The Importance of Interaction with Membrane Lipids through the Pleckstrin Homology Domain of the Guanine Nucleotide Exchange Factor for Rho Family Small Guanosine Triphosphatase, FLJ00018

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FLJ00018, a heterotrimeric guanosine 5′-triphosphate (GTP)-binding protein (G protein) Gβγ subunit-activated guanine nucleotide exchange factor for Rho family small GTPases, regulates cellular responses, including cell morphological changes and gene transcriptional regulation, and targets the cellular membranes. FLJ00018 contains a Dbl homology (DH) domain in addition to a pleckstrin homology (PH) domain. Here we show that the PH domain of FLJ00018 is required for FLJ00018-induced, serum response element-dependent gene transcription. Although the PH domain of KIAA1415/P-Rex1, another Gβγ subunit-activated guanine nucleotide exchange factor for Rho family small GTPases, binds to phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate, the PH domain of FLJ00018 binds to polyphosphoinositides including phosphatidylinositol 4,5-bisphosphate, and phosphatidic acid. These results suggest that FLJ00018 is targeted via its PH domain to cellular membranes.

Key words Rho family; pleckstrin homology domain; polyphosphoinositide

By cycling between an inactive guanosine 5′-diphosphate (GDP)-bound form and an active guanosine 5′-triphosphate (GTP)-bound form, Rho family small GTPases (Rho), including RhoA, Rac, and Cdc42, act as nucleotide-dependent molecular switches to induce changes in the organization of the actin cytoskeleton to promote a variety of cell responses, including morphogenesis, chemotaxis, axonal guidance, and cell-cycle progression.1,2) The turning on of this cycle is controlled by a large family of Rho-specific guanine nucleotide exchange factors (RhoGEFs) that stimulate the exchange of GDP for GTP. Typically, RhoGEFs are large multidomain proteins that are tightly regulated to control their function. RhoGEF can be subdivided into two main subfamilies. First, they possess a Dbl homology (DH) domain that is found in tandem with a pleckstrin homology (PH) domain. This family is currently represented by 69 members in mammalian genomes.3,4) Second, Dock180-related proteins containing the Dock homology region (DHR)-2 domain (also known as the Dock-ZH2 domain) form a subfamily of 11 mammalian members.5) The DH domain is responsible for catalytic activity, and the PH domain directs the subcellular localization and can modulate the DH domain function.3,4) FLJ00018/PLEKHG2 is a RhoGEF and contains DH and PH domains. FLJ00018 possesses a region that binds to heterotrimeric GTP-binding protein Gβγ subunits.6) The binding activates the RhoGEF activity of FLJ00018, thereby enhancing serum response element (SRE)-dependent gene transcription and affecting the spreading of cells.

It has been shown that PH domains determine the subcellular localization and/or activity of PH domain-containing proteins by interacting with particular phospholipids, including phosphoinositide. For example, the PH domain of Asef, a RhoGEF, binds to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) and targets Asef to the cell–cell adhesion sites in Madin-Darby canine kidney II (MDCK II) cells.7) Several reports show that the PH domain regulates the functions of some RhoGEFs.5–11) P-Rex1/KIAA1415 and P-Rex2 are phosphoinositide- and Gβγ subunit-dependent, Rac-specific RhoGEFs whose activities are inhibited by wortmannin, an inhibitor of phosphoinositide 3-kinase (PI3K).12,13) On the other hand, wortmannin does not attenuate FLJ00018-induced SRE-dependent gene transcription.6) These results suggested that the lipid requirement of FLJ00018 differs from that of KIAA1415/P-Rex1.

In the present study, to test the lipid requirement of FLJ00018, we have examined the functional significance of the PH domain of FLJ00018. We show here that the PH domain of FLJ00018, unlike that of P-Rex1, binds to phosphoinositide including phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) and phosphatidic acid (PA).

MATERIALS AND METHODS

Plasmids and Reagents A complementary DNA clone for FLJ00018 and human phospholipase D2 gene was isolated during the Kazusa human cDNA project, which aimed to accumulate information on the coding sequences of long cDNAs for unidentified human genes. Plasmids of pcDNA3.1-Gβ/δ and pcDNA3.1-Gβγ were from the Missouri S&T cDNA Resource Center, U.S.A. These genes and related mutant genes were subcloned pF4A-CMV or pFN21A vectors (Promega, U.S.A.) by restriction enzyme digestion or polymerase chain reaction amplification. The pSRE.L-luciferase reporter plasmid was purchased from Agilent Technologies and pRL-SV40 was purchased from Nippon Gene. Glutathione-coupled Sepharose 4B and a mouse monoclonal antibody against glutathione transferase (GST) were obtained from GE Healthcare. Isopropyl-1-thio-β-D-galactopyranoside and a mouse monoclonal antibody against Myc-epitope were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Rabbit anti-Gβ/δ antibody was pre-
pared as described previously and used to detect Gβ.\(^5\)

**Cell Culture, Transfection, and Immunoblot Analysis**

HEK293 cells were grown in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum at 37°C. Transient transfection was performed using LipofectAMINE Plus according to the manufacturer’s instructions (Life Technologies). Cells were transfected with DNA for 3 h and then treated with 1× insulin-transferrin-selenium-X (Life Technologies) for 6–8 h. Finally, the cells were washed twice with serum-free DMEM and then incubated for 16–18 h in DMEM. Transfected cells were washed with phosphate-buffered saline and lysed with 1% sodium dodecyl sulfate (SDS) in 20 mm Tris–HCl (pH 8.0) and 1 mm ethylenediamine tetraacetic acid (EDTA). Equal amounts of samples were subjected to SDS-polyacrylamide gel electrophoresis. They were then immunoblotted with various antibodies, employing a chemiluminescence reagent (GE Healthcare). Densitometry was achieved using an LAS-4000 mini-luminescent image analyzer (GE Healthcare).

**Assay of SRE-Dependent Gene Transcription**

HEK293 cells seeded on 24-well plates were co-transfected with the indicated expression plasmids (400 ng of total DNA/well) together with the pSRE-Luciferase reporter plasmid and the pRL-SV40 control reporter vector. After transfection, cells were washed once with phosphate-buffered saline and lysed with passive lysis buffer. Luciferase activities were determined with the Dual-Luciferase Reporter Assay System (Promega) as described previously. The activity of the experimental reporter was normalized against that of the control vector. All results are given as means±S.D. values. The significance of group differences was analyzed with Student’s t-test for single comparisons. A p value of <0.05 was considered significant.

**Protein-Lipid Overlay Assays**

Protein-lipid overlay assays were performed using PIP Strips\(^\text{TM}\) (Echelon Bioscience) following the manufacturer’s instructions. Briefly, PIP Strips were blocked in 3% fatty acid-free bovine serum albumin (BSA) in TBS at room temperature for 1 h. The strips were then incubated with GST, GST-FLJ00018PH (283–441 amino acids (aa)), and GST-KIAA1415PH (206–372 aa) overnight in the dark at 4°C. The strips were washed three times in TBS-T and incubated overnight with horseradish peroxidase-conjugated anti-GST antibody at 4°C. The signals were directed by enhanced chemiluminescence (ECL) (GE Healthcare).

**Cell Fractionation**

The cytosolic fraction was extracted using a ProteoExtract Subcellular Proteome Extraction kit (Calbiochem). Triton X-100 soluble materials were extracted with TNE buffer (25 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, and protease inhibitors) containing 1% Triton X-100. Insoluble materials were further extracted with TNE buffer containing 1% SDS.\(^1\)

**RESULTS AND DISCUSSION**

To determine whether or not the PH domain of FLJ00018 is important for the regulation of SRE-dependent gene transcription, we prepared and used two mutants of FLJ00018 lacking the PH domain (ΔPH1: deleted 269–450 aa; ΔPH2: deleted 342–450 aa) (Fig. 1A). To determine whether or not the PH domain of FLJ00018 was required for FLJ00018 activity, we tested the ability of the wild type, ΔPH1, and ΔPH2 to activate SRE-dependent gene transcription in HEK293 cells (Fig. 1B). In the ΔPH1-expressing cells, SRE-dependent gene transcription did not increase significantly. It was thought that ΔPH1 became inactive because the deletion region of ΔPH1 contained a part of the DH domain. SRE-dependent gene transcription in ΔPH2-expressing cells also showed no significant increase, although ΔPH2 was the mutant with the full sequence of the DH domain. Furthermore, Gβγ did not enhance SRE-dependent gene transcription in ΔPH1- or ΔPH2-expressing cells. In ΔPH2-expressing cells, SRE-dependent gene transcription was reduced significantly more than in Gβγ-expressed cells. The mechanism underlying this reduction remains unexplained. In contrast to ΔPH1- or ΔPH2-expressing cells, Gβγ enhanced SRE-dependent gene transcription in the wild-type-expressing cells. At this time, the expression levels of these mutants were not different from that of the wild type. Also, we reported previously that an FLJ00018 mutant, P2 (1–464 aa), acts as a constitutively active mutant and that the PH domain was required for the activation of this mutant.\(^6\) These results suggest that the PH domain of FLJ00018 is im-
important for its function.

Next, to investigate whether or not the PH domain of FLJ00018 would bind to specific phospholipids, we performed a protein-lipid overlay assay using a recombinant fusion protein consisting of the PH domain of FLJ00018 or KIAA1415/P-Rex1 and GST (GST-FLJ00018-PH or GST-KIAA1415-PH). As shown in Fig. 2, GST-FLJ00018-PH interacted with polyphosphoinositide and PA. The interaction was especially strong in PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3. The interaction between GST-FLJ00018-PH and phospholipids including polyphosphoinositide and PA is similar to that of the PH domain of another RhoGEF, Trio.15) In contrast, GST-KIAA1415-PH specifically interacted with PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. KIAA1415/P-Rex1 is known to be activated by PtdIns(3,4,5)P_3 and G_{pG}.13) This was confirmed by our results. On the other hand, FLJ00018-induced, SRE-dependent gene transcription was regulated in a wortmannin-insensitive manner.6) This suggested that the wortmannin-insensitive regulation mechanism of FLJ00018 might be related with FLJ00018-PH’s interaction with polyphosphoinositide and PA. Since the details of this mechanism are still unknown, further study is required. GST alone showed no specific interaction (data not shown). A recent report indicated that phospholipase D (PLD)-generated PA coupled epidermal growth factor receptor stimulation to Ras activation by Sos, a Ras-specific GEF.16) This suggested that FLJ00018 might also be regulated by PLD-generated PA via its PH domain in living cells.

To determine PLD’s effect on the intracellular localization of FLJ00018, we examined the subcellular localization of FLJ00018 in phospholipase D2 (PLD2)-co-expressing cells (Fig. 3). It is well known that the mammalian PLD family consists of two related gene products, PLD1 and PLD2. PLD2 is constitutively active in the presence of PtdIns(4,5)P_2, and localized to plasma membrane in most cell types.17) The subcellular localization of FLJ00018 and ΔPH2 in FLJ00018- or ΔPH2-expressing cells in the presence or absence of PLD2 was examined by biochemical methods. FLJ00018 was distributed equally in the cytosolic fraction and Triton X-100-insoluble fraction. In FLJ00018 and PLD2 co-expressing cells, the cytosolic distribution of FLJ00018 decreased, and much of the FLJ00018 was distributed in the Triton X-100-insoluble fraction. In the same cells, PLD2 was also distributed in the Triton X-100-insoluble fraction. ΔPH2 was distributed equally in the cytosolic fraction and Triton X-100-insoluble fraction in ΔPH2-expressing cells. However, ΔPH2 also was distributed equally in the cytosolic fraction and Triton X-100-insoluble fraction in ΔPH2 and PLD2 co-expressing cells. These results suggested that the PH domain of FLJ00018 plays an important role in the subcellular localization of FLJ00018. In general it is well-known that intracellular filamentous actin is distributed in the Triton-X 100-insoluble fraction. On the other hand, it is also well-known that the lipid raft/caveolae is distributed in the Triton-X 100-insoluble fraction. Our results suggest the possibility that the PH domain of FLJ00018 regulated the association with these intracellular components containing the lipid raft or filamentous actin. However, the details have not yet been made clear.

In the present study, we report for the first time that the PH domain of FLJ00018 interacts with polyphosphoinositide and PA. In contrast, the PH domain of another G_{pG}-dependent
RhoGEF, KIAA1415/P-Rex1, binds to PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. Further studies on the interaction between lipids and the PH domains of various RhoGEFs are required in order to unravel the physiological function of this signaling system of RhoGEF.

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