Modulation of the Neural Glutamate Transporter EAAC1 by the Addicsin-interacting Protein ARL6IP1*

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Addicsin (Ar6ip5) is a murine homologue of rat glutamate transporter-associated protein 3-18 (GTRAP3-18), a putative negative modulator of Na⁺-dependent neural glutamate transporter-excitatory amino acid carrier 1 (EAAC1). Here we report that ADP-ribosylation factor-like 6 interacting protein 1 (AR6IP1) is a novel addicsin-associated partner that indirectly promotes EAAC1-mediated glutamate transport activity in a protein kinase C activity-dependent manner. Like addicsin, AR6IP1 is expressed in numerous tissues and proved likely to be co-localized with addicsin in certain neurons in the matured brain. AR6IP1 was not translocated from the subcellular compartments under any of the test conditions and had no association with any molecules on the plasma membrane. Immunoprecipitation assay demonstrated that AR6IP1 bound directly to addicsin and that the hydrophobic region located at amino acids 103–117 of addicsin was crucial to the formation of the AR6IP1-addicsin heterodimer and addicsin homodimer. Glutamate transport assay revealed that increasing the expression of AR6IP1 in C6BU-1 cells markedly enhanced Na⁺-dependent EAAC1-mediated glutamate transport activity in the presence of 100 nM phorbol 12-myristate 13-acetate. Under these conditions, kinetic analyses demonstrated that EAAC1 altered glutamate transport activity by increasing its glutamate affinity but not its maximal velocity. Meanwhile, increasing expression of addicsin Y110A/L112A mutant lacking binding ability for AR6IP1 showed no enhancement of EAAC1-mediated glutamate transport activity, regardless of phorbol 12-myristate 13-acetate activation, suggesting that association between addicsin and AR6IP1 causes altered EAAC1-mediated glutamate transport activity. Our findings suggest that AR6IP1 is a novel addicsin-associated partner that promotes EAAC1-mediated glutamate transport activity by decreasing the number of addicsin molecules available for interaction with EAAC1.

Glutamate is the major excitatory neurotransmitter in excitatory synapses and the metabolic substrate for γ-aminobutyric acid synthesis in the inhibitory neurons in the mammalian central nervous system. Extracellular glutamate levels are strictly regulated by Na⁺-dependent high affinity excitatory amino acid transporters (EAATs), which convey L-glutamate, L-aspartate, and D-aspartate from the extracellular space into the cells by means of the Na⁺-K⁺-coupled transport system (1). EAATs can be classified into five different homologues, designated EAAT1 (glutamate/aspartate transporter (GLAST)), EAAT2 (glutamate transporter-2 (GLT-1)), EAAT3 (excitatory amino acid carrier 1 (EAAC1)), EAAT4, or EAAT5 (2). In the CNS, GLAST and GLT-1 mainly localize to the astrocytes and play a major role in protecting neurons from glutamate-induced toxicity (3) as well as terminating glutamatergic transmissions (4, 5). On the other hand, EAAC1 is widely expressed in the CNS and localizes in the somata and dendrites of certain neurons in the CNS (6, 7). EAAT4 is expressed in cerebellar Purkinje cells and EAAT5 in the retina (8–10).

The function of EAAC1 in the CNS has not been established. However, recent studies demonstrate that EAAC1 acts as a cysteine transporter and maintains neuronal glutathione metabolism (11), and that EAAC1 has a unique mitochondria-mediated anti-apoptotic function in injured motor neurons (12). Knock-out of EAAC1 in rodents leads to the development of epilepsy resulting from reduced synthesis of the neurotransmitter γ-aminobutyric acid (13), dicarboxylic aminoaciduria, and significant motor impairment (14). Changes in glutamate transport activity of EAAC1 are also associated with long term potentiation and fear conditioning (15). These pieces of evidence suggest that EAAC1 may contribute physiologically to multiple neurotic functions as distinct from glutamate clearance in the CNS. Furthermore, a number of reports demonstrate that intracellular signaling molecules and accessory proteins regulate the expression and function of EAAC1 (16). For instance, protein kinase C (PKC) and phosphatidylinositol 3-kinase regulate the cell surface expression and intrinsic activity of EAAC1 (17). Furthermore, GTRAP3-18 (glutamate transporter-associating protein 3-18 (GTRAP3-18)), a novel addicsin-associated partner that indirectly promotes EAAC1-mediated glutamate transport activity by increasing its glutamate affinity but not its maximal velocity. Meanwhile, increasing expression of addicsin Y110A/L112A mutant lacking binding ability for AR6IP1 showed no enhancement of EAAC1-mediated glutamate transport activity, regardless of phorbol 12-myristate 13-acetate activation, suggesting that association between addicsin and AR6IP1 causes altered EAAC1-mediated glutamate transport activity. Our findings suggest that AR6IP1 is a novel addicsin-associated partner that promotes EAAC1-mediated glutamate transport activity by decreasing the number of addicsin molecules available for interaction with EAAC1.

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4 The abbreviations used are: EAAT, excitatory amino acid transporter; EAAC1, excitatory amino acid carrier 1; PKC, protein kinase C; CNS, central nervous system; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; Tricine, N, N’-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; WB, Western blotting; IHC, immunohistochemistry; HRP, horseradish peroxidase; RT, reverse transcriptase; siRNA, small interfering RNA; IR, immunoreactivity.
associated protein 3-18) negatively modulates EAAC1-mediated glutamate transport by protein-protein interaction with EAAC1 (18). We have recently identified a novel mRNA encoding a protein designated "addicsin" that is highly up-regulated in amygdala nuclei in morphine-administered mice (19). Amino acid comparison reveals that addicsin is a murine homologue of GTRAP3-18, a putative modulator of EAAC1, and JWA, a human vitamin A-responsive factor (18, 19). The protein known in various papers as addicsin, GTRAP3-18, or JWA has recently been renamed Arl6ip5. Addicsin/GTRAP3-18 is known in various papers as addicsin, GTRAP3-18, or JWA has been particularly focused on the evidence that addicsin/GTRAP3-18 negatively modulates EAAC1-mediating glutamate transport activity in a PKC-activity-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Animals**—Male ddY mice (6 weeks old; 25–30 g in body weight; Japan SLC, Inc., Shizuoka, Japan) were maintained in individual cages (12-hour light-dark cycle; 23–24 °C) and used in the experiments. All animals received humane care in accordance with the National Institute of Advanced Industrial Science and Technology guidelines.

**Antibodies**—Polyclonal rabbit anti-Arl6ip1 antibody recognizing the 24-kDa monomeric Arl6ip1 (3.7 mg/ml, 1:250 dilution for Western blotting (WB) and immunohistochemistry (IHC)) was generated. It was raised against a synthetic peptide (NH$_2$-CGMAKERINLKLK QKE-CNONH$_2$; amino acids 185–199) conjugated to keyhole limpet hemocyanin through a cysteine residue in the N terminus. The polyclonal anti-addicsin antibody (1.7 mg/ml, 1:100 dilution for WB, 1:50 dilution for IHC) was a generous gift from Transgenic Co. Ltd. (Kumamoto, Japan). The monoclonal anti-c-Myc antibody (0.4 mg/ml, 1:500 dilution for WB) was obtained from Roche Applied Science. The monoclonal anti-FLAG M2 antibody (4.9 mg/ml, 1:2000 dilution for WB) was purchased from Sigma. The monoclonal anti-actin antibody (1.0 mg/ml, 1:200 dilution for WB) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (0.5 mg/ml, 1:2000 dilution for WB) were purchased from Chemicon (Temecula, CA). The HRP-conjugated goat anti-rabbit IgG antibody (1.0 mg/ml, 1:20,000 dilution for WB) was obtained from MP Biomedicals (Solon, OH). The Alexa Fluor 488 goat anti-mouse IgG antibody (2.0 mg/ml, 1:500 for IHC) was purchased from Molecular Probes (Eugene, OR).

**Materials**—Fetal bovine serum (FBS), Lipofectamine 2000, Hygromycin, Zeocin, and Mifepristone were purchased from Invitrogen. The anti-FLAG M2 affinity gel, anti-c-Myc affinity gel, avidin beads immobilized on cross-linked 6% beaded agarose, Dulbecco’s modified Eagle’s medium (DMEM) (high glucose with l-glutamine), HAT supplement, protease inhibitor mixture, l-glutamate, phorbol 12-myristate 13-acetate (PMA), and 4α-phorbol 12,13-didecanooate were purchased from Sigma. Protein A-Sepharose and EZ-Link sulfo-NHS-biotin were obtained from Pierce.

**Reverse Transcriptase (RT)-PCR Analysis**—Single-stranded cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) from total RNA isolated from various tissues (50 mg) by guanidine thiocyanate extraction. PCR was carried out using specific primers for the target cDNA. Amplification entailed 30 cycles after pretreatment at 95 °C for 12 min using the following protocols: Arl6ip1, 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s; addicsin and glyceraldehyde-3-phosphate dehydrogenase, 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s. The primers for Arl6ip1 (381 bp) were 5′-TGG CTG GTG TTT CCT GCT TT-3′ and 5′-GTT TGT TTA TCT CCC TTT TG-3′. The primers for addicsin (496 bp) were 5′-TGC CTG CGA CGA TTT CCT CC-3′ and 5′-ATC TTC CTG CTG TCT CCA GG-3′. The primers for glyceraldehyde-3-phosphate dehydrogenase (434 bp) were 5′-GTG GCA GTG CTG GGC GGC ACC ACC ACA-3′ and 5′-TTC AGT GCA GCC ACT-3′ and 5′-TTC ATG CAC CTC AAC TAC TAC-3′.

**Construction of Plasmid**—Full-length mouse Arl6ip1, addicsin, or EAAC1 cDNA was produced by RT-PCR using single-stranded cDNA synthesized from brain total RNA. Amplification entailed 30 cycles after pretreatment at 95 °C for 12 min using the following protocols: Arl6ip1, 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s; addicsin, 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s; EAAC1, 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 90 s. The primers for Arl6ip1 were 5′-AAC AAT TCA TGG CGG AGG GGG ATG ACC GCA-3′ and 5′-TTC TCG AGC AGC TCA TTG TT TTT TT TCT GTC-3′. The primers for addicsin were 5′-GCA TCC TGG CCG AGG GGG ATG ACC GCA-3′ and 5′-AGT GCA ATG GCA TGG ACT-3′ and 5′-TTC ATT GAC CTC AAC TAC TAC-3′.
Yeast Two-hybrid Screening—Matchmaker two-hybrid system 3 (Clontech) was used for the yeast two-hybrid screen. A full length of addicsin cDNA was subcloned into a bait vector, pGBK7, by fusion with the GAL4 DNA-binding domain. The oligo(dT)-primed cDNAs prepared from the amygdala in repeated morphine-administered mice were subcloned into a prey vector, pACT2, by fusion with the GAL4 activation domain. These constructs were co-transfected into the yeast AH109 strain using the lithium acetate method. After the transformants had grown on tryptophan-, leucine-, and histidine-deficient plates (SD-TLH) containing 20 mg/ml 5-bromo-4-chloro-3-indolyl-α-d-galactopyranoside (X-α-gal) (Clontech) at 30 °C, the resultant blue colonies were selected. The plasmids were isolated using Zymoprep (Zymo Research Corp., Orange, CA), and their cDNA sequences were then analyzed.

Cell Culture and Transfection—C6BU-1 and COS7 cells were maintained in DMEM supplemented with 10% FBS (10% FBS-DMEM) at 37 °C in 5% CO2. NG108-15 cells were maintained in 10% FBS-DMEM containing 1× HAT supplement at 37 °C in 5% CO2. For transient expression, cells were transfected with Lipofectamine 2000 and maintained for 48 h before the experiments. For creating a mifepristone-regulated expression system, C6BU-1 stable cell lines designated C6BU-1-pSw-Arl6ip1, C6BU-1-pSw-addicsin, and C6BU-1-pSw-addicsinY110A/L112A, respectively, were produced using the GeneSwitch System (Invitrogen). These cell lines were cultured in 10% FBS-DMEM containing 100 µg/ml Hygromycin and 250 µg/ml Zeocin for dual selection and were exposed to 10 nM mifepristone for 24 h to express conditionally V5-tagged Arl6ip1 (Arl6ip1-V5), V5-tagged addicsin (addicsin-V5), and V5-tagged addicsinY110A/L112A (addicsinY110A/L112A-V5), respectively. Each basal transcription level was undetectable by Western blotting analysis using the anti-V5 monoclonal antibody.

Western Blot Analysis—For distribution analysis, mouse brain S2 fractions were prepared as described previously (19). The S2 fractions (40 µg) suspended in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.005% bromophenol blue, 5% 2-mercaptoethanol) were subjected to 12% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Bio-Rad). For immunoprecipitation assay, whole cell lysates were prepared by dissolving in 100 µl of SDS sample buffer. The whole cell lysates (10 µl) were subjected to Tris-Tricine SDS-PAGE (16.5% T, 3% C) and then transferred to the polyvinylidene fluoride membrane. After the blots had been blocked with 10% dried milk in PBS containing 0.1% Tween 20 (PBS-T), they were incubated with specific antibodies for the target proteins. After incubation with HRP-conjugated secondary antibody, specific signals were detected by the enhanced chemiluminescence (ECL) system (BD Biosciences).

Immunoprecipitation Assay—For in vitro immunoprecipitation assay, at 48 h after transfection using Lipofectamine 2000, NG108-15 or COS7 cells were dissolved in the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) containing 0.1% protease inhibitor mixture. Cell extracts were incubated with 0.1 volume of anti-FLAG M2 affinity gel or anti-c-Myc affinity gel overnight at 4 °C. After washing five times with TBS buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), gels were suspended in SDS sample buffer and subjected to Western blotting. For in vivo immunoprecipitation assay, mouse whole brains were homogenized with a 10-fold volume of lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, and 0.1% protease inhibitor). After centrifugation at 100,000 × g for 1 h, the supernatant (250 µg/ml) was incubated with rabbit anti-Arl6ip1 serum or rabbit preimmune serum for overnight at 4 °C. The supernatant was further incubated with protein A-Sepharose for 1–2 h at 4 °C. After rinsing five times with the lysis buffer, the protein A-Sepharose was suspended in SDS sample buffer and then subjected to Western blotting.

Glycerol Gradient Centrifugation Analysis—Glycerol gradient centrifugation analysis was carried out as described previously (26). NG108-15 cells were co-transfected with pcDNA-Arl6ip1-FLAG and pcDNA-addicsin-myc using Lipofectamine 2000. At 24 h after transfection, cells were dissolved in RIPA buffer containing 0.1% protease inhibitor. 1 ml of cell lysate was layered onto 10 ml of a 10–40% linear glycerol gradient containing 10 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol. After centrifugation for 24 h at 130,000 × g in a Hitachi RPS40T rotor at 4 °C, 0.5-ml fractions were collected from the bottom of each centrifugation tube. Samples were analyzed by Western blotting using anti-FLAG M2 monoclonal antibody and anti-c-Myc monoclonal antibody.

Glutamate Transport Assay—The glutamate transport assay was performed as described previously (27). Briefly, C6BU-1-pSw-Arl6ip1, C6BU-1-pSw-addicsin, or C6BU-1-pSw-addicsinY110A/L112A cells were cultured on the PRIMARIA 24-well plate (BD Biosciences). After exposure to vehicle (2.2 mM ethanol) or 10 mM Mifepristone for 24 h at 37 °C, cells were further incubated in 10% FBS-DMEM supplemented with vehicle (1.3 mM DMSO) or 100 nm phorbol esters for 30 min at 37 °C. Cells were rinsed three times with 500 µl of sodium- or choline-containing buffer maintained at 37 °C (5 mM Tris-HCl, 10 mM HEPES, 2.5 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 1.2 mM K2HPO4, 10 mM glucose, 140 mM NaCl, or choline chloride) before incubation with 0.5 µM 1-[3H]glutamate (2.5 µCi/ml) (GE Healthcare) and 30 µM unlabeled glutamate for 5 min at 37 °C in a final volume of 400 µl/well. The glutamate uptake was terminated using triple washing with 500 µl of ice-cold choline-containing buffer. After cells were solubilized in 400 µl/well of 0.1 N NaOH, the radioactivity of 360 µl/well lysates was analyzed using an LS6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA). Moreover, a 20-µl of aliquot was used to determine its protein concentration using a DC protein assay kit (Bio-Rad). Five independent glutamate uptake experiments were performed in quintuplicate. For the saturation analysis, L-glutamate concentrations ranged from 10 to 500 µM. Kinetic parameters were determined using the Eadie-Hofstee equation. In the knockdown experiments, cells were grown in 12-well plates and transiently transfected with each double-stranded siRNA (60 pmol/well) for 24 h by using Lipofectamine 2000. After transfection, the cells were treated with 1.3 mM DMSO or 100 nM PMA for 30 min at 37 °C and then subjected to glutamate transport assay. The sense sequence for the siRNA control was 5'-AGCAGGGUGUUUUUCGAAATdT-3'. The sense sequence for siRNA 1 was 5'-UUCUCCGAACGUGUCAC-
GUDtD'T-3'. The sense sequence for siRNA 2 was 5'-UUUG-CAAGAAACACCUCGUdGdG-3'. The siRNA 1 and siRNA 2 knocked down about 50 and 15%, respectively, of the total addicsin mRNA.

**Biotinylation Assay**—Biotinylation assay was performed as described previously (17). In brief, C6BU-1-pSw-Arl6ip1, C6BU-1-pSw-addicsin, and C6BU-1-pSw-addicsin Y110A/L112A cells were cultured in 10% FBS-DMEM containing 100 μg/ml Hygromycin and 250 μg/ml Zeocin on 35-mm culture dishes (BD Biosciences). Cells were washed three times with phosphate-buffered saline containing Ca2+ and Mg2+ (PBS-Ca2+/-Mg2+; 138 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 9.6 mM Na2HPO4, 1 mM MgCl2, and 0.1 mM CaCl2, pH 7.3). The cells were then reacted with 0.5 mg/ml EZ-Link sulfo-NHS-biotin in PBS-Ca2+/-Mg2+ at 4°C for 20 min. Cells were incubated with 100 mM glycine in PBS-Ca2+/-Mg2+ at 4°C for 20 min to remove nonreacted biotin and dissolved in 250 μl RIPA buffer containing 0.1% protease inhibitor. Cell extracts were centrifuged at 16,500 g for 30 min at 4°C to remove insoluble materials and a 125-μl aliquot of total lysate fraction was obtained. The total lysate fraction was further incubated with equal volume of 50% slurry of avidin beads for 1 h and centrifuged at 16,500 g for 10 min at 4°C to recover the intracellular lysate fraction. The avidin beads were washed six times with 250 μl of RIPA buffer and were subjected to 65 μl of SDS sample buffer to elute the absorbed proteins while shaking for 30 min. All preserved samples were analyzed by Western blotting.

**Immunohistochemical Analysis**—For distribution analysis of Arl6ip1 and addicsin in vivo, fresh frozen coronal sections 16 μm thick were prepared from mouse adult brain (6-week-old male ddY mouse, Bregma +4.2, −1.8 or −5.3 mm). The sections were stained using the Vectastain®Elite ABC kit (Vector, Burlingame, CA) according to the manufacturer's protocol. In brief, the sections were fixed with acetone/methanol solution (w/w, 1:1) for 15 min and blocked with 1.5% normal goat serum for 20 min. They were then exposed to the rabbit anti-mouse Arl6ip1 IgG polyclonal antibody or rabbit anti-addicsin IgG overnight at 4°C. The sections were then incubated with biotinylated goat anti-rabbit IgG secondary antibody for 30 min and streptavidin-conjugated HRP complex for 30 min. Signals were detected using the 3,3'-diaminobenzidine solution. Images were acquired using a BX21 light microscope (Olympus, Tokyo, Japan). For immunostaining of C6BU-1-pSw-Arl6ip1 and C6BU-1-pSw-addicsin cells, cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.25% Triton X-100 in PBS containing 1% normal goat serum for 5 min. After blocking with 1% normal goat serum for 1 h, the cells were reacted with anti-V5 monoclonal antibody overnight at 4°C and then exposed to Alexa Fluor 488 goat anti-mouse IgG secondary for 1 h. After the cells had been covered in Gel/Mount (Biomedica, Foster City, CA), images were acquired using a Fluoview FV1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan).

**RESULTS**

**Identification of Arl6ip1 as an Addicsin-associated Factor**—To identify potential factors associated with mouse addicsin, a yeast two-hybrid screen was carried out using a full length of addicsin cDNA as bait. From a prey cDNA library prepared from the amygdala of repeatedly morphine-administered mice, 49 positive clones were obtained. Of these positive clones, two clones clearly displayed α-galactosidase activity. These two clones yielded an identical cDNA sequence encoding mouse Arl6ip1 corresponding to its 1–59 amino acids (GenBank™ accession number AF223953) (data not shown). The amino acid sequence analysis revealed that addicsin had no homology with Arl6ip1. However, the hydrophobic profile of addicsin was almost the same as that of Arl6ip1 (data not shown). Reconfirmation testing using a full length of mouse Arl6ip1 as prey or bait revealed specific interactions with addicsin (data not shown). Furthermore, to test this interaction with addicsin and Arl6ip1 in vitro, we prepared crude cell lysates from NG108-15 cells in which the FLAG-tagged Arl6ip1 (Arl6ip1-FLAG) and Myc-tagged addicsin (addicsin-myc) were expressed. Immunoprecipitation assay using these cell lysates demonstrated that Arl6ip1-FLAG specifically interacted with addicsin-myc in the cell extracts prepared from co-expression cells (Fig. 1A, lanes 4 and 8), but not from Arl6ip1-FLAG (lanes 2 and 6) or addicsin-myc single-expression cells (lanes 3 and 7). No proteins were immunoprecipitated with either anti-FLAG or anti-c-Myc antibodies in sham NG108-15 cell extracts (Fig. 1A, lanes 1 and 5), suggesting that there is no specific binding to immunobeads and no cross-reaction of antibodies. Glycerol gradient centrifugation analysis revealed that the elution profile of Arl6ip1-FLAG was very similar to that of addicsin-myc (Fig. 1B). The elution peaks of both proteins were observed at fraction 18 with a deduced molecular mass of 24 kDa (Fig. 1B). In addition, the elution peak of addicsin homodimer was detected in the fractions corresponding to average molecular mass of 44 kDa (Fig. 1B, lower panel). These observations support the proposition that addicsin can form both addicsin-Arl6ip1 heterodimer and addicsin-addicsin homodimer in vivo. Furthermore, to test their interactions in vivo, an antiserum was generated using synthetic peptides corresponding to amino acids 185–199 of mouse Arl6ip1. The resultant antiserum specifically recognized an ~24-kDa single band in crude mouse whole brain lysates, consistent with the calculated molecular weight of Arl6ip1 (Fig. 1C, left panel, I), whereas the preimmune serum completely failed to react to the single band (Fig. 1C, left panel, P). The in vivo immunoprecipitation assay using mouse matured whole brain crude lysates demonstrated that addicsin was immunoprecipitated with anti-Arl6ip1 antibody but not preimmune antibody (Fig. 1C, right panel). These results suggest that addicsin interacts with Arl6ip1 in the mouse matured CNS.

**Determination of the Arl6ip1-binding Region on Addicsin**—To determine the region of addicsin responsible for binding to Arl6ip1, we carried out immunoprecipitation assays using several addicsin truncations. Arl6ip1 was able to associate with a full length of addicsin (wild type), a deletion mutant lacking the C-terminal region at amino acids 145–188 (d1), a truncation of the N-terminal domain at amino acids 1–102 (d2), and a mutant deleting the region containing the C-terminal PKC phosphorylation motif at amino acids 136–144 (d3) (Fig. 2A and B). However, Arl6ip1 failed to interact with a mutant lacking a portion of the second hydrophobic region at amino acids 103–117 of addicsin (d4) (Fig. 2A and B). As in the results for
Arl6ip1, addicsin was able to interact with the wild type, d1, d2, and d3 mutant but not the d4 truncation (Fig. 2B). On the other hand, EAAC1 interacted with all the mutants (Fig. 2A). These results thus suggest that the hydrophobic region at amino acids 103–117 of addicsin is crucial to the homo- and hetero-multimerization of addicsin (Fig. 2C).

Distribution of Arl6ip1 mRNA and Protein in Vivo—To investigate the tissue distribution of Arl6ip1 transcript and protein using RT-PCR, we performed Western blotting and immunohistochemical analysis. RT-PCR analysis showed that Arl6ip1 mRNA was found in both the neuronal and non-neuronal tissues tested (Fig. 3A). Moreover, Arl6ip1 mRNA was co-expressed with addicsin mRNA in all the tissues examined (Fig. 3A). Western blot analysis demonstrated that Arl6ip1 was broadly expressed in various brain regions consistent with the ubiquitous localization of addicsin in matured CNS (Fig. 3B). Furthermore, immunohistochemical analysis revealed that Arl6ip1 was widely localized in matured brain and expressed in neuron-like cells (Fig. 3C, panels a, c, and e). For instance, in the olfactory bulb, Arl6ip1 immunoreactivity (IR) was detected in the mitral cell layer, glomerular layer, and granular layer (Fig. 3C, panel d). In the hippocampal formation, Arl6ip1 IR was observed in the granular layer of the dentate gyrus and stratum pyramidale of the CA1–3 fields (Fig. 3C, panel e). Arl6ip1 IR was also detected in the molecular layer, Purkinje cell layer, and granular cell layers of the cerebellum (Fig. 3C, panel f). Consistent with these results, addicsin IR also showed the same localization pattern as Arl6ip1 IR in these brain regions tested (Fig. 3C, panels b, d, and f), strongly suggesting that Arl6ip1 is co-localized with addicsin in the matured CNS.

Positive Modulation of EAAC1-mediated Glutamate Transport Activity by Arl6ip1—To investigate the effect of Arl6ip1 on EAAC1-mediated glutamate transport activity, we established stable C6BU-1 cell lines, designated C6BU-1-pSw-addicsin and C6BU-1-p5Sw-Arl6ip1, respectively. In these cell lines we were able to induce the V5-tagged addicsin (addicsin-V5) or
FIGURE 3. Distribution of Arl6ip1 in vivo. A expression of Arl6ip1 and addic- sin mRNA in various mouse tissues. Mouse Arl6ip1 and addiccin mRNAs pre- pared from various mouse tissues were analyzed by RT-PCR. The mouse glyc- eraldehyde-3-phosphate dehydrogenase (GAPDH) was detected as a positive control. B, expression of Arl6ip1 and addiccin in various mature brain regions. The supernatants (S2 fraction) prepared from various mature brain regions were resolved by SDS-PAGE, transferred to a polyvinylidene fluoride mem- brane, and immunoblotted with anti-Arl6ip1 antibody, anti-addiccin antibi- body, and anti-actin antibody, respectively. C, localization of Arl6ip1 and addiccin in mature mouse brain. Mouse coronal brain sections were sepa- rately labeled with the anti-Arl6ip1 antibody and anti-addiccin antibody. The typical staining patterns in the olfactory bulbs (panels a and b), hippocampus (panels c and d), and cerebellum (panels e and f) are presented. Neuron-like cells were ubiquitously immunostained with both antibodies. Scale bars cor- respond to 500 μm in panels a–d and 50 μm in panels e and f. Abbreviations used are as follows: CA1, hippocampus CA1 field; CA3, hippocampus CA3 field; DG, dentate gyrus; G, granular cell layer of the cerebellum; Gl, glo- merular layer of the olfactory bulb; Gro, granule layer of the olfactory bulb; M, molecular layer of the cerebellum; Mi, mitral cell layer of the olfactory bulb; P, Pur- kinje cell layer of the cerebellum. Cx, cerebral cortex; Cb, cerebellum; Hip, hippocampus; Ob, olfactory bulbs; Md, medulla oblongata; Ls, limbic system.

V5-tagged Arl6ip1 (Arl6ip1-V5) in a closely mifepristone-de- pending manner (Fig. 4, A and B). Next, to evaluate the effect of increasing expression of Arl6ip1-FLAG or addiccin-myc on EAAC1-mediated glutamate transport activity, we measured the EAAC1-mediated glutamate transport ability of C6BU-1- pSw-addiccin and C6BU-1-pSw-Arl6ip1 under various conditions. Compared with the control, the EAAC1-mediated glutamate transport activity was unchanged by up-regulation of addiccin-V5 or Arl6ip1-V5 (Fig. 4, A, lanes 1 and 2, and B, lanes 1 and 2). However, when these cells were stimulated with 100 nM PMA, the glutamate uptake activity in C6BU-1-pSw-addic- cin fell steeply with increasing expression of addiccin-V5 (Fig.

FIGURE 4. Positive modulation of EAAC1-mediated glutamate transport by Arl6ip1 in C6BU-1 cells. A glutamate transport activity in C6BU-1-pSw-addiccin cells. Glutamate transport assay was performed on C6BU-1-pSw-addiccin cells under the indicated conditions. Addiccin inhibited EAAC1-mediated glutamate transport activity in a PKC-dependent manner. Data are means ± S.E., n = 5; *, p < 0.05; **, p < 0.01, N.S., no significance. B, glutamate uptake activity in C6BU-1-pSw-Arl6ip1 cells. Glutamate transport assay was performed on C6BU-1-pSw-Arl6ip1 cells under the indicated conditions. Arl6ip1 altered EAAC1-mediated glutamate transport activity in a PKC-dependent manner. Data are means ± S.E., n = 5; **, p < 0.01, N.S., no significance. C, effect of decreased expression of addiccin on glutamate transport activity. Under PMA treatment, addiccin-specific siRNAs altered EAAC1-mediated glutamate transport activity. Data are mean ± S.E., n = 12 for siRNA 1 and n = 8 for siRNA 2; *, p < 0.05; **, p < 0.01; N.S., no significance. D, kinetic analysis of C6BU-1-pSw-Arl6ip1 cells. Both affinity for glutamate (Km) and maximum velocity (Vmax) were calculated using the Eadie-Hofstee equation based on EAAC1-mediated glutamate transport ranging from 10 to 500 μM of L-glutamate (n = 6). When cells conditionally expressed Arl6ip1, kinetic analysis of the cells treated with 100 nM PMA showed an increase in apparent affinity for glutamate without a shift in velocity as compared with cells treated with 1.3 mM DMSO.
4A, lanes 3 and 4). By contrast, the glutamate transport activity in C6BU-1-pSw-Arl6ip1 significantly increased with up-regulation of Arl6ip1-V5 (Fig. 4B, lanes 3 and 4). When these cells were exposed to 100 nm 4α-phorbol, a nonstimulating analogue of PMA, glutamate uptake activity in both cells remained at basal levels under all the conditions tested (Fig. 4, A, lanes 5 and 6, B, lanes 5 and 6). Furthermore, cell viability assay demonstrated that neither cell line displayed any cytotoxicity because of up-regulation of addicsin-V5 or Arl6ip1-V5 (data not shown). Thus, these data strongly support the proposition that Arl6ip1 acts as a positive modulator of EAAC1-mediated glutamate transport in a PKC activity-dependent manner. Furthermore, to investigate the effect of decreased addicsin expression on glutamate uptake activity, we performed a knockdown experiment by transient transfection of double-stranded siRNAs into C6BU-1-pSw-Arl6ip1. Cells treated with addicsin-specific siRNAs had about a 200% increase in glutamate transport activity compared with cells treated with control siRNA (Fig. 4C). Under DMSO treatment, cells transfected with siRNA 1 showed an increasing trend in basal glutamate transport activity (Fig. 4C). These data strongly support the results in Fig. 4B. Next, to evaluate the biochemical nature of altered EAAC1-mediated glutamate uptake, the kinetics of C6BU-1-pSw-Arl6ip1 was analyzed. When Arl6ip1-V5 was conditionally expressed, the PMA-treated cells showed an increase in affinity without any shift in maximal velocity ($K_m = 647 \mu M; V_{max} = 1.5 \times 10^4 \text{ pmol/mg/min}$) as compared with DMSO-treated cells ($K_m = 824 \mu M; V_{max} = 1.5 \times 10^3 \text{ pmol/mg/min}$) (Fig. 4D). These data indicate that Arl6ip1 alters glutamate transport activity by increasing the catalytic efficiency of EAAC1.

Analysis of Subcellular Localization of Arl6ip1 in C6BU-1 Cells—To examine the molecular mechanism of the altered glutamate transport activity in C6BU-1-pSw-Arl6ip1, the subcellular localization and protein-protein interaction between EAAC1 and Arl6ip1 was analyzed. Western blot analysis revealed that Arl6ip1-V5 levels were unchanged by PMA treatment (Fig. 5A). Furthermore, immunohistochemical analysis demonstrated that Arl6ip1-V5 was localized in cytoplasmic structures and showed no changes in localization pattern as a result of 100 nm PMA treatment (Fig. 5B, panels a and b). Cell surface biotinyl assay indicated that Arl6ip1 had no association with any molecules on the cell surfaces under any of the conditions tested (Fig. 5C). Moreover, immunoprecipitation assay indicated that Arl6ip1 failed to interact with EAAC1, whereas addicsin could associate with EAAC1 (data not shown). These results suggest that Arl6ip1 is localized in intracellular compartments and has no interaction with EAAC1.

On the basis of these results, we arrived at the hypothesis that Arl6ip1 might regulate the amount of addicsin homodimer by formation of Arl6ip1-addicsin heterodimer on the cytoplasmic structure (Fig. 7). To test this idea, we searched for an addicsin mutant that lacked interaction with Arl6ip1 but not with addicsin itself. Finally, we noticed two amino acids at positions 110 and 112 of addicsin. These amino acids are located in the addicsin and Arl6ip1 association region (Fig. 2C) and are completely conserved among species from Drosophila to humans (Fig. 6A). Immunoprecipitation assay demonstrated that a double-mutated form of addicsin, designated addicsin Y110A/L112A (addicsin YL), had markedly less binding activity to Arl6ip1 (39.5 ± 5.5% of wild-type addicsin), although it had the same binding activity to addicsin itself (113.9 ± 9.6% of wild-type addicsin) (Fig. 6B). Furthermore, to investigate the effect of addicsin YL on EAAC1-mediated glutamate transport activity, we established the C6BU-1-pSw-addicsin YL cell line that exclusively up-regulated the V5-tagged addicsin YL (addicsin YL-V5) in a mifepristone-dependent manner (Fig. 6D, inset). Cell surface biotinylation assay indicated that wild-type addicsin had a strong association with some molecules on the cell surface (Fig. 6C). However, in this cell line, addicsin YL had markedly less association with any molecules on the cell surfaces under all of the conditions tested (Fig. 6C), suggesting that addicsin YL had lost its binding ability to EAAC1. Interestingly, when cells were stimulated with 100 nm PMA, the glutamate uptake activity in C6BU-1-pSw-addicsin YL remained unchanged with increasing expression of addicsin YL-V5 (Fig. 6D, lanes 3 and 4). By contrast, glutamate uptake activity was sustained at basal levels under all the conditions tested when the
**FIGURE 6. Effect of binding ability of addicsin to Arl6ip1 on EAAC1-mediated glutamate transport activity.** A, comparison of amino acid sequences in the second hydrophobic region of addicsin from different species. Accession numbers are human AB052638, mouse AB240182, zebrafish AB052637, and *Drosophila* AB052636. The asterisk indicates an identical amino acid from *Drosophila* to humans. B, binding activity of the addicsinY110A/L112A (addicsin WT) or addicsin itself (addicsin YL) mutant to the wild type of Arl6ip1 or addicsin. To evaluate the binding ability of addicsin YL to Arl6ip1 (WT/YL) or Arl6ip1-FLAG, addicsin-YL/myc (WT/FLAG) and addicsin-FLAG/myc complexes were immunoprecipitated (IP) with an anti-FLAG antibody and analyzed by Western blotting. C, cell surface biotinylated addicsin and Actin were co-immunoprecipitated and analyzed by Western blotting. D, comparison of activities of glutamate transporters in the presence of Mifepristone (100 nM) or vehicle (DMSO). Data are means ± S.E., n = 5. *N.S. cells were stimulated with DMSO (Fig. 6D, lanes 1 and 2) or 100 nM 4α-phorbol (Fig. 6D, lanes 5 and 6). These data suggest that the interaction of Arl6ip1 and addicsin may be an important element in the expression of enhanced EAAC1-mediated glutamate transport activity in a PKC activity-dependent manner.

**DISCUSSION**

Arl6ip1 was first identified as an ADP-ribosylation factor-like 6 (ARL-6)-association factor by yeast two-hybrid screening (28). Arl6ip1 was also isolated as a down-regulatory factor during myeloid differentiation by differential display (29). However, the biological functions of Arl6ip1 have not been fully elucidated. Amino acid analysis demonstrates that Arl6ip1 has two putative casein kinase II phosphorylation motifs (amino acids 18–21 and 128–131) and protein kinase C phosphorylation motifs (amino acids 94–96, 115–117, and 128–130) (data not shown). Arl6ip1 also contains an N-glycosylation motif (amino acid 6–9), a prenyl group-binding motif (amino acids 72–75), and an endoplasmic reticulum retention signal (amino acids 200–203). Consistent with these data, Arl6ip1 appeared to be localized in subcellular compartments, including the endoplasmic reticulum in *C6BU*-1 cells, according to our immunohistochemical analysis (Fig. 5B). Furthermore, ARL-6, an Arl6ip1-associated partner, belongs to the Ras superfamily of small GTP-binding proteins and is essential for membrane-associated intracellular trafficking processes (30). In agreement with this information, Arl6ip1 is structurally similar to addicsin (data not shown), which is thought to be localized in intracellular compartments that are implicated in intracellular trafficking events. Thus, this evidence strongly suggests that Arl6ip1, like addicsin, may be implicated in trafficking events, including protein transport, membrane trafficking, and cell signaling.

The *C6BU*-1 glioma cell is a suitable model for analyzing the molecular mechanisms of EAAC1-mediated glutamate transport, because only EAAC1 is endogenously expressed as a glutamate transporter (31). The glutamate transport activity of EAAC1 can be explained by either changing the number of transporters expressed on the plasma membrane or by changing the catalytic efficiency of the transporter located on the plasma membrane. In *C6BU*-1-pSw-Arl6ip1 cells, PMA stimulation elicited the acceleration of glutamate transport activity with an increase in affinity but not in maximal velocity once cells expressed Arl6ip1-V5 (Fig. 4D). These data indicate that Arl6ip1 alters the catalytic efficiency of EAAC1 located on the plasma membrane. In *C6BU*-1-pSw-Arl6ip1 cells, Arl6ip1 could not directly interact with EAAC1 on the plasma membrane and stayed in the subcellular compartment as a result of myeloid differentiation by differential display (29). However, the biological functions of Arl6ip1 have not been fully elucidated.

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PKC might increase in addicsin-Arl6ip1 interaction, thereby decreasing the number of addicsin molecules available for interaction with EAAC1. The resulting decrease in the addicsin-EAAC1 complex causes a change in the catalytic efficiency of EAAC1.

The glutamate transport activity changes in the presence of PMA treatment in our experiments (Fig. 4, A and B), suggesting that PKC activation is essential for the dynamics of EAAC1-mediated glutamate transport. At present, this precise molecular mechanism remains unclear. However, EAAC1, addicsin, and Arl6ip1 themselves have putative PKC phosphorylation motifs (19, 29, 32). Thus, these molecules may be phosphorylated by PKC and modulate the function of EAAC1. Interestingly, the accumulated evidence reveals that PKCα regulates the redistribution of EAAC1 from the intracellular compartment to the plasma membrane and that PKCε controls EAAC1-mediated glutamate transport by a trafficking-independent mechanism in C6BU-1 cells (33). Thus, in C6BU-1-pSw-Arl6ip1 cells, PKCe may be involved in the enhancement of glutamate transport induced by increasing expression of Arl6ip1. However, further studies will be needed to elucidate the PKC-dependent modulation of EAAC1.

In summary, our biochemical and histological analyses show that Arl6ip1 can specifically interact with addicsin both in vitro and in vivo and bind a small portion of the hydrophobic region of addicsin, which is located at its amino acids 103–117. Furthermore, Arl6ip1 positively modulated EAAC1-mediated glutamate transport by increasing its affinity for glutamate, without any shift in maximal velocity, in a PKC activity-dependent manner. Arl6ip1 was localized in subcellular compartments and had no interacting ability to EAAC1 on cell surfaces in C6BU-1 cells. It is thus possible that Arl6ip1 is an addicsin-associated partner in vivo, and that Arl6ip1 promotes the catalytic efficiency of EAAC1 by reducing the interaction of addicsin with EAAC1 in a PKC activity-dependent manner.

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