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Prickle2 is localized in the postsynaptic density and interacts with PSD-95 and NMDA receptors in the brain

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The planar cell polarity (PCP) protein, Prickle (Pk), is conserved in invertebrates and vertebrates, and regulates cellular morphogenesis and movement. Vertebrate Pk consists of at least two family members, Pk1 and Pk2, both of which are expressed in the brain; however, their localization and function at synapses remain elusive. Here, we show that Pk2 is expressed mainly in the adult brain and is tightly associated with the postsynaptic density (PSD) fraction obtained by subcellular fractionation. In primary cultured rat hippocampal neurons, Pk2 is colocalized with PSD-95 and synaptophysin at synapses. Moreover, immunoelectron microcopy shows that Pk2 is localized at the PSD of asymmetric synapses in the hippocampal CA1 region. Biochemical assays identified that Pk2 forms a complex with PSD proteins including PSD-95 and NMDA receptor subunits via the direct binding to the C-terminal guanylate kinase domain of PSD-95. These results indicate that Pk2 is a novel PSD protein that interacts with PSD-95 and NMDA receptors through complex formations in the brain.

Keywords: NMDA receptor/planar cell polarity/Prickle/PSD-95/synapse.

Abbreviations: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; APMSF, *p*-amidinophenyl methansulphonyl fluoride; CNS, central nervous system; Dsh, *Drosophila* Dishevelled; EPSC, excitatory postsynaptic current; Fmi, *Drosophila* flamingo; Fz, *Drosophila* frizzled; GK, guanylate kinase; KO, knockout; MAGUK, membrane-associated guanylate kinase; NMDA, *N*-methyl D-aspartate; PCP, planar cell polarity; Pk, Prickle; PSD, postsynaptic density.

Planar cell polarity (PCP) is a fundamental property of cells required for asymmetric cell morphology, membrane trafficking and macromolecular distribution (*1–3*). PCP proteins were first discovered in *Drosophila melanogaster*, where they were shown to regulate the precise arrangement of hairs and bristle on the surface of the wing, eye and epidermis (*4*). From genetic and molecular studies, the core PCP genes, including *flamingo* (*Fmi*), *frizzled* (*Fz*), *dishevelled* (*Dsh*) and *prickle* (*Pk*), have been shown to establish polarization of cell morphology by the asymmetric localization of these proteins (*4–6*). Subsequent studies have established that the core PCP proteins are conserved in vertebrates as well as invertebrates, and globally function as regulators of cell morphology and behaviour (*4–6*).

In the central nervous system, a wide variety of PCP proteins are expressed during developmental processes (*7*), and studies of mutant mice confirm the role of the PCP pathway in neural tube closure and neuronal migration (*6*). However, PCP proteins are not only highly expressed in the immature brain, but also in the mature adult brain, and accumulating evidence suggests a broader role for the PCP pathway in neuronal polarity, axon guidance, dendritic morphogenesis and synaptic plasticity (*6–9*). For example, Fz3- (an orthologue of *Drosophila* Fz) and Celsr3- (an orthologue of *Drosophila* Fmi) deficient mice showed defects in axon fibers (*9*, *10*). In addition, the apicobasal polarity protein Scribble1, a component of the PCP pathway, has recently been shown to be localized in dendritic spines and to play a crucial role in spine morphology (*8*). Indeed, heterozygous mutant mice of Scribble1 exhibit enhanced learning and impaired social behaviour, indicating the contribution of Scribble1 to neuronal plasticity (*8*). These findings suggest that PCP proteins play a pivotal role, not only in early neural formation, but also in formation, maintenance and function of complex neural networks in mature neurons. However, little is known about the expression and exact localization of PCP proteins at the synapse level.

To further investigate the role of PCP proteins during brain function, we focused on Pk2, a mouse homolog of *Drosophila* Pk. We first cloned Pk1 and Pk2 from a mouse brain cDNA library, and raised a polyclonal antibody specific to Pk2. We found that Pk2 was concentrated in the PSD fraction, using subcellular fractionation analysis, and that it localized to the PSD of asymmetric synapses in the adult mouse brain using post-embedding electron microscopy. Biochemical analysis showed that the PSD proteins, such as PSD-95 and NMDA receptor subunits were co-immunoprecipitated with Pk from brain fractions, and that Pk2 directly bound to the GK domain of

PSD-95. These results indicate that Pk2 is a novel PSD protein that forms protein complexes with PSD-95 and NMDA receptors in the brain.

Experimental Procedures

Molecular cloning of Pk cDNAs

Total RNA from the hippocampus and amygdaloid corpus of 10-week-old male mice was extracted using QIAzol lysis reagent (QIAGEN). The total RNA extract was reverse transcribed using random primer (nonamer) and AMV reverse transcriptase XL (TaKaRa) according to the manufacturer's instructions. The full-length fragments of Pk1 and 2 were cloned from cDNA by PCR with oligonucleotides as follows: Pk1-forward, 5′-GAGACG CGTATGCCTTTGGAGATGGAACCG-3′; Pk1-reverse, 5′-GAG ACGCGTTTAAGAAATGATACAGTTTTT-3′; Pk2-forward, 5′-GAGGAATTCATGGTAACAGTGATGCCGCTG-3′; Pk2-reverse, 5′-GAGGTCGACTTACGAAATGATACAGTTTTT-3′; These primers included a MluI restriction site in the Pk1-forward and -reverse primers, a EcoRI restriction site in the Pk2-forward primer and a SalI restriction site in the Pk2-reverse primer. PCR products were subcloned in pCIneo-Myc and pCAEGFP (*11*) for transfection.

Construction of PSD-95

The full-length cDNA of rat PSD-95 was kindly provided by Dr S. Okabe and subcloned in pBluescript II-KS (Stratagene) to use as the template for PCR to prepare various constructs. Expression vectors were constructed in pGEX (GE healthcare) and pCIneo-Myc vectors by standard molecular biological methods. The constructs of PSD-95 contained the following amino acid residues: PSD-95-SH3 (amino acid 394–533), PSD-95-SH3-GK (amino acid 412–724), PSD-95-GK (amino acid 499-stop) and Myc-PSD-95 (amino acid 1-stop).

Expression of Pk2 in HEK293 cells

HEK293 cells in 6-cm dishes were transfected with the indicated expression vectors using LipofectAMINE 2000 (Invitrogen). After transfection (48 h), cells were collected and protein was extracted using 400 µl of lysis buffer [20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% (w/v) Triton X-100, 10 µg/ml leupeptin, 10 µM APMSF] at 4°C for 1 h. The samples were centrifuged at 20,000*g* at 4°C for 20 min to collect the supernatant and pellet fraction.

Subcellular fractionation

To obtain the P2 and PSD fractions, subcellular fractionation of rat brain was prepared as described previously (*12*, *13*). In brief, brain homogenates were centrifuged at 1,400*g*. The supernatant (S1) was centrifuged at 12,000*g*, and the resultant pellet was washed once. The washed pellet (P2 fraction) was further purified using a set of sucrose density gradients consisting of 0.85, 1.0 and 1.2 M sucrose, and centrifugation at 82,500*g*. The band between the 1.0 and 1.2 M sucrose gradients was collected and diluted four times and centrifuged at 32,800*g*. The synaptosome fraction was lysed by stirring in 6 mM Tris–Cl (pH 8.1). The synaptosome membrane fraction was centrifuged at 48,200*g* and then purified using a second set of sucrose density gradients prepared as described above. The band between the 1.0 and 1.2 M sucrose gradient was collected and used as the SM3 fraction. The SM3 fraction contained pre- and postsynaptic membranes, including the PSD and docked vesicles. The SM3 fraction was treated with 1% (w/v) Triton X-100 and further centrifuged at 48,200*g*. The resultant pellet was used as the PSD fraction.

Antibodies

A rabbit antiserum against Pk2 was raised against specific segments of recombinant Pk2 (amino acid 365–618) that differed from Pk1. The antiserum was affinity purified as previously described (*14*). Anti-CAST antibody (1 : 200, rabbit polyclonal) was obtained as previously described (*15*). Anti-PSD-95 (1 : 500, mouse monoclonal, Affinity Bioreagents), anti-GluN1 (1 : 500, mouse monoclonal, BD Transduction), anti-synaptophysin (1 : 2,000, mouse monoclonal, CHEMICON), anti-Myc (1 : 500, mouse monoclonal, Roche Applied Science) and anti-GFP (1 : 500, rabbit polyclonal, Invitrogen) antibodies were purchased from commercial sources.

Immunoprecipitation

Proteins were extracted from the P2 fraction with 1% (w/v) deoxycholate, followed by dilution and dialysis with a Triton X-100-based buffer as previously described (*16*). The extract (1 mg of protein) was incubated with 3 µg of the control IgG anti-Pk2 antibody or the anti-PSD-95 antibody at 4°C for 2 h. After the beads were extensively washed with lysis buffer, the bound proteins were eluted by boiling the beads in SDS sample buffer [60 mM Tris–Cl, pH 6.7, 3% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 5% (w/v) glycerol] for 5 min. The samples were then analysed by western blotting.

Pull down assay

HEK293 cells expressing EGFP-Pk2 in 10-cm dishes were lysed in 1.0 ml of lysis buffer at 4°C for 1 h. The sample was centrifuged at 20,000*g* at 4°C for 20 min to collect the supernatant. In brief, the lysate of HEK293 cells expressing each EGFP-Pk2 fusion protein was incubated with 20 µl of glutathione-sepharose beads containing GST-PSD-95-SH3, -SH3-GK or -GK at 4°C for 1 h. After the beads were extensively washed with lysis buffer, the bound proteins were eluted by boiling the beads in SDS sample buffer for 5 min. The samples were then analysed by western blotting.

Generation of Pk2 KO mice

Pk2 KO mice were generated by homologous recombination in embryonic stem (ES) cells. The open reading frame (776–1,651 bp) of Pk2 in the sixth exon was replaced by a neomysin resistance cassette. Recombinant ES cell clones were analysed by Southern blotting and positive clones were injected into mouse blastcysts to obtain highly chimeric mice that transmitted the mutation through the germ line. Germ-line transmission of the mutation was confirmed by Southern blotting and genotyping (data not shown).

Primary neuron cultures and immunostaining

Primary cultures of rat hippocampal neurons were prepared as described previously (*17*). Cells were fixed with 2% (w/v) paraformaldehyde/4% (w/v) sucrose/0.01% (w/v) saponin in PBS (pH 7.4) at room temperature for 20 min. Non-specific binding was blocked with 4% (w/v) Block Ace (Dainippon Sumitomo Pharmaceutical) containing 0.01% (w/v) saponin for 1 h. The cells were incubated with primary antibodies for 1 h, followed by secondary antibodies. Stained cells were analysed by confocal laser microscopy using a 100× oil immersion objective lens (LSM510, Carl Zeiss MicroImaging, Inc).

Immunohistochemistry and immunoelectron microscopy

Under deep pentobarbital anesthesia (100 mg/kg of body weight, intraperitoneal), mice were perfused transcardially with 4% (w/v) paraformaldehyde/0.1 M sodium phosphate buffer (PB, pH 7.2) for light microscopic immunohistochemistry or with 4% (w/v) paraformaldehyde/0.1% (v/v) glutaraldehyde/0.1 M PB for post-embedding immunogold electron microscopy. For light microscopic immunohistochemistry, paraffin sections were first subjected to pepsin pretreatment for antigen exposure, *i.e.*, incubation in 1 mg/ml of pepsin (DAKO) in 0.2 N HCl for 10 min at 37°C. Sections were immunoreacted with primary antibodies (1 µg/ml) for immunoperoxidase and immunofluorescence detection, as described previously (*18*). For staining of nuclear DNA, TOTO-3 (Molecular Probe) was added to the secondary antibody solution.

For post-embedding immunogold microscopy, microslicer sections (400 µm) were cryoprotected with 30% (w/v) sucrose in 0.1 M PB, and frozen rapidly with liquid propane in a Leica EM CPC unit. Frozen sections were immersed in 0.5% (v/v) uranyl acetate in methanol at −90°C in a Leica AFS freeze-substitution unit, infiltrated at −45°C with Lowicryl HM-20 resin (Lowi) and polymerized with UV light. After etching with saturated sodium-ethanolate solution for 3 s, ultra-thin sections on nickel grids were treated successively with 1% (v/v) human serum albumin (Wako)/0.1% (v/v) Tween-20 in Tris-buffered saline (H-TBST pH 7.5) for 1 h, primary antibodies (15 µg/ml) in H-TBST overnight and colloidal gold (10 nm)-conjugated anti-rabbit IgG (1 : 100, British Bio Cell International) in H-TBST for 2 h. Finally, grids were stained with

uranyl acetate for 15 min. Electron micrographs were taken using a H7100 electron microscope (Hitachi).

Results

Molecular cloning of Pk cDNAs and characterization of the anti-Pk2 antibody

To isolate mammalian orthologues of *Drosophila* Pk, we first searched the GenBank database and found at least two family members of Pk (Pk1 for NM_001033217, Pk2 for NM_001134459) (*19*). By screening the mouse cDNA library, we obtained the full-length cDNAs of Pk1 and Pk2. The cDNAs showed a similar domain structure to *Drosophila* Pk (Fig. 1A). Pk1 and Pk2 had a conserved domain (77% identity) consisting of a PET (for Pk, Espinas and Testin) (*19*) and three LIM (for Lin1, Islet-1, Mec-3) domains (*20*), and showed 48% identity over the entire sequence (data not shown). Although there was no particular difference in gene expression throughout various organs, mRNA of Pk2 was highly concentrated in the hippocampus (*7*, *21*). Given these data, we proceeded to analyse Pk2 in more detail.

To characterize the biochemical properties of Pk2, we first produced an antibody against Pk2 that could recognize the distinct region after the third LIM domain (Fig. 1A). To confirm the specificity of the anti-Pk2 antibody, we expressed the Pk1 and Pk2 protein in HEK293 cells. Western blot analysis indicated that the expressed protein (Myc-Pk1 and -2) showed a mobility similar to that of native Pk2 (~100 kDa) following SDS-PAGE, and that the anti-Pk2 antibody specifically detected Pk2 but not Pk1 (Fig. 1B). Moreover, the signals by the anti-Pk2 antibody were almost completely diminished in the Pk2 KO mice (Fig. 4B), indicating that this antibody specifically recognizes endogenous Pk2.

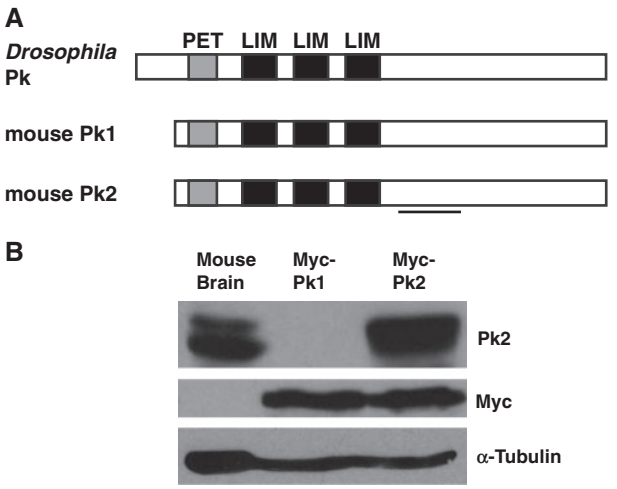

Fig. 1 Characterization of the anti-Pk2 antibody. (A) Structure of the Pk homologue. Grey box, PET domain. Black box, LIM domain. The anti-Pk2 antibody was raised against specific segments of recombinant mouse Pk2 (amino acid 365–618, underline) that differ from mouse Pk1. (B) Specificity of the anti-Pk2 antibody. The pCIneo-Myc vector containing Pk1 or Pk2 was transfected in HEK293 cells. The lysate of HEK293 cells expressing each Pk was analysed by western blotting using the anti-Pk2 antibody as well as Myc and α-Tubulin antibodies for controls. The anti-Pk2 antibody specifically detected Pk2 but not Pk1.

Tissue and subcellular distribution of Pk2

Tissue distribution analysis using the anti-Pk2 antibody showed a major protein band of ~100 kDa in the rat brain (Fig. 2A), consistent with previous gene expression analysis (*21*, *22*). As well as in the brain, it was reported that there was predominant mRNA expression in the lung (*21*). However, it was not detected in our protein analysis, presumably because the protein expression was lower than that in the brain. In addition, we noticed a protein band in the liver. Although there may be a smaller splice variant in the liver, their properties are currently unknown.

Subcellular distribution analysis in the rat brain showed that Pk2 was concentrated in the PSD fraction. The subcellular distribution pattern of Pk2 was similar to that of the PSD-95, NMDA receptor, AMPA receptors and CAST (*17*) (Fig. 2B). The synaptic vesicle protein, synaptophysin was mainly detected in the CSV fraction, but not in the PSD fraction (Fig. 2B). These results indicate that Pk2 is mainly expressed

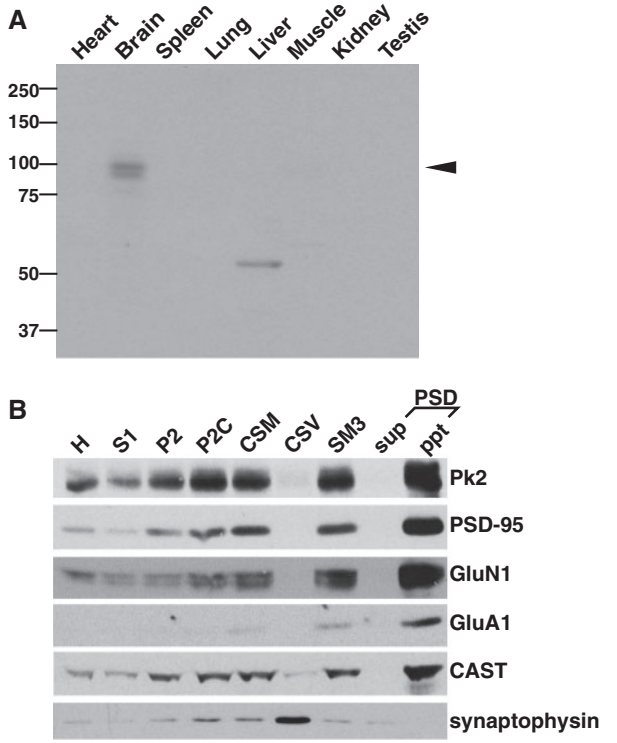

Fig. 2 Tissue and subcellular distribution of Pk2. (A) The homogenates of various rat tissue (20 μg of protein each) were analysed by western blotting using the anti-Pk2 antibody. Arrowhead indicates Pk2. (B) Subcellular distribution. The homogenates of rat brain tissue were subjected to subcellular fractionation. An aliquot of each fraction (5 μg of protein each) was analysed by western blotting with the indicated antibodies. H, homogenate; S1, crude synaptosomal fraction; P2, crude membrane fraction; P2C, synaptosomal fraction; CSM, crude synaptic membrane fraction; CSV, crude synaptic vesicle fraction; SM3, synaptic membrane; PSD, postsynaptic density fraction; sup, 1% (w/v) Triton X-100-soluble fraction of PSD; ppt, 1% (w/v) Triton X-100-insoluble fraction of PSD. PSD-95, GluN1 and GluA1 were used as PSD proteins, CAST was used as a pre-synaptic active zone protein, synaptophysin was used as synaptic vesicle protein.

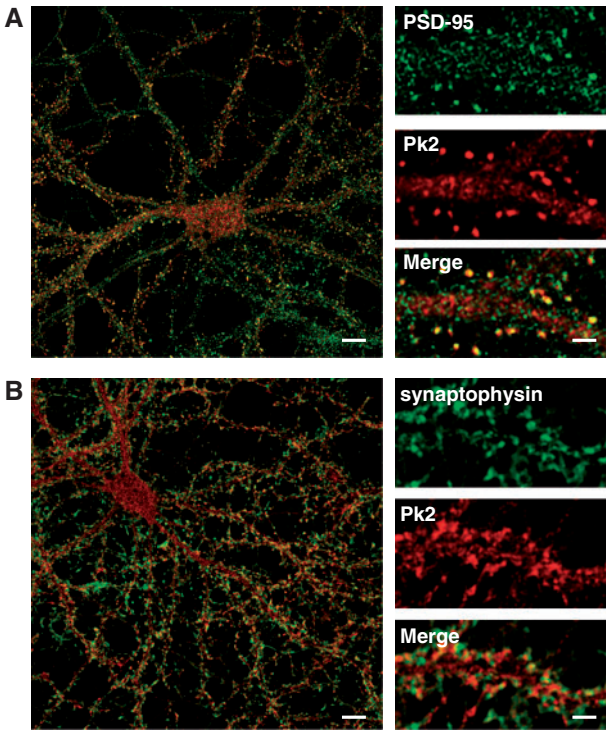

Fig. 3 Synapse specific localization of Pk2 in cultured neurons. Primary cultured rat hippocampal neurons at 21 days of culture were double labelled using the anti-Pk2, anti-PSD-95 (A) or anti-synaptophysin antibodies (B). Bars, 10 μm, 40 μm.

in the brain and tightly associates with synaptic junctions.

Localization of Pk2 in the PSD

We next examined the spatial distribution of Pk2 in primary cultured rat hippocampal neurons. Pk2 was entirely expressed in the cell soma and dendritic shaft, and the punctate signals concentrated along the dendrites were co-localizing with PSD-95 and synaptophysin (Fig. 3). We further examined the more precise localization of Pk2 in the mouse brain. The antigen-unmasking method with pepsin pretreatment was essential for immunohistochemical detection of Pk2, as was the case for NMDA receptor subunits and PSD-95 protein family proteins (*23*, *24*). With pre-treatment, immunohistochemical signals for Pk2 appeared widely in the brain, with the highest levels in the hippocampus (Fig. 4A), which was consistent with mRNA expression data (*7*). In addition, the signals were almost completely diminished in the Pk2 KO mice (Fig. 4B). In the CA1 region, immunohistochemical signals for Pk2 were punctate and densely distributed in the neuropil of the striatum oriens and radiatum, whereas expression in the perikarya and dendritic shafts were very low (Fig. 4C), indicating exclusive localization in dendritic spines, a postsynaptic protrusion forming excitatory synapses. To confirm this notion, we next performed post-embedding immunogold electron microscopy and revealed intense labeling of Pk2 in the PSD in the stratum radiatum of the CA1 region (Fig. 4D). Together with the biochemical

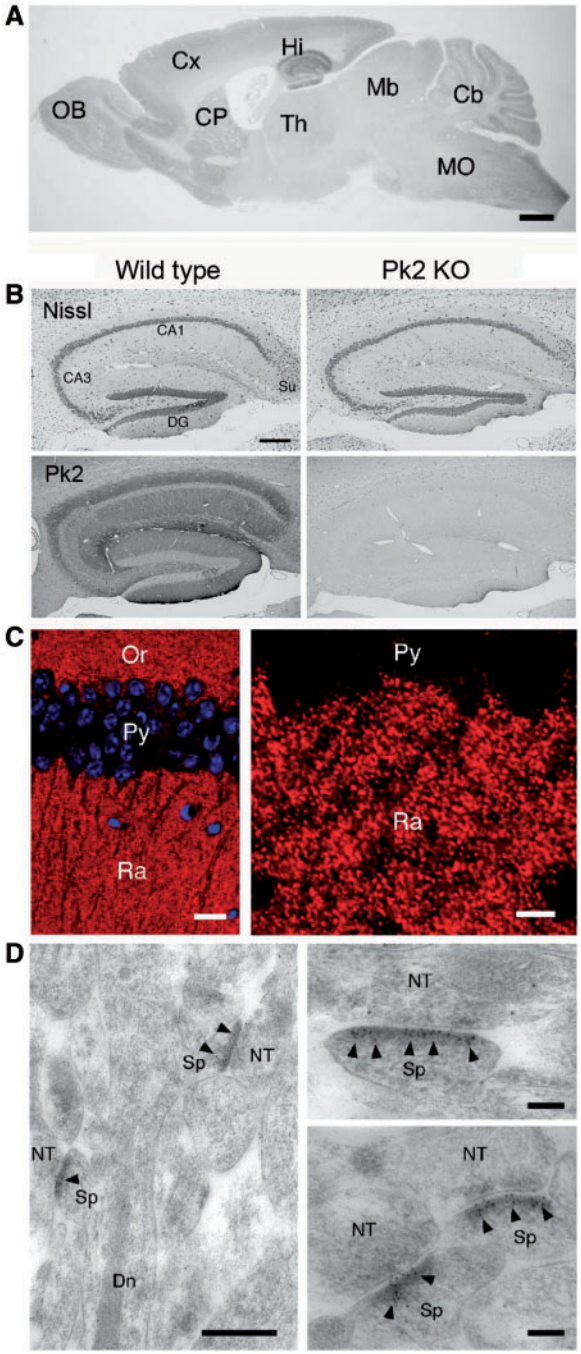

Fig. 4 Immunohistochemical distribution and postsynaptic localization of Pk2 in mouse brain. (A) Immunoperoxidase showing overall distribution of Pk2 in the adult mouse brain. (B) Immunoperoxidase showing anti-Pk2 antibody specificity at the hippocampus of wild-type and Pk2 KO mice. Nissl staining was used to detect the cell bodies of neurons. (C) Immunofluorescence showing punctate labelling for Pk2 (red) in the neuropil of the hippocampal CA1 region. Sections are counterstained for the nucleus with TOTO3 (blue). (D) Post-embedding immunogold electron microscopy. Arrowheads indicate immunogold particles in the PSD at asymmetrical axo-spinous synapses in the hippocampal CA1 region. Cb, cerebellum; CP, caudate putamen; Cx, cortex; DG, dentate gyrus; Dn, dendrites; Hi, hippocampus; Mb, midbrain; MO, medulla oblongata; NT, nerve terminal; OB, olfactory bulb; Or, stratum oriens; Py, pyramidal cell layer; Ra, stratum radiatum; Sp, spine; Su, subiculum; Th, thalamus. Scale bars, A, 1 mm; B, 200 μm; C, 20 μm (left), 5 μm (right); D, 500 nm (left), 100 nm (right).

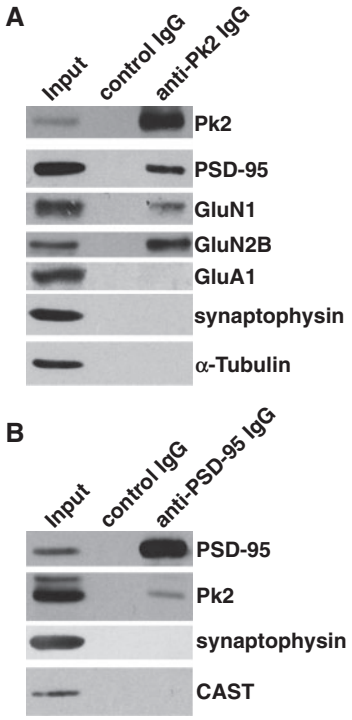

Fig. 5 Complex formation of Pk2, PSD-95 and NMDA receptors. Extracts of the P2 fraction were subjected to immunoprecipitation using the anti-Pk2 (A) or anti-PSD-95 antibody (B). The immunoprecipitates were analysed by western blotting using various antibodies against the indicated proteins. Input contains 8.0% of the extract used for the assay.

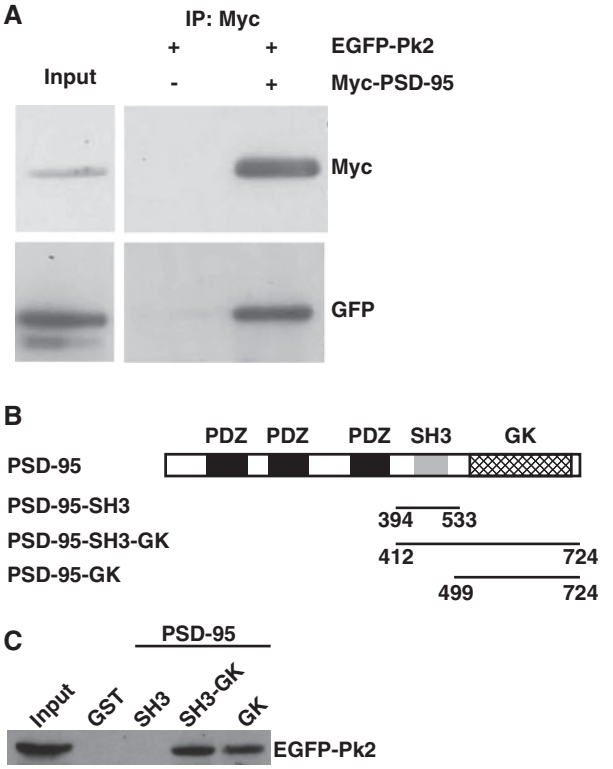

Fig. 6 Direct binding of Pk2 to the GK domain of PSD-95. (A) Immunoprecipitation using the anti-Myc antibody for PSD-95. Expression plasmid of EGFP-Pk2 and Myc-PSD-95 were transfected into HEK293 cells. Proteins were extracted, followed by immunoprecipitation using the anti-Myc antibody. The immunoprecipitate was then analysed by western blotting using the indicated antibodies. Input contains 8.0% of the extract used for the assay. (B) Constructs of PSD-95 for GST fusion proteins. (C) Pull down assay for the Pk-binding domain of PSD-95. The GST fusion proteins containing various regions of PSD-95 as well as GST alone were immobilized to glutathione-sepharose beads. The extract of HEK293 cells expressing EGFP-Pk2 was then incubated with the beads. Proteins that bound to the beads were analysed by western blotting using the anti-GFP antibody. Equal amounts of the GST fusion proteins were loaded and assessed by protein staining with Coomassie brilliant blue (data not shown).

data, these results indicate that Pk2 is a novel component of the PSD.

A complex formation of Pk2 with PSD-95 and NMDA receptors

To investigate the function of Pk2 at the PSD, we examined the binding of Pk2 to PSD proteins. For this purpose, we first immunoprecipitated Pk2 using the antibody from the extract of the P2 fraction of the rat brain. Among the proteins examined, PSD-95, GluN1 and GluN2B were co-immunoprecipitated with Pk2, but neither GluA1, synaptophysin, nor α-Tubulin were co-immunoprecipitated (Fig. 5A). Moreover, using the anti-PSD-95 antibody, Pk2 was also co-immunoprecipitated with PSD-95 (Fig. 5B). In contrast, neither synaptophysin nor CAST was co-immunoprecipitated (Fig. 5B). These results clearly indicate that Pk2 is associated with the PSD-95 and NMDA receptor complex in the brain.

Direct binding of Pk2 and PSD-95

Considering the unique localization of Pk2 at the PSD and the immunoprecipitation data, we speculated that Pk2 directly interacted with PSD proteins. One such candidate includes PSD-95. To examine the binding of Pk2 and PSD-95, we transfected expression plasmids of EGFP-Pk2 and Myc-PSD-95 into HEK293 cells and extracted proteins, followed by an immunoprecipitation assay using an anti-Myc antibody for PSD-95. Interestingly, EGFP-Pk2 was co-immunoprecipitated with Myc-PSD-95 (Fig. 6A). This result indicates that Pk2 directly interacts with PSD-95.

To further examine the mode of Pk2 binding to PSD-95, we next performed a pull down assay using GST-fusion proteins containing SH3 and/or GK domains of PSD-95 (Fig. 6B). The extract of HEK293 cells expressing EGFP-Pk2 was incubated with glutathione-sepharose beads containing the above GST fusion proteins. EGFP-Pk2 bound to the constructs that had the GK domain, but not to the constructs that only had the SH3 domain (Fig. 6C). Taken together, these data indicate that the PCP protein Pk is a PSD protein at mature synapses that forms a complex with PSD-95 and NMDA receptors through the direct binding to the GK domain of PSD-95.

Discussion

In this study, we found that one of the PCP proteins, Pk2, is tightly associated with the PSD fraction using biochemical subcellular analysis, and is colocalized

with other synaptic proteins in primary cultures of rat hippocampal neurons. Post-embedding immunogold electron microscopy also showed that Pk2 is specifically localized at the PSD of asymmetric synapses. Moreover, Pk2 forms a protein complex with PSD-95 and NMDA receptors by direct binding to the C-terminal GK domain of PSD-95. Accordingly, we propose that the PCP gene product Pk2 functions as a PSD protein in the brain, indicating a possible role of Pk2 in PSD-95/NMDA receptor-mediated synaptic function.

Mode of localization of Pk2 at the PSD

How Pk2 correctly and specifically localizes to the PSD of excitatory synapses remains unclear. One possibility is that Pk2 could be recruited to the PSD by its binding partner PSD-95. Indeed, PSD-95 has been shown to function as a scaffold for many synapse proteins, and some of the proteins directly bind to the GK domain of PSD-95 (*25*). Currently, we have not yet determined a PSD-95-binding domain in Pk2. By using a deletion mutant of Pk2, which lacks the PSD-95-binding region, the importance of binding to the GK domain of PSD-95 for the localization of Pk2 to the PSD would be identified.

Possible function of Pk2 at the PSD

Revealing molecular processes to control synaptic responses during synaptic activity is critical for understanding brain function, such as learning and memory. Excitatory synaptic strength is mainly due to incorporation or retrieval of AMPA receptors from the synaptic site, and these changes are maintained by the synthesis of appropriate gene products activated via NMDA receptors (*26–28*). Several lines of evidence have also suggested that PSD-95 is a key component for the targeting of AMPA receptors to synaptic membranes (*29–32*), and a mutant mouse lacking PSD-95 indicates the contribution to NMDA receptor-dependent synaptic plasticity (*33*). Similar to other members of the family of synaptic membrane-associated GK proteins (MAGUKs), PSD-95 has multiple protein-protein interaction motifs, including three PDZ domains and C-terminal SH3 and GK domains (*34*). While the PDZ domains are important for direct binding to NMDA receptors, the SH3 and GK domains interact with several proteins that may influence intracellular signaling cascades. Xu *et al*. (*29*) examined the function of PSD-95 by AMPA and NMDA receptor mediated EPSCs in lentivirus-mediated molecular replacement, and demonstrated not only the N-terminal PDZ domain, but also the C-terminal SH3 and GK domains are necessary for localization of PSD-95 to the synaptic site and for scaffolding other signaling proteins (*35*). Our biochemical data in this article clearly show that Pk2 forms a protein complex with NMDA receptors and PSD-95 in the brain (Fig. 5, 6), suggesting that a PCP protein may play a role in NMDA receptor-mediated synaptic plasticity and PSD-95 localization. Moreau *et al*. (*8*) have recently reported that the apicobasal polarity protein Scribble1, known to have a function in the PCP pathway (*6*), is localized to dendritic spines. Heterozygous mutant mice of Scribble1 show an increased number of enlarged spines and synaptic plasticity, indicating a crucial role for the PCP pathway for spine morphology and function (*8*). Currently, the molecular interaction between Pk2 and Scribble1 is unclear, but it would be of great interest for future research to determine if Pk2 is involved in Scribble1-mediated synaptic plasticity in mature neurons. Such observations would lead to the discovery that the PCP signaling pathway contributes to regulation of synaptic plasticity and higher brain functions such as learning and memory, emotion, and consciousness.

Contribution of Pk2 in neuronal polarity

Polarity is a major feature of neurons, but its molecular and cellular mechanisms underlying trigger and maintenance of axon and dendrite formation (axon/dendrite polarization) are still obscure. Recent evidence indicates that a serine/threonine kinase SAD is essential for proper axon formation and subsequent synapse maturation (*36–38*). In addition, studies using cultured hippocampal neurons have shown that other signalling molecules, such as PI3K, PTEN, Ras small G proteins, GSK3β and CRMP2, regulate neuronal polarity (*39*). However, there is no direct evidence that PCP proteins are involved in this process, although PCP proteins were originally characterized as regulators for polarization during early development. Recently, Pk1 and Pk2 have been shown to promote neurite outgrowth (*21*, *40*). Knockdown of Pk1 or Pk2 effectively decreased neurite outgrowth of mouse neuroblastoma Neuro2a cells (*21*), and over-expression of Pk1 or Pk2 induced striking neurite-like process formation in C1300 cells (*40*). Of course, these data alone are not adequate to conclude that the Pk family regulates axon/dendrite polarization, since these cells do not show polarity characteristics of neurons. Thus, genetic studies using KO mice would be useful for understanding the role of the Pk family in neuronal polarization during development. Interestingly, Pk1 KO mice show early embryonic lethality associated with loss of apicobasal polarity of epiblast cell (*41*). Therefore, conditional KO mice of Pk1 and/or Pk2 would reveal a direct engagement of the Pk family in establishment and maintenance of neuronal polarity.

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Conflict of interest

None declared.

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