 5*A*, the amount of coimmunoprecipitated GABARAP in PRIP-DKO mice was lower than that of WT mice (60.5 ± 1.4%), indicating that PRIP molecules are required to facilitate the binding of GABARAP to $GABA_A$ receptors via the γ2 subunits.

To examine the effect of the peptide on native neuronal receptors, we analyzed cultured rat hippocampal neurons transfected with the PRIP-binding peptide or scrambled peptide. Transfected neurons could be visualized because of the coexpression of GFP fluorescence from the pIRES vector (Fig. 5*B*). We used electrophysiological experiments to examine whether the interference of the interaction between PRIP and the β subunits by the peptide alters the response to diazepam. The I_{GABA} responses at 0.1, 1, and 10 μM of GABA in neurons containing either peptide were similar (data not shown). When we examined the sensitivity to diazepam as shown in Figure 5*B*, diazepam (1 μM) potentiated I_{GABA} elicited by 1 μM GABA in hippocampal neurons transfected with the scrambled peptide (66 ± 6.2% increase). In contrast, the potentiation of I_{GABA} in neurons expressing the PRIP-binding peptide was reduced markedly (34 ± 5.8%). These results suggest that the disruption of the interaction between PRIP and the $GABA_A$ receptor β subunits leads to a reduction of cell surface benzodiazepine-sensitive (α/γ2 subunit-containing) $GABA_A$ receptors and additionally suggest that PRIP molecules are involved in regulating the cell surface number of γ2 subunits by a mechanism dependent on β subunits of $GABA_A$ receptors and GABARAP.

Discussion

PRIP-1 first was identified as a novel inositol 1,4,5-trisphosphate binding protein (Kanematsu et al., 1992, 1996, 2000, 2005; Yoshida et al., 1994; Takeuchi et al., 1996, 1997, 2000; Matsuda et al., 1998; Yamamoto et al., 1999; Murakami et al., 2006). Additional studies to find binding partners, including protein phosphatase 1 (Yoshimura et al., 2001; Terunuma et al., 2004; Yanagihori et al., 2006) and GABARAP (Kanematsu et al., 2002), prompted us to examine the possible involvement of PRIP-1 in $GABA_A$ receptor signaling. PRIP-1 and the γ2 subunit bind to GABARAP in a competitive manner (Kanematsu et al., 2002), and GABARAP is reported to facilitate the membrane transport of γ2 subunit-containing receptors to the cell surface (Leil et al., 2004; Chen et al., 2005); we therefore predicted that a gene knock-out of PRIP-1, which would eliminate PRIP-dependent competition of GABARAP binding to $GABA_A$ receptors, would result in an increased cell surface expression of γ2 subunit-containing receptors. Contrary to this prediction, studies on PRIP-1 KO mouse phenotypes indicated a functionally decreased expression of the γ2 subunit-containing $GABA_A$ receptors, as analyzed by electrophysiological and behavioral studies (Kanematsu et al., 2002). We expected that gene compensation at the level of PRIP-2 proteins might account for these unexpected findings because PRIP-2 also binds GABARAP. To this end we produced PRIP-1/PRIP-2 DKO mice to investigate the functional consequences of removing all PRIP protein expression from the nervous system. Although a locomotor activity test using PRIP-DKO mice was in reverse, PRIP-DKO mice exhibited a basically similar pharmacological and behavioral phenotype as the PRIP-1 KO mice; they were also functionally less sensitive to diazepam. It could be speculated that compensation by PRIP-2 might be restricted to certain neuron cell types of PRIP-1 KO mice. Some difference in the distribution between PRIP-1 and PRIP-2 genes was observed in an *in situ* hybridization analysis that used rat brain; PRIP-2 mainly localizes at the external granular cell layer of cerebral cortex and granular cell layer of dentate gyrus (Uji et al., 2002; our unpublished data).

We performed several sets of experiments in PRIP-DKO mice to understand better the role of PRIP proteins in $GABA_A$ receptor trafficking at the molecular level. Subunit expression of the receptors was altered in PRIP-DKO mice; the total amounts of each α1, α2, β1, and β2 subunit were upregulated in quantitative real-time RT-PCR. The levels of α4, β3, γ2, and δ subunits were similar. It is known that insensitivity to diazepam is produced by receptors containing α4 or α6 subunits combined with γ2 subunits (Korpi et al., 2002); however, this is unlikely, because no significant change in the abundance of α4 and γ2 subunits was observed between WT and PRIP-DKO brains, and little change was exhibited in the level of diazepam-insensitive benzodiaz-

epine receptors by a ligand-binding assay that used [^{3}H]Ro15-4513 and diazepam (Homanics et al., 1997; Sur et al., 1999) in the hippocampus of PRIP-DKO mice (data not shown).

Based on our findings, the phenotype of PRIP-DKO mice includes a reduction in surface expression of diazepam-sensitive $GABA_A$ receptors (α1, 2, 3, 5/β/γ2). In agreement with this, the ligand-binding assay using [^{3}H]flumazenil confirmed the reduced expression of diazepam-sensitive $GABA_A$ receptors on the cell surface with the upregulation of muscimol binding. These results indicate that PRIP is involved in facilitating the trafficking and/or insertion into the surface membrane of the γ2 subunit-containing receptors, the processes of which probably require GABARAP to bind to the γ2 subunit (Leil et al., 2004; Chen et al., 2005). However, it is difficult to assume how readily PRIP molecules are implicated in the GABARAP-mediated transport of the γ2 subunits, because we previously elucidated that PRIP molecules bind GABARAP in a competitive manner with the γ2 subunits (Kanematsu et al., 2002; Uji et al., 2002). The results obtained in this study show that the amount of association between GABARAP and $GABA_A$ receptors was reduced significantly in the PRIP-DKO brain, and the exogenous addition of PRIP-1 and GABARAP facilitated the trafficking of γ2 subunit-containing receptors to the cell surface in the recombinant cell system. Disrupting the interaction between PRIP and β subunits in a rat cultured hippocampal neurons (WT) resulted in a decrease of cell surface-expressed γ2 subunits, which resembled the phenotype of PRIP-DKO mice, indicating that the interaction of PRIP with the β subunits plays an important role in this process. However, in previous work (Terunuma et al., 2004), we also observed a very weak direct interaction between PRIP and the γ2 subunit in the GST-γ2 pull-down assay with recombinant PRIP-1, expressed and labeled by an *in vitro* transcription/translation method. The γ2 subunit and β subunits might share the same binding region in the PRIP. To examine this issue, we performed a competition assay using recombinant PRIP-1 incubated with GST-β2 (1 μM) and various concentrations of His-γ2 (0–2 μM). The interaction between PRIP-1 and β2 subunit was not inhibited by the γ2 subunit (supplemental figure, available at www.jneurosci.org), indicating that the interaction of PRIP to the β subunit is stronger than that to the γ subunit.

Physiological pentameric $GABA_A$ receptors in brain have been assumed to be those comprising three subunits (i.e., α1/β2/γ2) or associating with a fourth subunit (Fritschy and Möhler, 1995; Whiting et al., 1999). Because fewer γ2 subunit-containing $GABA_A$ receptors have been observed in PRIP-DKO neurons, the γ2 subunit might be replaced by other subunits (i.e., δ, ε, θ, and π subunits). A candidate subunit to replace could be assumed from their regional localizations in the brain. The δ subunit is widely distributed in the rodent brain, but the ε and θ subunits have the most restricted distribution; they are abundant in the hypothalamus and brainstem regions (Korpi et al., 2002). The π subunit seems rare or nonexisting in the brain (Hedblom and Kirkness, 1997). Therefore, the δ subunit might be a candidate for the compensation in hippocampus and cerebral cortex neurons of PRIP-DKO. However, the responses to THIP, a δ subunit-preferring hypnotic drug, were not different in hippocampal neurons of WT and PRIP-DKO mice, and La^{3+} similarly enhanced the THIP-gated current, indicating little alteration of δ subunit-containing receptors in DKO mice. The enhancement by La^{3+} in mouse CA1 hippocampus neurons was observed previously by Shen et al. (2005); La^{3+} (300 μM) increased the current by 30% in wild-type rat CA1 hippocampal neurons. Furthermore, there was no significant difference in the result of the experiment that used the neurosteroid THDOC, the target for which is the δ subunit (Wohlfarth et al., 2002; Bianchi and Macdonald, 2003). These results suggest that fewer γ2 subunits in $GABA_A$ receptors on the cell surface observed in PRIP-DKO mice do not appear to be compensated by other third subunit candidates. Very recently, Mortensen and Smart (2006) found the presence of $GABA_A$ receptors composed of only α/β subunits, lacking a third subunit in rat hippocampal pyramidal neurons. Therefore, it might be plausible that α/β pentamers lacking a third subunit are expressed in the PRIP-DKO hippocampal neurons.

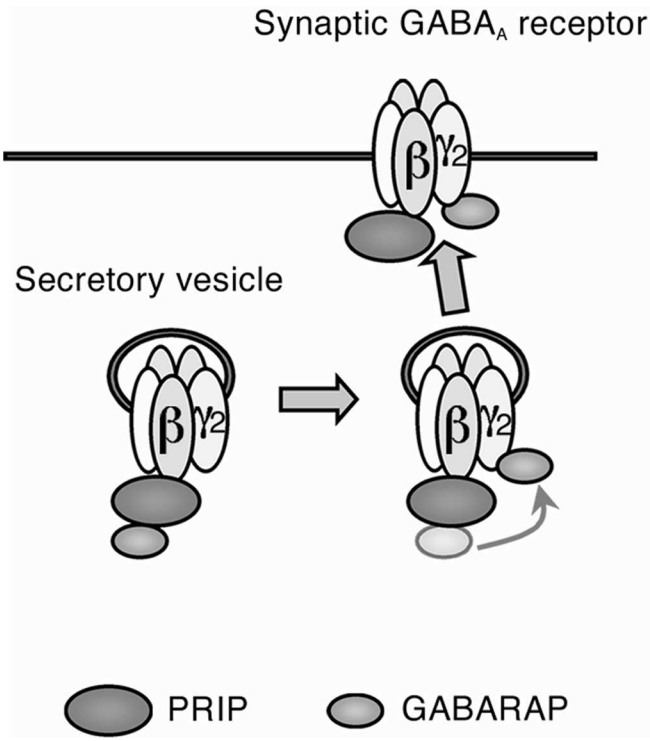

Figure 6. Possible role of PRIP molecules as a scaffold protein for GABARAP. PRIP molecules, interacting with GABARAP, bind to the β subunits of $GABA_A$ receptor and deliver it to the γ2 subunit at an appropriate moment. Then the receptor is transported to the surface membrane because of GABARAP (Leil et al., 2004; Chen et al., 2005).

Disruption of the interaction of PRIP with the β subunits by the peptide did not cause the alteration of the subunit assembly pattern, probably excluding the possibility that deletion of PRIP molecules induces preferential assembly between only α and β subunits. Reduced binding of GABARAP with pentameric $GABA_A$ receptors as assessed by coimmunoprecipitation suggests that PRIP and GABARAP cooperate to promote the trafficking of γ2 subunit-containing receptors. Considering these results, we would propose a tentative conclusion that the formation of triplet complexes among the β subunits, PRIP, and GABARAP would facilitate the association of GABARAP with the γ2 subunit to be transported at the right place at the right time (Fig. 6); alternatively, the association between β subunits and PRIP would promote primarily the association of GABARAP to the γ2 subunit. However, precise molecular mechanisms by which PRIP facilitates the association of GABARAP with the γ2 subunit remain to be elucidated. Furthermore, there are many other proteins reported to interact directly with $GABA_A$ receptor subunits and thus able to modulate the numbers of cell surface receptors (Brandon et al., 2000, 2002, 2003; Kittler et al., 2000; Bedford et al., 2001; Beck et al., 2002; Chen and Olsen, 2007).

The $GABA_A$ receptors containing the $\gamma 2$ subunits play physiologically important roles in brain function at many aspects, because homozygous deletion of the $\gamma 2$ subunit in mice leads to death within a few days after birth, and heterozygotes result in severe growth retardation, sensorimotor and behavioral dysfunctions, and drastic reduction in life span (Günther et al., 1995). In addition to the physiological importance, the $\gamma 2$ subunit is also clinically important because of being a target for benzodiazepine-type drugs, which are widely used as therapeutic medication, including as anxiolytics, hypnotics, anticonvulsants, and muscle relaxants (Günther et al., 1995; Möhler et al., 2001). Therefore, the mechanisms for regulating the cell surface number of $\gamma 2$ subunits need to be elucidated fully. Our findings here that PRIP is important for expressing the $\gamma 2$ subunit-containing receptors by interacting with the β subunits may be one of the essential factors of the complicated system of dynamic regulation of $GABA_A$ receptors at inhibitory synapses.

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