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Phospholipase Cδ4 Associates with Glutamate Receptor Interacting Protein 1 in Testis

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We reported previously that phospholipase C (PLC) δ4 is required for calcium mobilization in the zona pellucida–induced acrosome reaction in sperm. Here we focused on the function of the C2 domain of PLCδ4 and report that glutamate receptor–interacting protein1 (GRIP1) was identified as a binding protein of the PLCδ4-C2 domain on yeast two-hybrid screening. Physiological interaction of GRIP1 with PLCδ4 in mouse testis was confirmed by immunoprecipitation with anti-PLCδ4 antibodies and the association seemed to correlate with the maturation stage of sperm. We also determined that a PDZ-binding motif at the C-terminus of the PLCδ4-C2 domain is responsible for GRIP1 binding, whereas the sixth or seventh PDZ domain of GRIP1 is essential and sufficient for association with the PLCδ4-C2 domain. These results indicate that PLCδ4 binds *via* its C2 domain to the PDZ6 or PDZ7 domain of GRIP1, and that this association may play a role in spermatogenesis.

Key words: C2 domain, calcium, glutamate receptor–interacting protein 1, PDZ domain, phospholipase Cδ4.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid; BAPTA-AM, *O*,*O*′-bis (2-aminophenyl) ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, enhanced green fluorescent protein; GRIP1, glutamate receptor–interacting protein 1; IP_3, inositol 1,4,5-triphosphate; KBD, kinesin-binding domain; PDZ, PSD95/discs large/ZO-1; PH, pleckstrin homology; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; SOC, store-operated channel.

Phospholipase C (PLC) catalyzes a critical step in receptor-mediated signal transduction by generating two second messenger molecules, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium from intracellular stores, and DAG mediates the activation of protein kinase C (PKC), leading to various cellular responses (*1*, *2*). The thirteen PLC isozymes are divided into six classes, β(1–4), γ(1, 2), δ(1, 3, 4), ε, ζ and η (1, 2) on the basis of the activation mechanism and structural features (*3–8*). All PLC isozymes contain catalytic X and Y domains as well as various regulatory domains, including a C2 domain, a EF-hand motif, and a pleckstrin homology (PH) domain (*9*, *10*). Among PLC isozymes, PLCδ is evolutionarily the most primitive isoform, and is expressed in plants, molds, and yeasts.

We recently generated knockout mice of PLCδ4 and found that PLCδ4 is a critical enzyme in the zona pellucida-induced acrosome reaction in sperm (*11*). Our data indicated that PLCδ4 is involved in sustained calcium influx through store-operated channels (SOCs) as well as intracellular calcium mobilization (*12*). In addition, it was recently reported that the C2 domain of PLCβ binds to the TRPM7 ion channel, leading to inactivation of this channel (*13*). Therefore, we tried to identify intracellular binding partners of the C2 domain of PLCδ4 to clarify the function(s) of PLCδ4. We performed yeast two-hybrid screening with a mouse testis library and the C2 domain of PLCδ4 as bait. We identified the glutamate receptor-interacting protein (GRIP1) as a binding partner of the C2 domain of PLCδ4.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen—A cDNA fragment encoding the C2 domain of mouse PLCδ4 was subcloned into the pGBKT7 vector (BD Biosciences, Palo Alto, CA, USA) and used as bait to screen a mouse testis Matchmaker cDNA library (BD Biosciences). DNA sequences from positive clones were determined with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Construction of Plasmids—Full-length mouse GRIP1 cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from mouse brain RNA. Mouse GRIP1 deletion constructs in pGAD424 were generated by PCR with the following primer pairs: PDZ1–3, forward, 5′-GCAGATCTATGCCGGGCTGGAAGAAG-3′, and reverse, 5′-GCCTCGAGTCACGTGACAGGGTCAGC-3′; PDZ4–5, forward, 5′-GCGGATCCGGCTTCGGAATCCAACTG-3′, and reverse, 5′-GCCTCGAGTCACCCCCCATAGCGCTT-3′;

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PDZ6–7, forward, 5′-GCGAATTCCCCCTTGGCATCACAATT-3′, and reverse, 5′-GGCGTCGACCTATAGTGTGTTAGTGGG-3′; and KBD, forward, 5′-GCGAATTCGATGCCCAATCTGCATCA-3′, and reverse, 5′-GCCTCGAGTCACTCCATGCCAGAGTC-3′. For PDZ6, the forward primer is identical to the forward primer for PDZ6-7, and the reverse primer is 5′-GGCGTCGACTCATGTCTGTTTCTTAATTTT-3′. For PDZ7, the forward primer is 5′-GCGGATCCGACTTCGGGTTCAGTGTG-3′, and the reverse primer is identical to the PDZ6-7 reverse primer. The primers for KBD+PDZ6 are the KBD forward primer and PDZ6 reverse primer, and those for KBD+PDZ7 are the KBD forward primer and PDZ7 reverse primer. The C2 domains of PLCs in pGBKT7 were generated by PCR with the following primer pairs: PLCδ1-C2, forward, 5′-GCGGATCCAAGCTCCGAGTCTGGATCATC-3′, and reverse, 5′-GC CTCGAGTTAGAGGTGGACATGGCGGTACC; PLCδ3-C2, forward, 5′-GCGAATCCACTCTGGCAATCCAGGTG-3′, and reverse, 5′-GCCTCGAGTTACAGGTGGATGTGGCGGTA-3′; PLCδ4-C2, forward, 5′-GCGAATTCATCCTTGTAGTCCAGGTGATC-3′, and reverse, 5′-GCCTCGAGTTACAAGGATACATGTCGGTAGC-3′; PLCβ1-C2, forward, 5′-GCGAATTCACTCTATCTGTTAAGATT-3′, and reverse, 5′-GCCTCGAGTCACAGGCAGATGTAGTGATA-3′; and PLCγ1-C2, forward, 5′-GCGAATTCGTCATTTGCATTGAGGTG-3′, and reverse, 5′-GCCTCGAGTCACAAAGGCACTGCTCTATA-3′. PCR was carried out for 25 cycles of 15 s at 94°C, 15 s at 55°C, and 90 s at 74°C. cDNAs were cloned into pENTR vectors (Invitrogen, Carlsbad, CA, USA) and then subcloned into pGAD424 or pGBKT7.

Binding Analysis between the C2 Domains of PLCs and GRIP1 Fragments—A yeast two-hybrid system was used to examine the interaction between the C2 domains of PLCs and GRIP1 fragments. Yeast cells co-transformed with vectors expressing the C2 domains of PLCs and various fragments of GRIP1 were grown on double-dropout medium (-Trp-Leu) and then selected for growth on triple-dropout medium (-Trp-Leu-His). The yeast cells were also transformed with a deletion construct of the C-terminal HVSL of PLCδ4-C2 to narrow the region of PLCδ4-C2 that binds GRIP1.

Antibodies—Anti-PLCδ4 serum was described previously (*11*). Anti-PLCδ1 monoclonal antibody was a gift from Dr. Pann-Ghill Suh (POSTECH, Korea). Anti-GRIP1 monoclonal antibody was purchased from BD Biosciences. Pre-immune purified IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG antibody was from Sigma (St. Louis, MO, USA) and fluorescent secondary antibodies were from Invitrogen.

Immunoprecipitation and Western Blotting—Mouse testes were homogenized in a buffer comprising 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 0.2 mM DTT, and 0.1% NP-40. The homogenates were cleared by centrifugation at 15,000 × *g* for 15 min at 4°C, and the resulting supernatants were passed through silica membranes (Promega A9282, Madison, WI, USA) to remove fat. The supernatants were incubated with anti-PLCδ1 or anti-PLCδ4 antibodies, followed by incubation with Protein A Sepharose™ CL-4B (Amersham Biosciences, Piscataway, NJ, USA). The precipitates were subjected to SDS-PAGE and then transferred electrophoretically on PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then probed with antibodies against PLCδ1, PLCδ4, or GRIP1, and incubated with appropriate HRP-conjugated secondary antibodies (DAKO, Tokyo, Japan), and then immunocomplexes were visualized by mean of enhanced chemiluminescence (ECL, Amersham).

Cell Culture and Cell Staining—COS-7 cells were maintained in DMEM supplemented with 10% FBS (Invitrogen) at 37°C under 5% CO_2. The GFP-tagged PLCδ4-C2 domain and FLAG-tagged PDZ6-7 of GRIP1 were transiently expressed in COS-7 cells with the use of Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, the cells were fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Then the cells were incubated with anti-FLAG antibody (Sigma), and treated with Alexa594-conjugated secondary antibody (Invitrogen). The cells were examined by laser confocal microscopy (Leica Microsystems, Wetzlar, Germany).

Cell Culture and Treatment with BAPTA-AM—Rat C6 glioma cells were purchased from the Health Science Research Resources Bank (HSRRB, Osaka, Japan), and maintained in Ham's F-10 (Invitrogen) supplemented with 15% horse serum and 2.5% fetal bovine serum (FBS) at 37°C under 5% CO_2. 90% confluent cells in a 100 mm dish were treated with BAPTA-AM (Dojindo, Kumamoto, Japan) for 1 h and then harvested by trypsinization. The cell pellets were lysed with a buffer comprising 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, protease inhibitor cocktail (Roche), and 1% NP40 with (BAPTA-treatment) or without (control) 1 mM EDTA. The cell lysates were centrifuged at 15,000 × *g* at 4°C, and the supernatants were immunoprecipitated with anti-PLCδ4 antibody. The precipitates were subjected to SDS-PAGE and Western blotting.

RESULTS AND DISCUSSION

GRIP1 Interacts with the C2 Domain of PLCδ4—The C2 domain of PLCδ4 was used as bait for yeast two-hybrid analysis to screen a mouse testis cDNA library for proteins that bind to the C2 domain. Fifteen plasmids containing fragments with the same sequence, which was identical to that of the multi-PDZ domain protein GRIP1, were isolated independently. GRIP1 protein was originally identified as a protein that interacts with AMPA receptor subunits GluR2 and GluR3 (*14*), and seems to play an important role in the distribution of its binding proteins. For example, the interaction between GluR2 and GRIP1 is crucial for precise apposition of GluR2 to the postsynaptic membrane. Phosphorylation of GluR2 by PKC, one of the signaling molecules downstream of PLC, causes GluR2 to release GRIP1, leading to long-term depression (LTD) in the cerebellum via internalization of GluR2 and modulation of the AMPA receptor channel function (*15*, *16*). Although the function of GRIP1 has been studied intensively in the brain, little has been reported for testis except for the suggestion that GRIP1 binds to DNA polymerase β and acts as a scaffold protein during meiosis (*17*).

Yeast two-hybrid technology was used to verify the specificity of the binding of GRIP1 to the C2 domains of other PLC isozymes. Yeasts were co-transformed with vectors expressing GRIP1 and the C2 domains of various

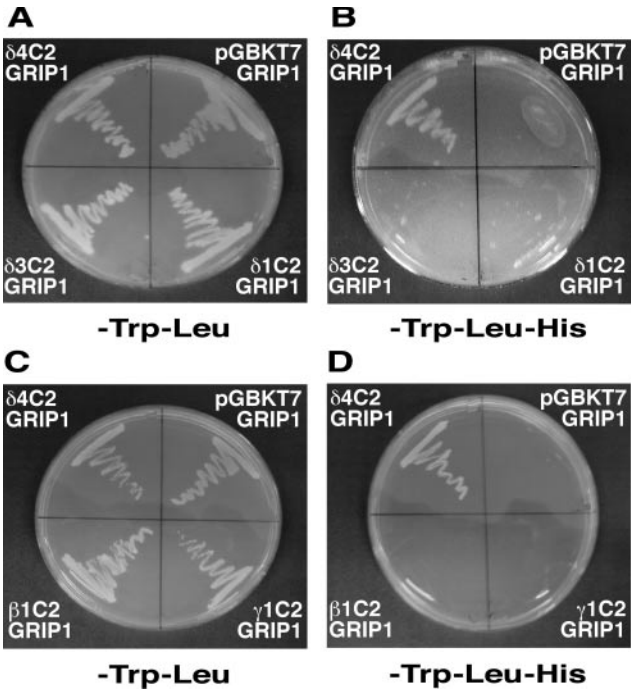

Fig. 1. **GRIP1 interacts with the C2 domain of PLCδ4.** Yeasts were co-transformed with vectors expressing GRIP1 and the C2 domain of PLC-δ type (A and B) or another PLC (C and D), and grown on double-dropout plates (A and C) or on triple-dropout plates (B and D). pGBKT7 is a negative control vector.

PLCs, grown on double-dropout medium (-Trp-Leu), and then selected for growth on triple-dropout medium (-Trp-Leu-His). All yeast transformants grew on double-dropout medium (Fig. 1, A and C). Yeasts transformed with vectors expressing the PLCδ4-C2 domain and GRIP1 grew on triple-dropout medium, whereas those expressing PLCδ1-C2 or PLCδ3-C2 and GRIP1 failed to grow on triple-dropout medium (Fig. 1B). Furthermore, yeasts transformed with vectors expressing the C2 domain of mouse PLCβ1 or PLCγ1, and GRIP1 did not grow on triple-dropout medium (Fig. 1D). These results provide strong evidence that GRIP1 binds specifically to the C2 domain of PLCδ4 in a yeast two-hybrid system.

PLCδ4 Interacts with GRIP1 in Testis—We next examined whether PLCδ4 binds to GRIP1 by co-immunoprecipitation assays of extracts of mouse testis. GRIP1 was immunoprecipitated with anti-PLCδ4 antibody but not anti-PLCδ1 antibody (Fig. 2, right panel). Most PLCδ1 or PLCδ4 was immunoprecipitated with anti-PLCδ1 or anti-PLCδ4 antibody, respectively (Fig. 2, left and middle panels). These data indicate that PLCδ4 specifically interacts with GRIP1 in testis. GRIP1 was also precipitated from brain lysates with anti-PLCδ4 antibody (data not shown); however, we failed to detect PLCδ4 when lysates were immunoprecipitated with anti-GRIP1 antibody or on co-localization of both proteins by immunostaining. This may be due to the low quantity of PLCδ4 in the testis. These data indicate that PLCδ4 interacts with GRIP1 and suggest that GRIP1 serves as a scaffold protein that is involved in signal transduction or localization of PLCδ4 in testis.

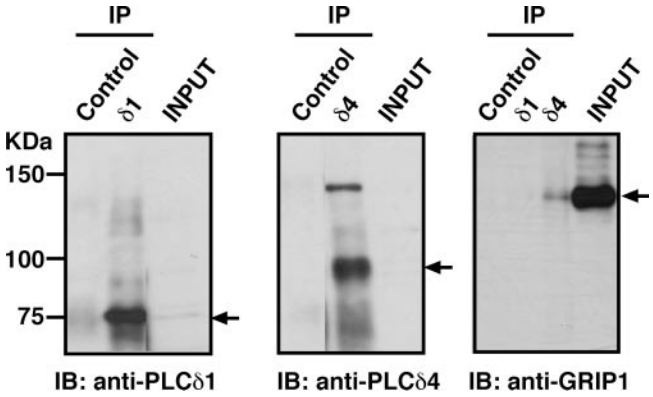

Fig. 2. **Co-immunoprecipitation of GRIP1 with PLCδ4 in testis.** GRIP1 in lysates of mouse testis was immunoprecipitated with either pre-immune IgG (control), anti-PLCδ1 (δ1), or anti-PLCδ4 (δ4) antibody. Immunoprecipitates were analyzed by SDS-PAGE, and immunoblotted (IB) with anti-PLCδ1, anti-PLCδ4, and anti-GRIP1 antibodies, respectively. Aliquots of the same lysates (10% INPUT) were directly subjected to SDS-PAGE and probed with antibodies as described above.

PDZ6 or PDZ7 of GRIP1 Is Sufficient for Binding to the C2 Domain of PLCδ4—GRIP1 is composed of seven PDZ domains and a kinesin-binding domain (KBD) that is located between the sixth and seventh PDZ domains. It has been reported that GRIP1 binds to glutamate receptor 2 *via* PDZ domains 4 and 5 (*14*), to EphB2/EphA7 receptors, the ephrin B1 ligand, and liprin-α *via* PDZ domain 6 (*18*, *19*), to RasGEF and GRIP1-associated protein1 (GRASP-1) *via* PDZ domain 7 (*20*), and to kinesin via the KBD (*21*). To determine the location of the PLCδ4-C2 domain–binding site in GRIP1, we generated a variety of plasmids encoding truncation mutants of GRIP1 and examined their binding abilities by mean of yeast two-hybrid analysis. As shown in Fig. 3, PDZ6-7, PDZ6+KBD, KBD, PDZ6, PDZ7, PDZ7+KBD and full-length GRIP1 bound to the C2 domain of PLCδ4. In contrast, PDZ1-3, PDZ4-5 and the KBD of GRIP1 showed no interaction. These results indicate that either the PDZ6 or PDZ7 domain of GRIP1 is necessary and sufficient for binding to the C2 domain of PLCδ4, and that GRIP1 is likely to act as a scaffold protein to promote interaction between signaling proteins.

The C2 Domain of PLCδ4 Has a PDZ-Binding Motif at the Extreme C-Terminus—PDZ domains commonly recognize C-terminal peptides in the interacting proteins (*22*, *23*). The C-terminal tetrapeptide of the C2 domain of PLCδ4 (HVSL) is conserved in rat, mouse, and human (Fig. 4A). HVSL is a class II PDZ target motif with hydrophobic amino acids at positions 0 and –2 (XϕXϕ, where ϕ is a hydrophobic amino acid, and X is any amino acid). To determine whether or not the HVSL of the C2 domain of PLCδ4 is a PDZ-binding motif, we constructed a deletion mutant of these four amino acids. This mutant failed to associate with GRIP1, confirming that this region of the C2 domain of PLCδ4 is a PDZ-binding motif (Fig. 4B). However, despite the high sequence homology among the C2 domains of PLCδ type isoforms, the C2 domains of PLCδ1 and PLCδ3 exhibited virtually no interaction with GRIP1. These results are similar to the relationship

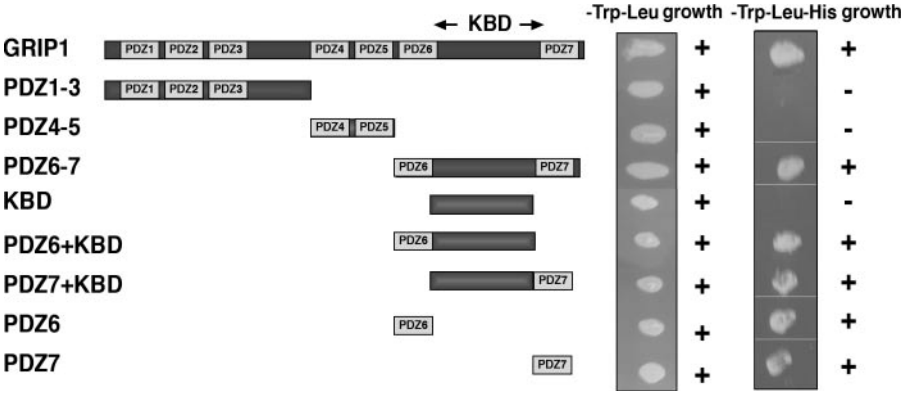

Fig. 3. **Identification of the domain in GRIP1 that binds to PLCδ4.** Yeasts were co-transformed with vectors expressing the C2 domain of PLCδ4 and various domains of GRIP1 as described, and were grown on double-dropout plates or on triple-dropout plates. + indicates growth on selective plates, – indicates no growth. KBD, kinesin-binding domain.

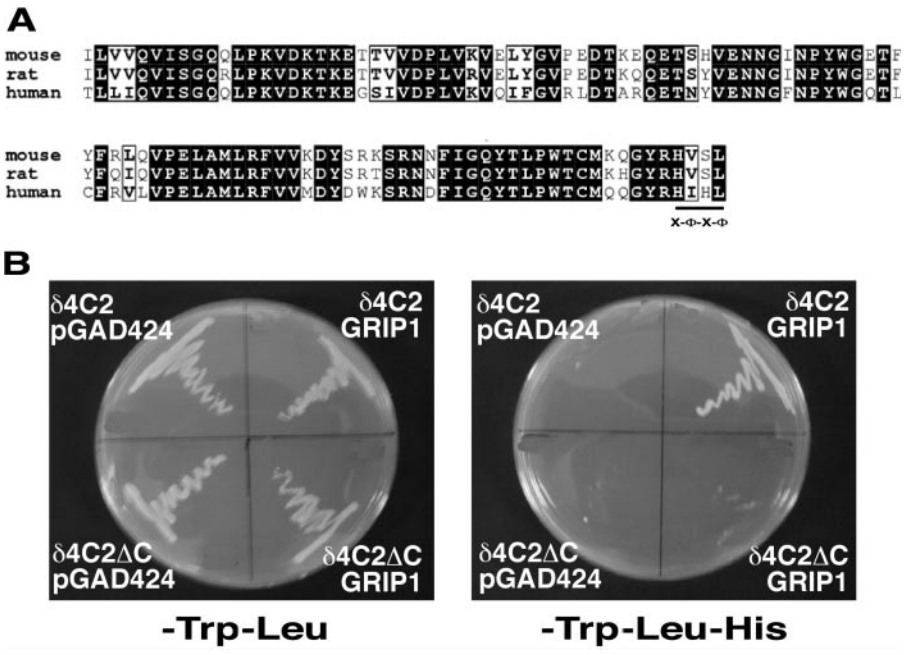

Fig. 4. **A C-terminal four amino acid peptide of the PLCδ4-C2 domain is responsible for binding to the PDZ domain of GRIP1.** (A) Comparison of the amino acid sequences of the C2 domains of mouse, human, and rat PLCδ4. The underline indicates the PDZ-binding motif. Φ, hydrophobic amino acid; X, any amino acid. (B) The wild-type (δ4C2) and a mutant with deletion of the C-terminal four amino acids (δ4C2ΔC) were examined for interaction with GRIP1 or a control vector (pGAD424). Transformants were grown on double-dropout selective plates, and then selected for growth on triple-dropout selection plates.

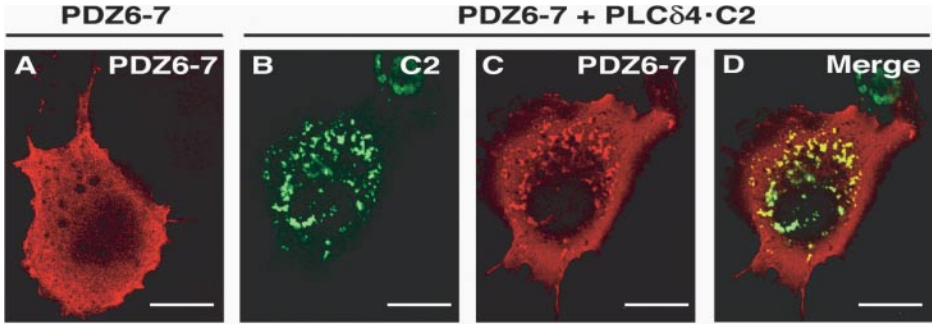

Fig. 5. **PLCδ4-C2 domain and PDZ6-7 of GRIP1 are detected together as dot-like structures.** COS-7 cells were transfected with pFLAG-GRIP1 PDZ6-7 (A) or pFLAG-GRIP1 PDZ6-7 and the GFP-PLCδ4-C2 domain (B–D), and then treated with anti-FLAG antibody to visualize PDZ6-7 of GRIP1. The distribution of the PLCδ4-C2 domain (B), PDZ6-7 of GRIP1 (C), and a merged image of the two signals (D) are shown. Scale bar, 20 μm.

between mGluR2 or mGluR3 and GRIP1. Although mGluR2 includes a PDZ binding motif as well as mGluR3, GRIP1 only bound to mGluR3, but not to mGluR2 (*24*). Taken together with this finding, our data suggest that conformational requirement of amino acids located upstream of the PDZ binding motif may be necessary for the binding to GRIP1 in addition to the extreme C-terminus of the C2 domain of PLCδ4.

Ectopically Expressed C2 Domain of PLCδ4 and PDZ6-7 of GRIP1 Are Co-localized in Cells—Because we could not detect endogenous PLCδ4 on immunostaining, which may be due to the low expression of PLCδ4 in C6 glioma cells, we next tried to confirm the association of the C2 domain of PLCδ4 and PDZ6-7 of GRIP1 by transient expression of both proteins in COS-7 cells. When FLAG-tagged PDZ6-7 of GRIP1 was expressed, it was detected throughout cells

(Fig. 5A). When we expressed the green fluorescent protein (GFP)–tagged C2 domain of PLCδ4 and FLAG-tagged PDZ6-7 of GRIP1 together, the C2 domain of PLCδ4 was observed as dot-like staining at intracellular membranes (Fig. 5B). Although most PDZ6-7 of GRIP1 was localized in the cytosol, we observed partial co-localization of PDZ6-7 of GRIP1 with the C2 domain of PLCδ4. These results strongly suggest interaction between the C2 domain of PLCδ4 and PDZ6-7 of GRIP1 in cells (Fig. 5, C and D).

Interaction between PLCδ4 and GRIP1 Depends on the Intracellular Calcium Concentration—Members of the PLCδ subfamily are believed to be the PLCs most sensitive to calcium. In addition, the C2 domain of PKC or Ferlin has been reported to bind to phospholipids in a calcium-dependent manner (*25*, *26*). We then examined whether or not the calcium concentration affects the association of these proteins. As shown in Fig. 6, the amount of GRIP1 that interacted with PLCδ4 was higher in rat C6 glioma cells, which express endogenous PLCδ4 and GRIP1, when intracellular calcium stores were depleted by BAPTA-AM than when the interaction occurred in the presence of calcium. This result suggests that the interaction between PLCδ4 and GRIP1 is more stable with relatively low calcium concentrations.

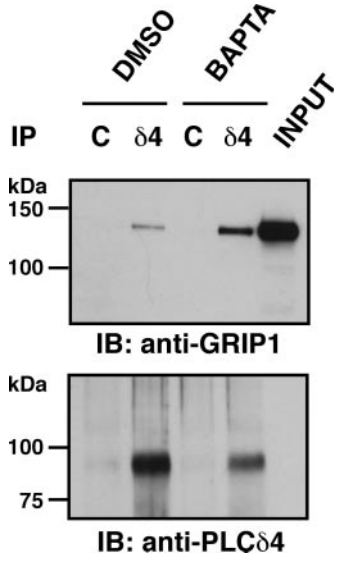

Fig. 6. **The effect of calcium on the association of PLCδ4 and GRIP1.** C6 glioma cells were treated with (BAPTA) or without (DMSO) BAPTA-AM to decrease the intracellular calcium level. Cell lysates were immunoprecipitated (IP) with pre-immune IgG (C) or anti-PLCδ4 antibodies (δ4). The precipitates were analyzed by Western blotting with anti-GRIP1 antibody (upper panel) or anti-PLCδ4 antibody (bottom panel). INPUT indicates aliquots of the lysates (10%).

Interaction between PLCδ4 and GRIP1 Seems to Correlate with the Maturation Stage of Sperm—To understand the physiological role of the interaction between GRIP1 and PLCδ4, the change in the association during spermatogenesis was examined. Spermatogenesis is a productive and highly organized process that generates virtually an unlimited number of sperm during adulthood. The regulative mechanisms for this continuous proliferation and differentiation of germ cells have remained unclear because of a lack of suitable models. First, spermatogenesis is synchronously initiated at around 3 weeks after birth and we first detected mature sperm in testis at 5–6 weeks after birth (data not shown), whereas spermatogenesis in adults is not synchronized. As shown in Fig. 7, the amount of GRIP1 that interacted with PLCδ4 was much higher at 3 or 4 weeks of age than at 5 to 7 weeks after birth. We can easily detect the tight association at age of 3 or 4 weeks after birth even when the signals are visualized by ECL with a very short-time exposure. This finding suggests that the interaction plays a role in the early stage of spermatogenesis. Since PLCδ4 is highly concentrated in spermatogonia in testes (*11*), stem cells of sperm, as well as acrosomes of sperm, the interaction/dissociation might be responsible for the balance between proliferation and differentiation of the germ cells.

Although the role of GRIP1 has been intensively examined in the brain, little has been reported for testis. We found in the present study that GRIP1 interacts with the C2 domain of PLCδ4 in testis on yeast two-hybrid analysis, cell staining, and immunoprecipitation. In addition, we suggested that the interaction occurs in an age-dependent manner. Because it is clear that PDZ domains are involved in targeting proteins to specific membrane compartments, our results suggest that GRIP plays an important role in spermatogenesis by assembling functional proteins, including PLCδ4. It will be important to elucidate how these complexes function in the testis.

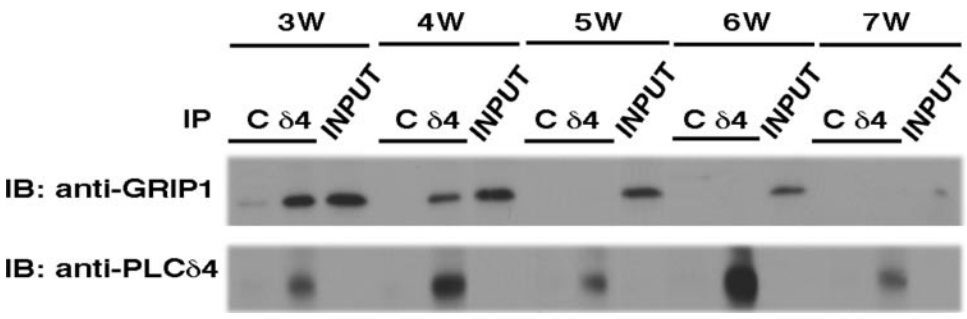

Fig. 7. **GRIP1 binds to PLCδ4 in an age-dependent manner.** GRIP1 in lysates of testes from mice of various ages (3–7 weeks after birth) was immunoprecipitated (IP) with either pre-immune IgG (C) or anti-PLCδ4 (δ4) antibody. The immunoprecipitates were analyzed by SDS-PAGE, and then immunoblotted (IB) with anti-PLCδ4 and anti-GRIP1 antibodies, respectively. Aliquots of the same lysates (10% INPUT) were directly subjected to SDS-PAGE and probed with antibodies as described above.

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