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Roles of SAM and DDHD domains in mammalian intracellular phospholipase A_1 KIAA0725p☆

Hiroki Inoue [1], Takashi Baba [1], Seiichi Sato, Ryuya Ohtsuki, Aya Takemori, Takuya Watanabe, Mitsuo Tagaya, Katsuko Tani *

School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo, 192-0392, Japan

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ABSTRACT

Members of the intracellular phospholipase A_1 family of proteins have been implicated in organelle biogenesis and membrane trafficking. The mammalian family comprises three members: phosphatidic acid-preferring phospholipase A_1 (PA-PLA_1)/DDHD1, p125/Sec23ip and KIAA0725p/DDHD2, all of which have a DDHD domain. PA-PLA_1 is mostly cytosolic, while KIAA0725p and p125 are more stably associated with the Golgi/endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) and ER exit sites, respectively. Here we show that KIAA0725p and p125 are novel phosphoinositide-binding proteins. Deletion and mutational analyses of KIAA0725p suggested that a sterile alpha-motif (SAM), which is also present in p125, but not in cytosolic PA-PLA_1, and the following DDHD domain comprise a minimal region for phosphatidylinositol 4-phosphate (PI(4)P)-binding. A construct with mutations in the positively charged cluster of the SAM domain is defective in both phosphoinositide-binding and Golgi/ERGIC targeting. Consistent with the view that the PI(4)P-binding is important for the membrane association of KIAA0725p, expression of phosphoinositide phosphatase Sac1 reduces the association of expressed KIAA0725p with membranes. In addition, we show that deletion of the DDHD domain or introduction of point mutations at the conserved aspartate or histidine residues in the domain abolishes the phospholipase activity of KIAA0725p and PA-PLA_1. Together, our results suggest that KIAA0725p is targeted to specific organelle membranes in a phosphoinositide-dependent manner, and that its SAM and DDHD domains are essential for its phosphoinositide-binding and phospholipase activity.

1. Introduction

Phosphoinositides including phosphatidylinositol 4-phosphate (PI(4)P) play pivotal roles in membrane trafficking [1,2]. Each phosphoinositide is abundant in specific organelle membranes; PI(4)P, phosphatidylinositol 3-phosphate (PI(3)P), and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) are mainly localized in the Golgi apparatus, endosomes and the plasma membrane, respectively. Phosphoinositides exert their effects by recruiting effector proteins that have phosphoinositide-binding domains, such as the pleckstrin homology (PH) domain and epsin N-terminal homology domain.

The intracellular phospholipase A_1 ($iPLA_1$) family is a recently identified phospholipase family and has been implicated in organelle biogenesis and membrane trafficking. While most eukaryote families only comprise one species, the mammalian family comprises three members, phosphatidic acid (PA)-preferring phospholipase A_1 (PA-PLA_1)/DDHD1/$iPLA_1\alpha$, p125/Sec23-interacting protein (Sec23ip)/$iPLA_1\beta$, and KIAA0725p/DDHD2/$iPLA_1\gamma$. PA-PLA_1 was purified as a PA-preferring $iPLA_1$ highly expressed in mature bovine testis [3–5]. p125 was identified as a protein that interacts with mammalian Sec23 [6,7], which is one of the coat protein complex II components involved in protein export from the endoplasmic reticulum (ER) [8]. p125 is localized in ER exit sites and plays a role in maintaining their integrity in HeLa cells [9,10]. Our recent study demonstrated that p125 ablation causes male subfertility in mice [11]. In contrast to the robust PLA_1 activity of PA-PLA_1, PLA_1 activity has not been detected for p125 so far [12]. KIAA0725p was found through a database search as a protein that is more homologous to p125 than

Abbreviations: DOPA, 1,2-dioleoyl-sn-phosphatidic acid; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; GST, glutathione-S-transferase; iPLA1, intracellular phospholipase A_1; LPA, 1-oleoyl-lysophosphatidic acid; LUV, large unilamellar vesicles; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PH, pleckstrin homology; PI(3)P, phosphatidylinositol 3-phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI(5)P, phosphatidylinositol 5-phosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PLA_1, phospholipase A_1; PS, phosphatidylserine; SAM, sterile alpha-motif; TLC, thin-layer chromatography; WT, wild-type

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* Corresponding author at: School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo, 192-0392, Japan. Tel./fax: +81 42 676 6905.
E-mail address: tani@toyaku.ac.jp (K. Tani).
[1] These authors contributed equally to this work.

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PA-PLA$_1$, and it exhibits PLA$_1$ activity toward PA and several other phospholipids. KIAA0725p is localized in the *cis*-Golgi and perhaps the ER-Golgi intermediate compartment (ERGIC) [12–14], and its overexpression causes dissociation of coat and tethering proteins from the Golgi as well as the aggregation of ER membranes. RNA interference experiments have shown that KIAA0725p functions in the transport of cholera toxin B from the Golgi to the ER [13], and of vesicular stomatitis virus G protein from the Golgi to the plasma membrane [14], although its participation in the former transport process has not been confirmed [14].

In this study, we examined the mechanism underlying the association of KIAA0725p with membranes. Despite the absence of typical phosphoinositide-binding domains, KIAA0725p was found to bind to phosphatidylinositol phosphate (PIP). We show that a tandem SAM-DDHD domain, which is located in the C-terminal region of KIAA0725p, is responsible for this PIP binding. In addition, we revealed the role of the DDHD domain, which is commonly present in iPLA$_1$ family proteins and some phosphatidylinositol transfer proteins [15–17].

2. Experimental procedures

2.1. Plasmids and mutagenesis

The mammalian expression vector for FLAG-tagged human KIAA0725p (pFLAG-CMV-KIAA0725p) was described previously [12]. The open reading frame of KIAA0725p was amplified by PCR from pFLAG-CMV-KIAA0725p and inserted into pEBG [18] and a version of pFastBac (Invitrogen, Carlsbad, CA) modified by inserting the coding sequence for glutathione-S-transferase (GST), resulting in expression constructs for an N-terminal GST-fusion form of KIAA0725p in mammalian cells and Sf9 insect cells, respectively. Deletion and point mutants of KIAA0725p or PA-PLA$_1$ were constructed by standard PCR-based methods and inverse PCR [19], respectively, using corresponding primer sets (Supplemental Table S1). The sequences of all PCR-amplified open reading frames in the constructs were confirmed using an automated ABI DNA sequencer. Sac1 expression constructs were kindly donated by Dr. P. Mayinger.

2.2. Antibodies

A polyclonal antibody against KIAA0725p was raised in rabbits against bacterially expressed, purified GST-tagged KIAA0725p (amino acids 1–120), and affinity-purified by using a protein comprising the same region of KIAA0725p but tagged with maltose-binding protein instead of GST. A mouse monoclonal antibody against ERGIC-53 was generously provided by Dr. H. P. Hauri. Rabbit polyclonal (F7425) and mouse monoclonal (clone M2, F1804) antibodies against a FLAG tag were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-GST (sc-459) and mouse monoclonal anti-GM130 (clone 35, 610822) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosciences (Franklin Lakes, NJ), respectively. Horseradish peroxidase- or tetramethylrhodamine isothiocyanate-conjugated goat secondary antibodies against rabbit or mouse IgGs were obtained from Jackson ImmunoResearch (West Grove, PA). An Alexa Fluor 488-conjugated goat secondary antibody was purchased from Invitrogen.

2.3. Cell culture and transfection

HeLa and 293T cells were maintained in Eagle's minimum essential medium (α modification) (Sigma-Aldrich) and Dulbecco's modified Eagle's medium (Invitrogen), respectively, supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C with 5% CO_2. Cells were plated 1 day before transfection with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were used for assays 18 hours after transfection.

2.4. Immunofluorescence and microscopy

Cells were fixed with PBS containing 4% paraformaldehyde and 165 mM glucose, and PBS containing 2% bovine serum albumin and 0.04% NaN_3 was used for blocking and antibody dilution. Confocal microscopy was performed using an Olympus Fluoview 1000 laser-scanning confocal microscope with a 60×, 1.35-numerical-aperture UPlanSApo oil immersion lens (Tokyo, Japan).

2.5. Expression of GST fusion proteins in Sf9 cells and purification

Expression of GST-tagged proteins in Sf9 cells and their purification were carried out according to the manufacturer's instructions for a Bac-to-Bac baculovirus expression system (Invitrogen) and Glutathione Sepharose 4B (GE Healthcare, Piscataway, NJ).

2.6. Lipid overlay assay

PIP-strips membranes were purchased from Echelon Bioscience (Salt Lake City, UT). Self-made PI(4)P/phosphatidylcholine (PC) membranes were prepared as follows. PI(4)P (Echelon; diC16) and PC (Avanti Polar Lipids, Alabaster, AL; 1,2-stearoyl-PC) were serially diluted with $CHCl_3$/methanol/50 mM HCl (5:10:4), and then spotted onto Hybond-C extra nitrocellulose membranes (GE Healthcare) at between 25 and 200 pmol/spot. The membranes were immersed in Tris-buffered saline plus 0.1% Tween 20 containing 3% fatty acid free-bovine serum albumin (Sigma-Aldrich) for 1 h and then incubated with 0.5 pmol/ml purified GST fusion proteins in the buffer overnight at 4 °C. Alternatively, 100,000*g* supernatants of cell lysates of 293T cells transiently expressing the GST fusion proteins were used instead of the purified proteins. After extensive washing, proteins bound to lipids on the membranes were detected with an anti-GST antibody and an ECL immunodetection system (GE Healthcare).

2.7. Liposome sedimentation assay

All lipids were purchased from Avanti Polar Lipids. Liposomes (large unilamellar vesicles (LUV)) were prepared as described previously [20] with slight modifications. Briefly, for phosphatidylinositol (PI)-containing LUVs, a mixture of 40% PC (chicken egg), 25% phosphatidylethanolamine (PE) (bovine liver), 15% phosphatidylserine (PS) (porcine brain), 10% PI (porcine brain), and 10% cholesterol was dried under a nitrogen gas flow, rehydrated with reconstitution buffer (200 mM sucrose, 20 mM Hepes, pH7.4, 20 mM KCl) to give 5 mM total lipids, vortexed, and then subjected to five freeze-thaw cycles in liquid nitrogen and water at room temperature. The lipid suspension was finally passed through an extruder equipped with 0.1 μm pore filter (Avanti Polar Lipids) 11 times to prepare uniformly-sized LUVs. For PI(4)P- or PI(4,5)P_2-containing LUVs, 10% PI was replaced by 5% PI and 5% PI(4)P or 5% PI(4,5)P_2. 1 μM purified GST fusion proteins were incubated with 1 mM of the prepared LUVs in PBS at 30 °C for 10 min and then ultra-centrifuged at 100,000*g* to collect the LUVs. After a wash with PBS, the precipitated materials were analyzed by SDS–PAGE and immunoblotting.

2.8. GST-pulldown assay

GST-pulldown assays using GST-KIAA0725p and GST-PA-PLA$_1$, and their mutants with FLAG-tagged constructs in 293T cells were carried out as described previously [6].

2.9. Preparation of ^{32}P-labeled 1,2-dioleoyl-sn-phosphatidic acid (DOPA)

1,2-Dioleoyl-*sn*-glycerol (Sigma-Aldrich) was phosphorylated with diacylglycerol kinase (Sigma-Aldrich) and [γ-^{32}P] ATP (6000 Ci/mmol; MP Biomedicals, Solon, OH) in DGK buffer (0.36% Triton X-100, 20 mM imidazole, pH7.5, 20 mM NaCl, 5 mM $MgCl_2$, 0.4 mM EGTA, and 0.7 mM DTT) for 15 min at 37 °C. The product, DOPA, was extracted with $CHCl_3$/methanol/1 N HCl (67:33:100), and followed by with $CHCl_3$/methanol (2:1). The organic phase was recovered, evaporated under a nitrogen gas flow, dissolved in $CHCl_3$, and then separated by thin-layer chromatography (TLC) on a Silica gel 60 TLC plate (EMD-Merck, Rockland, MA) with $CHCl_3$/methanol/acetic acid (26:6:3) as the developing solvent. The spot corresponding to the labeled DOPA was scraped from the TLC plate and the DOPA was extracted with $CHCl_3$/methanol/water (16:32:13). The extract was mixed with 0.53 volumes of $CHCl_3$/0.58 N acetic acid (1:1), and the organic phase containing the labeled DOPA was recovered, dried and finally dissolved in $CHCl_3$.

2.10. PLA_1 assay

GST-KIAA0725p and GST-PA-PLA_1, and their mutants were expressed in 293T cells and partially purified as described in the "GST-pulldown assay" section except for the use of the lysis buffer without Triton X-100 for the washing. The GST fusion proteins bound to the beads were used directly as enzymes for the PLA_1 assays. The reactions were started by adding 100 μl of reaction buffer [50 mM Tris–HCl, pH7.5, 100 mM KCl, 100 μM DOPA (Avanti Polar Lipids) and 4.2 nM ^{32}P-labeled DOPA] to the enzyme-containing tubes. A mixture of the cold and labeled DOPAs was applied as liposomes produced by sonication. The reactions were conducted at 37 °C for 15 min, and stopped by adding 200 μl of 1 N HCl and 400 μl of $CHCl_3$/methanol (2:1). The substrate DOPA and the product 1-oleoyl-lysophosphatidic acid (LPA) were recovered from the organic phase, dried, dissolved in $CHCl_3$ and then separated by TLC as described above. The radioactive DOPA and LPA on the TLC plate were quantified with a Bioimage Analyzer FLA-9000 (Fuji-Film, Tokyo, Japan).

2.11. Others

Prediction of domain structures and coiled-coil motifs for human $iPLA_1$ family proteins was carried out using the ExPASy Motifscan (http://hits.isb-sib.ch/cgi-bin/ PFSCAN) and COILS (http://www.ch.embnet.org/software/COILS_form.html) programs.

3. Results

3.1. KIAA0725p and p125 are novel PIP-binding proteins

To obtain a clue as to the mechanism by which KIAA0725p is targeted to Golgi/ERGIC membranes, we first examined its lipid-binding properties. Purified GST or GST-KIAA0725p was incubated with nitrocellulose membranes spotted with various phospholipids, and binding was detected with an anti-GST antibody. GST-KIAA0725p specifically bound to three PIPs, i.e. PI(3)P, PI(4)P and phosphatidylinositol 5-phosphate (PI(5)P) (Fig. 1A, middle panel), while negative control GST did not bind to any lipid (left panel). The binding affinities to the PIPs were similar although binding to PI(4)P seemed to be slightly tighter than that to the others (Supplemental Fig. S1). These interactions are independent of its phospholipase activity because the GST-KIAA0725p S351A mutant, which has no detectable phospholipase activity [12], bound to the phosphoinositides as well as the KIAA0725p wild-type (WT) did (Fig. 1A, right). p125, the other membrane-associated member, also bound to the three PIPs, whereas cytosolic isoform PA-PLA_1 did not (Fig. 1B). To confirm the PIP binding of KIAA0725p, we performed a liposome binding assay (Fig. 1C). In this experiment, we used the lipase-defective mutant to exclude possible cleavage of lipids in the liposomes during the assay incubation and centrifugation. For comparison, the GST-PH domain of phospholipase C δ1 was used. The GST-KIAA0725p S351A mutant more tightly bound to liposomes containing PI(4)P (Fig. 1C, lane 4) compared with to control liposomes without PIP (lane 2). However, in contrast to the results obtained with a membrane strip assay, the GST-KIAA0725p S351A mutant also bound to $PI(4,5)P_2$, although the extent of the binding was much less than that of the GST-PH domain of phospholipase C δ1 (lane 6). These different results regarding the binding to $PI(4,5)P_2$ in membrane strip and liposome binding assays may be ascribable to different states of $PI(4,5)P_2$ in nitrocellulose

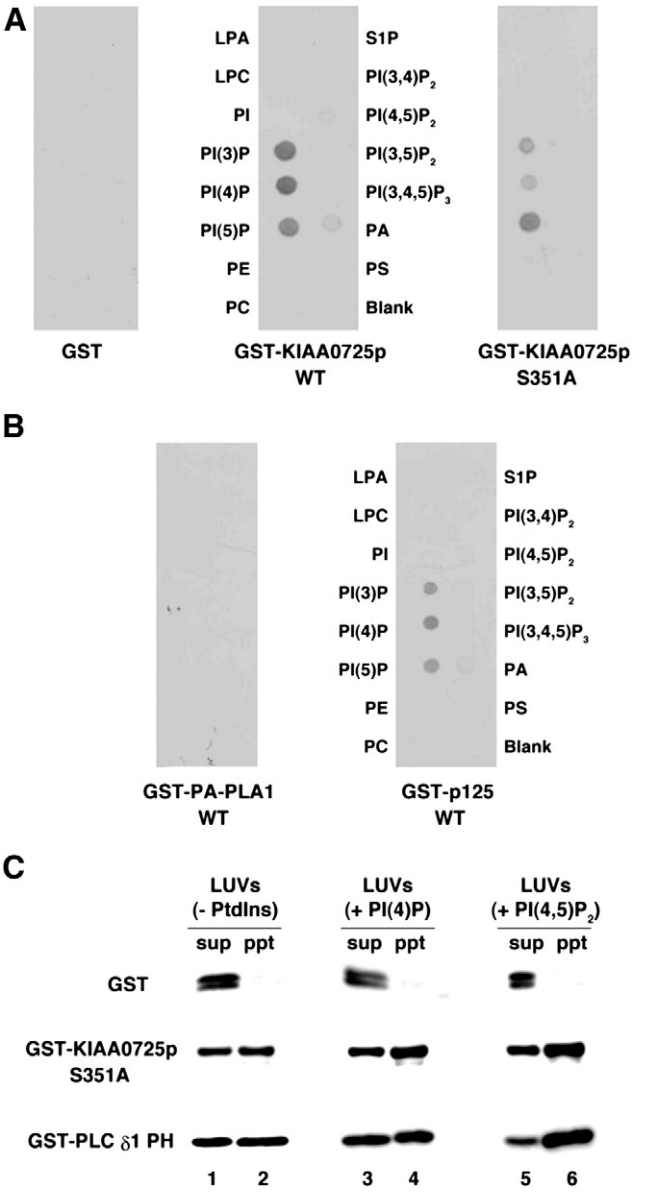

Fig. 1. KIAA0725p and p125 are novel PIP-binding proteins.A, GST (negative control), GST-KIAA0725p and GST-KIAA0725p S351A expressed in and purified from Sf9 cells were incubated overnight at 4 °C with PIP strips (Echelon Bioscience). The binding of the proteins to spotted lipids was detected using an anti-GST antibody. LPC and S1P denote lysophosphatidylcholine and sphingosine 1-phosphate, respectively. B, GST-PA-PLA_1 and GST-p125 were expressed and purified, and their lipid binding activity was examined as described in A. C, purified GST (top), GST-KIAA0725p S351A (middle), and the GST-phospholipase Cδ1 PH domain (bottom) were incubated for 10 min at 30 °C with LUVs containing no PIP (lanes 1 and 2), PI(4)P (lanes 3 and 4), or $PI(4,5)P_2$ (lanes 5 and 6), and then the binding was detected by immunoblotting using an anti-GST antibody.

membranes and liposomes. These results suggest that KIAA0725p binds to PIPs including PI(3)P, PI(4)P, PI(5)P and possibly PI(4,5)P_2.

3.2. The SAM-DDHD domain is the element responsible for PIP binding of KIAA0725p

As KIAA0725p does not have any well-known phosphoinositide-binding domain such as a PH domain, we attempted to determine the minimal domain(s) of KIAA0725p for binding to phosphoinositides. Given that the WWE and SAM domains are present in membrane-associated forms (p125 and KIAA0725), but absent in a cytosolic species (PA-PLA_1) (Fig. 2A), these domains are candidates for a novel PIP-binding domain. We first constructed four mutants (Fig. 2B) and performed a PI(4)P-binding assay using nitrocellulose strips. The C-terminal half comprising the SAM and DDHD domains (GST-SAM-DDHD) bound to PI(4)P (Fig. 2C, top right panel) as or more strongly than the full-length KIAA0725p WT (middle left panel), while the DDHD domain (GST-DDHD) (middle right panel), the SAM domain (GST-SAM) (bottom right panel), and the N-terminal half containing the WWE domain (GST-WWE-Lipase) (bottom left panel) exhibited no binding activity. These results suggest that the tandem SAM-DDHD domain is a minimal region for PIP-binding.

The PH domains of OSBP and FAPP can themselves be targeted specifically to the *trans*-Golgi network [21,22]. Thus, we examined whether or not the SAM-DDHD domain could itself be localized in the Golgi/ERGIC. Immunofluorescence microscopy showed that the SAM-DDHD domain of KIAA0725p tagged with GST was not in the Golgi/ERGIC, but diffusely spread over the cytoplasm (Supplemental Fig. S2, top right panels), as for other deletion mutants, suggesting that the SAM-DDHD domain itself does not operate as a Golgi/ERGIC-targeting module.

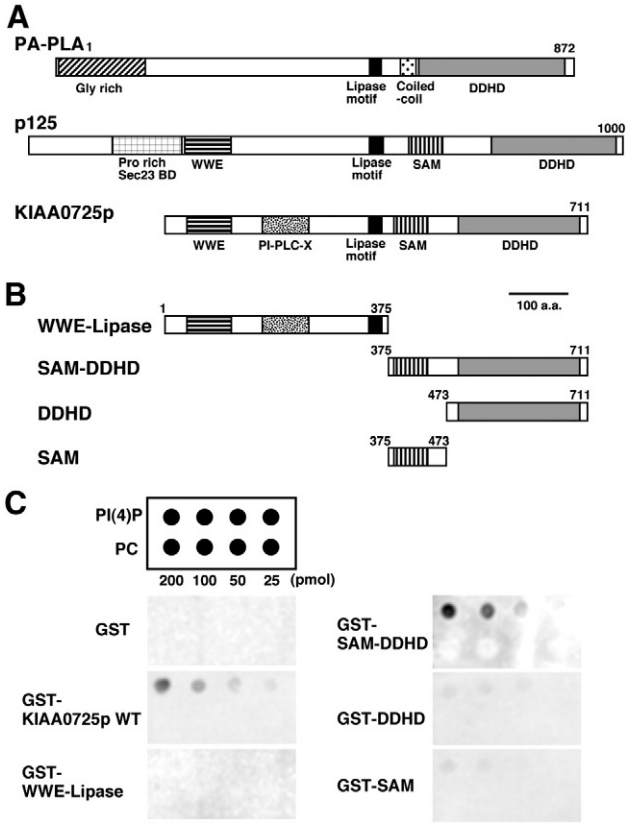

Fig. 2. The SAM-DDHD domain of KIAA0725p is a novel PIP-binding domain.A, schematic representation of the domain structure of $iPLA_1$ family proteins. The number at the upper right indicates the number of amino acid residues in each protein. B, schematic representation of KIAA0725p deletion mutants. C, PI(4)P and PC were spotted onto nitrocellulose membranes in the indicated amounts, and then the membranes were incubated with GST, GST-KIAA0725p WT or the deletion mutants expressed in 293T cells as described under "Experimental procedures." The binding of the proteins to lipids was detected as described for Fig. 1A.

3.3. A positively charged cluster in the SAM domain is required for the PIP binding and targeting of KIAA0725p to the Golgi/ERGIC

Negatively charged PIPs interact with a cluster of positively charged residues in typical PIP-binding proteins [23,24]. Since a positively charged Arg-434/Lys-435/Lys-436 cluster is present in the SAM domain of KIAA0725p, we substituted each of the residues with alanines (3RKA). The 3RKA mutant exhibited no PI(4)P-binding activity (Fig. 3A, bottom panel) and was not localized in Golgi/ERGIC membranes (Fig. 3B, lower row). The mislocalization of the mutant was not due to a gross conformational change induced by the mutation because its PLA_1 activity was comparable to or higher than that of the KIAA0725p WT (Fig. 5B). These results suggest that the R434/K435/K436 cluster of KIAA0725p is important for both PI(4)P-binding and membrane targeting.

3.4. Overexpression of KIAA0725p causes enlargement of the ERGIC in the perinuclear region

We previously reported that overexpression of KIAA0725p caused the dispersion of the Golgi visualized with β-COP and p115 staining, and the aggregation of the ER visualized with calnexin staining [12]. Here we examined the distribution of an ERGIC marker, ERGIC-53, in cells overexpressing the WT and mutants of KIAA0725p. As reported previously [25], ERGIC-53-positive punctate structures were found to be accumulated in the perinuclear region, with some at the periphery of the cells (Fig. 3C). Overexpression of KIAA0725p WT caused enlargement or aggregation of the perinuclear ERGIC concomitantly with a decrease or disappearance of the peripheral ERGIC puncta (Fig. 3C, top row). Peripheral ERGIC puncta, if any remained, were also enlarged. These effects were also observed in cells expressing the KIAA0725p S351A mutant (middle row). In contrast, the 3RKA mutant, which cannot be targeted to the Golgi/ERGIC, had no effect on the ERGIC morphology (bottom row). Quantitation showed that more than 50% of the cells expressing the KIAA0725p WT and KIAA0725p S351A mutant exhibited an aggregated ERGIC pattern, while essentially no such pattern was observed for cells expressing the 3RKA mutant (Fig. 3D).

3.5. Sac1 phosphoinositide phosphatase regulates KIAA0725p localization

PI(4)P is abundant in the Golgi and ER exit sites, and plays important roles in membrane trafficking events, including the recruitment of coat proteins and cargo sorting [26–28]. To confirm that PIP is important for the association of KIAA0725p with Golgi/ERGIC membranes, we manipulated the PIP levels in Golgi/ERGIC by using Sac1. Sac1 is a phosphoinositide phosphatase that prefers PI(3)P and PI(4)P, and is predominantly localized in the ER and Golgi under nutrient-rich and starvation conditions, respectively [27]. When WT Sac1 was expressed, the population of expressed KIAA0725p colocalized with the perinuclear ERGIC (perinuclear aggregation) was slightly decreased (Fig. 4A, second row, and quantitation in B). Because Sac1 is cycled between the ER and Golgi, some fraction of the expressed Sac1, as a consequence of overexpression, may be localized in ERGIC/Golgi membranes, leading to the dissociation of expressed KIAA0725p from aggregated ERGIC membranes. The expression of the Sac1 K2A mutant, which is deficient in the ER retrieval signal and considerably remains in the Golgi, caused drastic redistribution of KIAA0725p from both the Golgi and the perinuclear aggregates to the cytoplasmic punctate structures (Fig. 4A, third row, and B). These effects were not observed with the

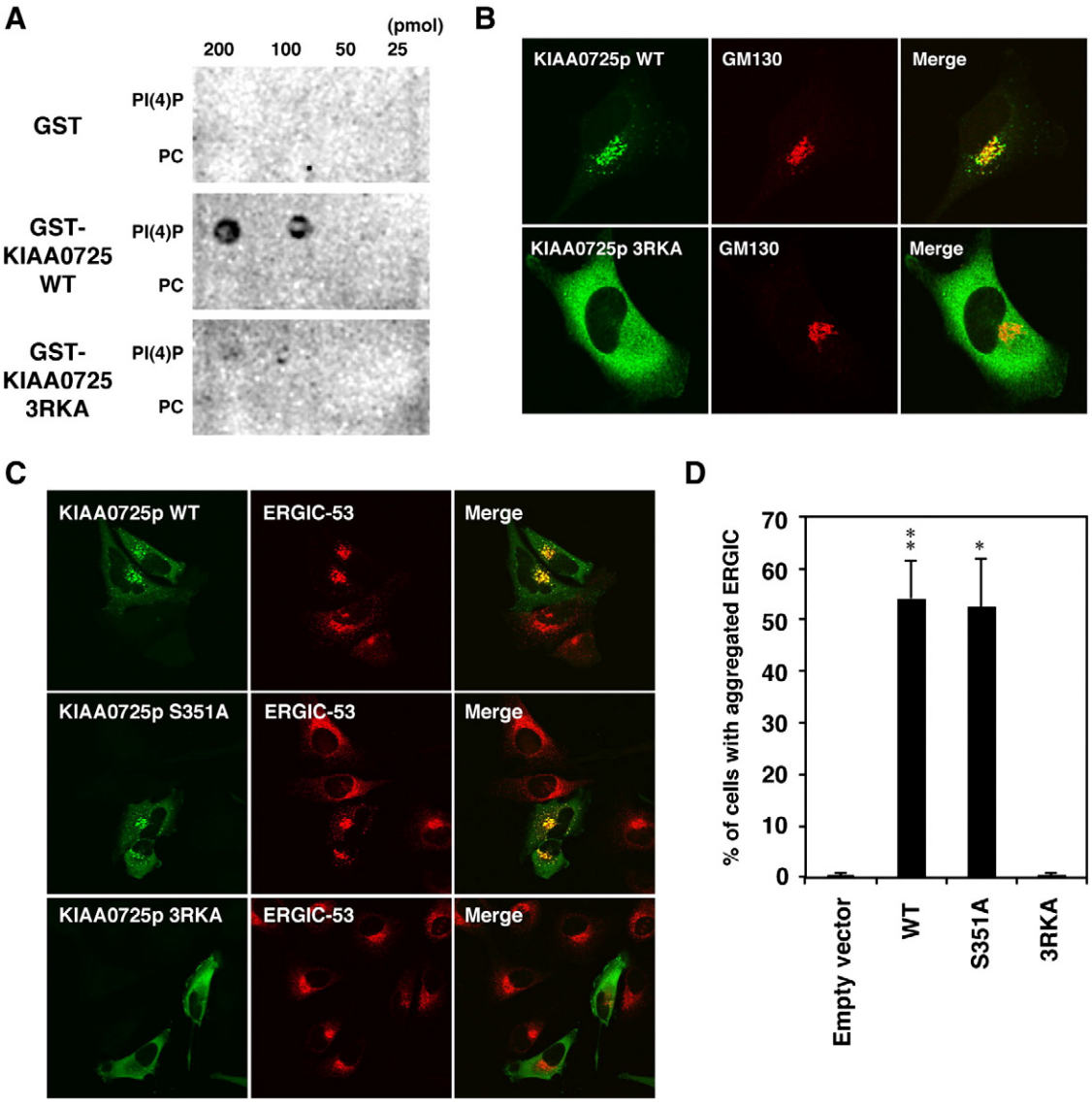

Fig. 3. A positively charged cluster in the SAM-DDHD domain is necessary for the targeting of KIAA0725p to the Golgi/ERGIC.A, GST (top panel), GST-KIAA0725p WT (middle panel), and the 3RKA mutant (bottom panel) expressed in 293T cells were incubated with PI(4)P- and PC-spotted nitrocellulose membranes, and then the binding was detected with an anti-GST antibody as described in Fig. 2C. B, the expression plasmid for FLAG-tagged KIAA0725p WT or the 3RKA mutant was transiently transfected into HeLa cells. After 18 h, the expressed proteins and GM130, a Golgi marker, were visualized with antibodies to a FLAG tag and GM130, respectively, followed by appropriate fluorophore-conjugated secondary antibodies. C, FLAG-tagged KIAA0725p WT (top row), S351A (middle row), or the 3RKA mutant (bottom row) was transiently expressed in HeLa cells, and then visualized together with ERGIC-53 as described in B. D, quantitative analysis of the ERGIC aggregation induced by overexpression of KIAA0725p WT, S351A and 3RKA mutants. In the experiments shown in C, the ratios of the cells with aggregated ERGIC among the KIAA0725p WT or mutant-transfected cells were calculated and expressed as means ± S.E. The experiments were repeated 4 times, and at least 100 cells were counted in each experiment. The asterisks indicate statistically significant differences; $^{*}P<0.05$; $^{**}P<0.01$, with Student's *t*-test in comparison with empty vector-transfected cells.

phosphatase-deficient mutant Sac1 C/S (Fig. 4A, bottom row, and B). These results suggest that the levels of phosphoinositides including PI(4)P regulate the recruitment of KIAA0725p to the Golgi/ERGIC.

3.6. The DDHD domain of KIAA0725p and PA-PLA$_1$ is essential for PLA$_1$ activity

The DDHD domain is a ~250-amino acids stretch found in iPLA$_1$ family proteins and some phosphatidylinositol transfer proteins [15–17]. The function of this domain remains unknown. To understand the role of the DDHD domain in the context of PLA$_1$ activity, we first constructed a series of deletion mutants of KIAA0725p and PA-PLA$_1$ (Fig. 5A), and measured their PLA$_1$ activities. In terms of KIAA0725p, neither of the deletion mutants showed PLA$_1$ activity (Fig. 5B). In the case of PA-PLA$_1$, on the other hand, the deletion mutant 142-DDHD and 348-DDHD, which lack the N-terminal 141 and 347 amino acids, respectively, exhibited a less but substantial activity compared with the WT enzyme (Fig. 5C). Deletion of the C-terminal 235 amino acids (Gly-CC) or 25 amino acids (Gly-847) severely abrogated the activity (Fig. 5C). It should be noted that the latter construct lacks only the C-terminal 11 amino acids of the DDHD domain, suggesting the importance of the DDHD domain for PLA$_1$ activity. The DDHD domain is named after four conserved residues, three aspartates and one histidine. To evaluate the significance of these residues in PLA$_1$ activity, these residues (Asp-662, Asp-820, His-839 and Asp-847) were individually substituted with an alanine. Of the four substitutions, those of two aspartates and the histidine (Asp-662, Asp-820 and His-839) markedly abolished the PLA$_1$ activity (Fig. 5D). These results suggest that the integrity of the DDHD domain is necessary for the PLA$_1$ enzymatic activity.

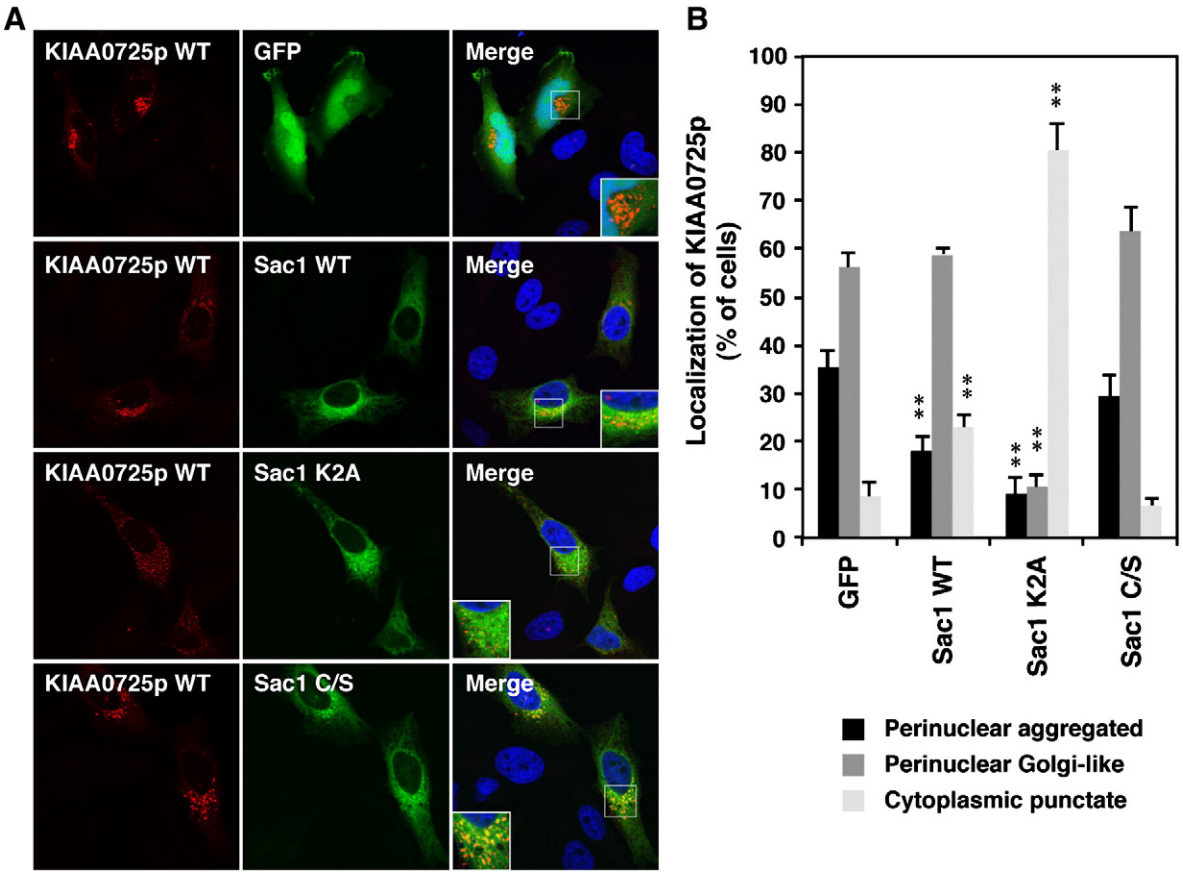

Fig. 4. Sac1 phosphatase reduces the perinuclear accumulation of KIAA0725p-positive vesicles.A, FLAG-tagged KIAA0725p WT was transiently expressed in HeLa cells together with GFP-tagged Sac1 WT, ER-retention signal defective mutant K2A or phosphatase activity-negative mutant C/S. After 18 h, the cells were fixed, stained, and analyzed by confocal microscopy. Enlarged images are shown in insets. B, quantitative analysis of the effect of Sac1 expression on KIAA0725p localization. In the experiments shown in A, the ratios of the cells with the indicated localization of KIAA0725p among the GFP-positive cells were calculated and expressed as means ± S.E. The experiments were repeated 4 times, and at least 100 cells were counted in each experiment. The asterisks indicate statistically significant differences; $^{**}P<0.01$, with Student's *t*-test in comparison with GFP-transfected cells in each data set.

3.7. KIAA0725p forms preferentially homooligomers between the N-terminal half and DDHD domain

PA-PLA$_1$ forms homooligomers, probably homotetramers [4]. We examined whether or not KIAA0725p also forms homooligomers and, if so, whether or not it also forms heterooligomers with PA-PLA$_1$. GST-tagged PA-PLA$_1$ or KIAA0725p was expressed with FLAG-tagged versions in 293T cells, and their interactions were analyzed by GST pulldown assays and immunoblotting. As shown in Fig. 6A, both PA-PLA$_1$ and KIAA0725p preferentially formed homooligomers (lanes 4 and 9, top panel), although small amounts of heterooligomers were also formed (lanes 6 and 7, top panel).

To determine which region(s) are responsible for the oligomer formation, we first mutated the coiled-coil region, which is present between the lipase motif and the DDHD domain of PA-PLA$_1$ (Fig. 5A). We constructed two mutants in which the coiled-coil structure was destroyed by a deletion or point mutations. The COILS program predicted complete abrogation of the coiled-coil structure of the two mutants (data not shown). GST pulldown assaying showed that both mutants formed homooligomers comparable with WT PA-PLA$_1$ (Fig. 6B, lanes 3 and 4, top panel), suggesting that the coiled-coil domain is not necessary for oligomer formation by PA-PLA$_1$. We then used a series of deletion mutants (Figs. 2A and 5A). Unexpectedly, each of the tested deletion mutants except for the SAM domain construct of KIAA0725p and the Gly-rich domain construct of PA-PLA$_1$ interacted with its own full-length form (Fig. 6C, top panel). These results suggest that multiple sites are involved in the oligomer formation. To test this possibility, we examined whether or not the DDHD domain of KIAA0725p (Fig. 2B) binds to the N-terminal half of the molecule lacking the DDHD domain (WWE-SAM; Fig. 5A). A GST pulldown assay demonstrated that the DDHD domain more strongly associates with the WWE-SAM construct than with the full-length form (Fig. 6D, lane 3, top panel). In contrast, no binding between the DDHD domains was observed (lane 4, top panel). The interaction between the WWE-SAM and the DDHD domain was not modulated by PI(4)P (Fig. 6E). These results suggest that KIAA0725p forms oligomers through multiple interactions between different regions, including an interaction between the DDHD domain and the N-terminal half of the molecule in a PI(4)P-independent manner.

4. Discussion

In this study, we showed that KIAA0725p is a novel phosphoinositide-binding protein. Deletion and point mutation analyses demonstrated that the minimal region for the binding is a unique tandem SAM-DDHD domain. The R434/K435/K436 positively charged cluster in the SAM domain is critical for both PIP binding and targeting of KIAA0725p to the Golgi/ERGIC, but neither for its PLA$_1$ activity nor its homooligomer formation. On the other hand, the DDHD domain is essential for its enzymatic activity and oligomer formation.

Specific phosphoinositides are accumulated in particular organelle membranes, and modulate membrane sorting and deformation machineries [1,2]. PI(4)P is present in the ER and Golgi apparatus. Recent studies suggested that PI(4)P plays an important role in membrane trafficking from the ER to the Golgi [26,29,30]. Our *in vitro* lipid overlay and liposome sedimentation assays showed that KIAA0725p interacts with three PIPs, i.e. PI(3)P, PI(4)P and PI(5)P,

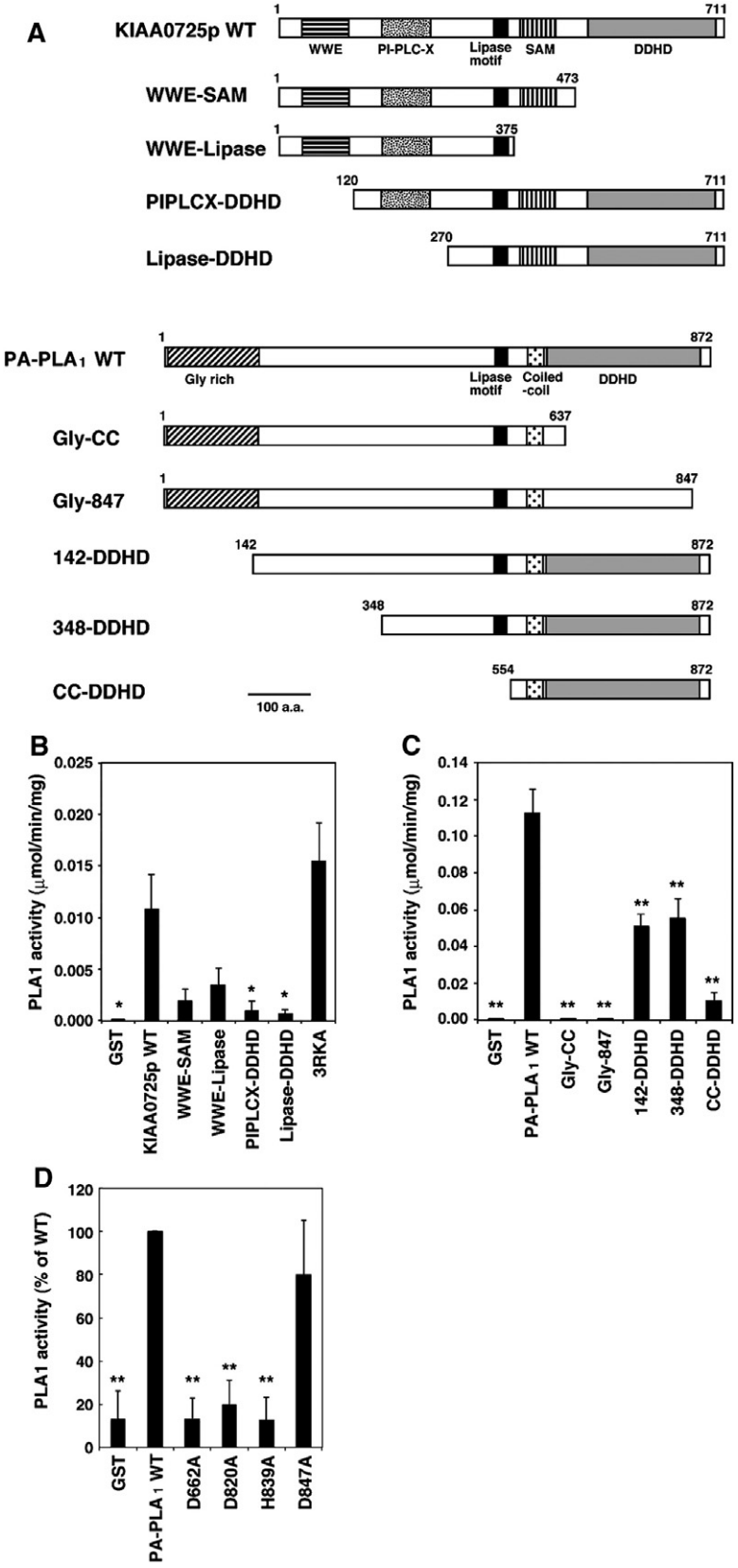

Fig. 5. The DDHD domain and conserved residues D, D and H are important for PLA_1 activity.A, schematic representation of the deletion mutants of KIAA0725p and PA-PLA_1 used for PLA_1 assaying. The DDHD domain of PA-PLA_1 encompasses amino acids 611–858. The Gly-847 mutant lacks the C-terminal 11 amino acids of the DDHD domain. GST-tagged KIAA0725p deletion mutants (B), PA-PLA_1 deletion mutants (C), or PA-PLA_1 point mutants (D) were expressed in 293T cells and partially purified, and then their PLA_1 activities were measured using ^{32}P-labeled DOPA as a substrate. The asterisks indicate statistically significant differences; $^{*}P<0.05$; $^{**}P<0.01$, with Student's *t*-test in comparison with WT.

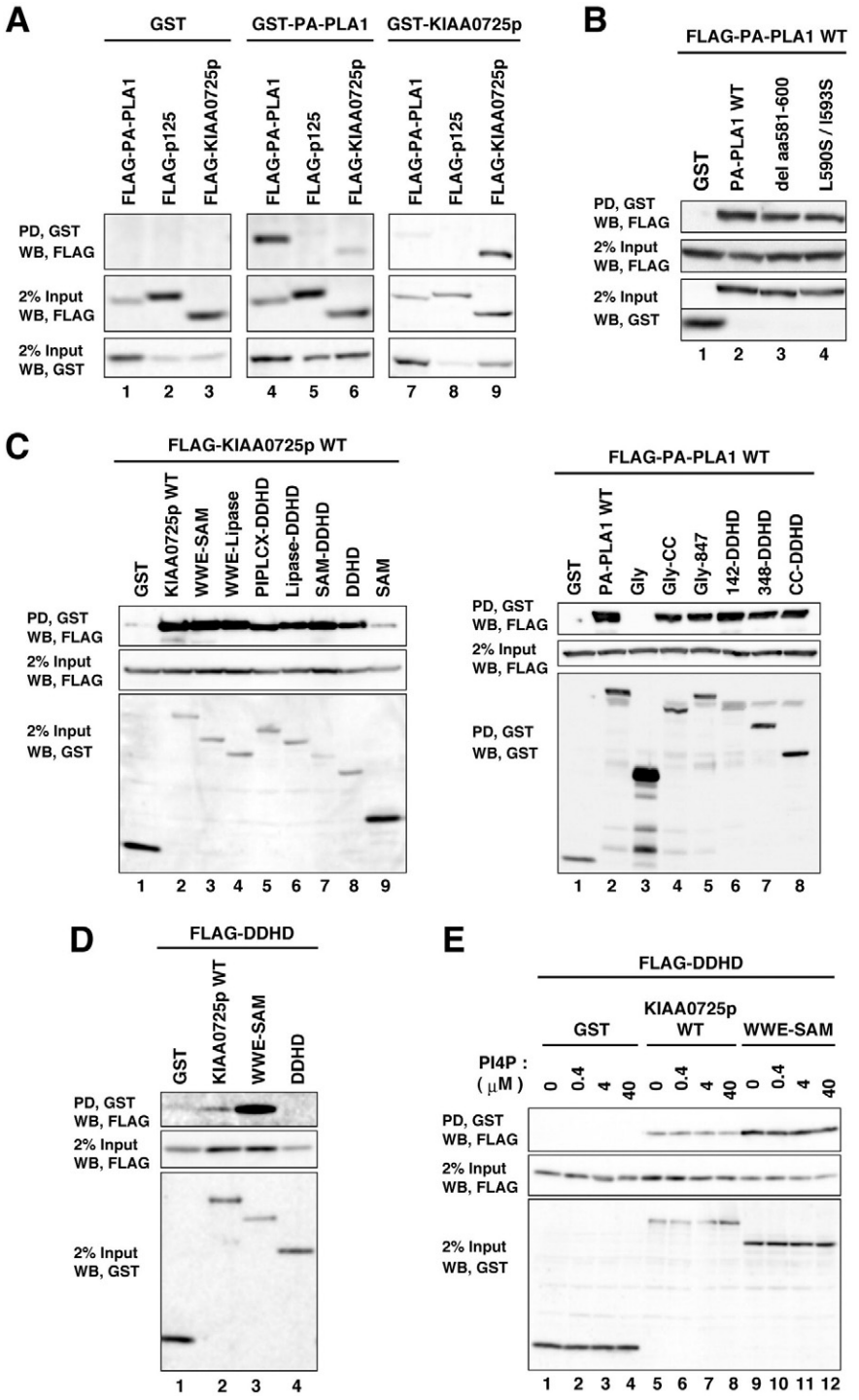

Fig. 6. KIAAA0725p forms homooligomers through interaction between the DDHD domain and the N-terminal half of the molecule.A, the expression vector encoding GST, GST-PA-PLA_1 or KIAA0725p was transfected in 293T cells together with the vector for FLAG-PA-PLA_1, p125 or KIAA0725p. 18 h after transfection, the cells were harvested and lysed, and GST-tagged proteins were pulled down (PD) using glutathione beads. The precipitated materials were analyzed by immunoblotting with an anti-FLAG antibody (top panel). 2% input was also analyzed by immunoblotting with antibodies to FLAG (middle panel) and GST (bottom panel). B, GST, GST-PA-PLA_1 or GST-PA-PLA_1 mutants with deletion or point mutations in the coiled-coil region were transiently expressed together with FLAG-PA-PLA_1 WT in 293T cells, and then GST pulldown experiments were performed as described in A. C, GST-tagged deletion mutants of KIAA0725p or PA-PLA_1, shown in Figs. 2B and 5A, and the Gly-rich domain construct (amino acids 1–176) of PA-PLA_1 were transiently expressed in 293T cells together with their WT FLAG-tagged constructs, and then GST pulldown assays were performed as described in A. D, GST, GST-KIAA0725p, or the WWE-SAM or DDHD construct was transiently expressed together with FLAG-DDHD in 293T cells, and then GST pulldown experiments were performed as described in A. E, GST pulldown experiments were conducted as described in D in the presence of the indicated concentrations of PI(4)P.

with similar affinities. As KIAA0725p was found to be associated with the Golgi/ERGIC, it is tempting to speculate that a primary target of KIAA0725p is PI(4)P. However, it should be noted that the affinities of KIAA0725 for PIPs may not be high enough to target it to membranes *in vivo*. The tandem SAM-DDHD domain, which is responsible for PIP binding, failed to be targeted to the Golgi/ERGIC. In addition to PI(4)P, KIAA0725p may require proteins residing in the Golgi/ERGIC for its association with membranes. Alternatively, oligomerization may contribute to stable binding of KIAA0725 to target membranes.

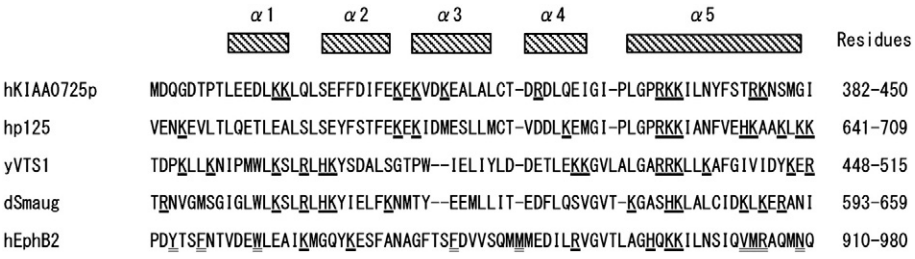

Fig. 7. Alignment of SAM domains.Positively charged residues and the residues of EphB2 involved in hydrophobic interactions for oligomer formation are underlined and double-underlined, respectively. Hatched bars indicate the five α-helices in the SAM domain. h, human; y, yeast; d, *Drosophila*.

The SAM domain is a widespread motif in signaling and nuclear proteins. SAM domains are among the most abundant protein–protein interaction motifs, including in protein oligomerization and RNA binding [31]. Some studies suggested that SAM domains also interact with lipids [32–34], but the physiological significance of this remains unknown. In the present study, we demonstrated that the SAM domain of KIAA0725 constitutes a part of the PIP binding site. This finding is consistent with that both KIAA0725 and p125 bind to PIPs, whereas PA-PLA_1 does not. The former two homologues contain a SAM domain, whereas the latter has a coiled-coil region instead of a SAM domain (Fig. 2A).

Fig. 7 shows alignment of the SAM domains involved in PIP binding (human KIAA0725p and p125), RNA binding (yeast VTS1 and *Drosophila* Smaug), and oligomer formation (human EphB2). R434/K435/K436, mutations of which abolish membrane targeting, are located in the N-terminus of helix α5. This positively charged cluster is well conserved and constitutes a part of the RNA-binding site in yeast VTS1 and *Drosophila* Smaug. The other RNA binding motifs in these proteins are a positively charged cluster located in a region encompassing the C-terminus of helix α1, the ensuing loop, and most of helix α2 [35–39]. $iPLA_1$ family members lack positively charged residues in helix α2 and the preceding loop. It is possible that one positively charged cluster may be enough for binding to PIPs. Although a conserved positively charged cluster at the C-terminus of the SAM domains is present in $iPLA_1$ family members, the amino acid residues responsible for oligomer formation between the SAM domains are not conserved. The hydrophobic residues found in EphB2 (Fig. 7, double-underline), which are involved in two types of interactions for oligomer formation [40,41], are replaced by hydrophilic or charged residues in $iPLA_1$ family members. This may explain the lack of ability of the SAM domain of KIAA0725p to mediate protein–protein interactions (Fig. 6C).

The DDHD domain is conserved in $iPLA_1$ family proteins and some phosphatidylinositol transfer proteins [15–17]. We assumed that this domain of $iPLA_1$ family proteins participates in their phospholipase activity. Our mutational analysis demonstrated this assumption to be correct; substitutions of the three conserved residues in the DDHD domain severely impaired the PLA_1 activity of PA-PLA_1. In addition, our study showed that this domain plays two more roles: in PIP binding and oligomerization. To exhibit phospholipase activity, almost the full-length KIAA0725p protein, which encompasses from the N-terminal WWE domain to the C-terminal DDHD domain, is necessary. As WWE domains mediate protein–protein interactions in general [42], this domain in KIAA0725p may contribute to the expression of PLA_1 activity through an interaction with the C-terminal DDHD domain or any other domains. While the ExPASy Motifscan program does not predict the existence of a WWE domain in the N-terminal region of PA-PLA_1 because of a short insertion, a typical sequence for a WWE domain [W-(bulky)-(hydrophobic)-X(5–10)-W/F/Y-X-X-W/F/Y-X(5–16)-(aliphatic)-E/D/Q/R] appears to be present in a region following the Gly-rich domain. Since the mutant 348-DDHD, which lacks this region, exhibited a PLA_1 activity comparable to that of the mutant 142-DDHD, this WWE-like domain may not function. Alternatively, this WWE-like domain and a coiled-coil region located on the N-terminal side of the DDHD domain, both of which are unique in PA-PLA_1, may be involved in the regulation of this enzymatic activity by PI [43].

In conclusion, we have demonstrated a novel mechanism by which KIAA0725p is targeted to the Golgi/ERGIC. KIAA0725p binds to phosphoinositides through a positively charged cluster in the SAM domain. The DDHD domain in PLA_1 family proteins is a multifunctional domain for PI(4)P binding, oligomer formation and enzymatic activity.

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