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The PI3K effector Arap3 interacts with the PI(3,4,5)P_3 phosphatase SHIP2 in a SAM domain-dependent manner

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Abstract

Arap3 is a phosphoinositide (PI) 3 kinase effector that serves as a GTPase activating protein (GAP) for both Arf and Rho G-proteins. The protein has multiple pleckstrin homology (PH) domains that bind preferentially phosphatidyl-inositol-3,4,5-trisphosphate (PI(3,4,5,)P_3) to induce translocation of Arap3 to the plasma membrane upon PI3K activation. Arap3 also contains a Ras association (RA) domain that interacts with the small G-protein Rap1 and a sterile alpha motif (SAM) domain of unknown function. In a yeast two-hybrid screen for new interaction partners of Arap3, we identified the PI 5′-phosphatase SHIP2 as an interaction partner of Arap3. The interaction between Arap3 and SHIP2 was observed with endogenous proteins and shown to be mediated by the SAM domain of Arap3 and SHIP2. In vitro, these two domains show specificity for a heterodimeric interaction. Since it was shown previously that Arap3 has a higher affinity for PI(3,4,5,)P_3 than for PI(3,4)P_2, we propose that the SAM domain of Arap3 can function to recruit a negative regulator of PI3K signaling into the effector complex.

Keywords: Arap3; SHIP2; PI3K; SAM domain; GTPase activating protein; Actin cytoskeleton

1. Introduction

The phosphoinositide (PI) 3 kinase (PI3K) pathway plays an important role in various signaling pathways, such as insulin signaling, membrane trafficking and the regulation of cell dynamics, via production of the second messenger PI(3,4,5,)P_3 [1,2]. Arap3 (*A*rf GAP, *R*ho GAP, *A*nkyrin repeat and *P*H domains) is a PI3K effector protein that was first identified through its ability to bind PI(3,4,5,)P_3 lipids [3]. Upon binding of PI(3,4,5,)P_3 lipids to one of its pleckstrin homology (PH) domains, Arap3 translocates to the plasma membrane and is activated to serve as a dual GTPase activating protein (GAP) for Arf and Rho G-proteins [4]. Arap3 is implicated in the regulation of the actin cytoskeleton, lamellipodia formation and cell spreading [5,6]. In addition, Arap3 contains an RA domain that binds specifically to the small G-protein Rap1 and a SAM domain of unknown function [4] (Fig. 1A). The SAM domain is a 60–70 amino acid motif that mediates protein–protein, protein–RNA and protein–lipid interactions [7,8]. SAM domains are found in over 1000 proteins with diverse cellular functions and in organisms from yeast to man [9]. They mediate protein–protein interactions by either homo- or heterodimerization or through oligomerization [10–12]. Thus far, the function and the binding partner of the Arap3 SAM domain are unknown. Besides PI(3,4,5,)P_3 lipids and $Rap1^{GTP}$, the only known interaction partner for Arap3 is the adaptor protein CIN85 that is involved in the internalization of monoubiquitinated membrane proteins [13,14]. This interaction is mediated by a proline–arginine motif in Arap3 that is specific for the CIN85 SH3 domain (Fig. 1A).

The SH2 domain-containing inositol 5′-phosphatase SHIP2 hydrolyzes PI(3,4,5)P_3 to PI(3,4)P_2 [15,16]. It is ubiquitously expressed and, together with SHIP1 and PTEN,

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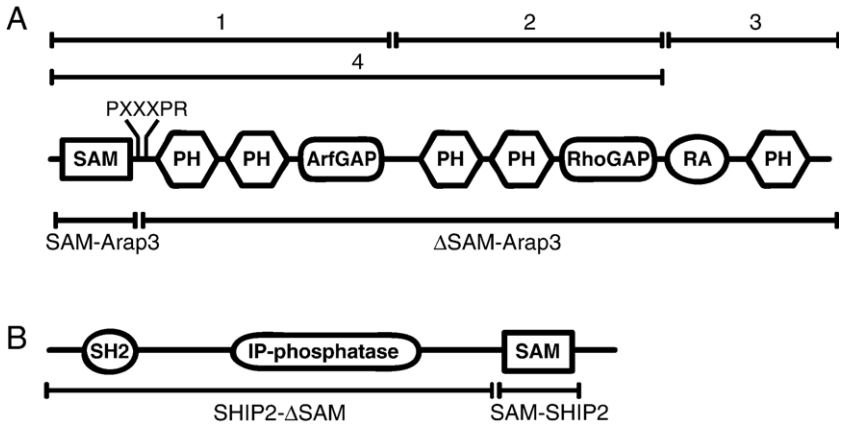

Fig. 1. Domain composition of Arap3 and SHIP2. Schematic representation of the domain structure of (A) Arap3 and (B) SHIP2. Numbers above indicated parts of Arap3 show the truncation mutants used in the Y2H screen. Both Arap3-1 and Arap3-4 interacted with SHIP2. The CIN85-binding proline–arginine motif in Arap3 is indicated as well.

inhibits PI3K-activated signaling pathways [17]. Studies in SHIP2 knockout mice suggest that SHIP2 plays a role in controlling insulin sensitivity and obesity, probably by decreasing the level of active protein kinase B (PKB) [18,19]. Like Arap3, SHIP2 has several protein–protein interaction domains. Besides its SH2 domain that mediates the recruitment of SHIP2 to activated receptor tyrosine kinases [20,21] and its catalytic phosphatase domain, SHIP2 has a proline-rich region followed by a C-terminal SAM domain. Several interaction partners are known for SHIP2, including the HGF receptor c-Met [21], the E3 ubiquitin ligase Cbl and Cbl-associated protein (CAP) [22]. In addition to its function in down regulating the insulin pathway, SHIP2 is also linked to the regulation of the actin cytoskeleton and cell adhesion, mainly by its ability to bind proteins such as filamin [23], vinexin [24], p130Cas [25] and Shc [26,27]. A role in endocytosis and the down regulation of the EGF and EphA2 receptors has also been proposed for SHIP2 [28,29]. Furthermore, after growth factor stimulation or adhesion, SHIP2 becomes phosphorylated and can relocalize to membrane ruffles [26,28]. Thus, SHIP2 can regulate changes in PI(3,4,5,)P_3 levels and is involved in the organization of the actin cytoskeleton. Until recent, binding partners were known only for the SHIP2 SH2 domain [21,25,27] and proline-rich region [22–24], not for its SAM domain, but it has now been shown that the EphA2 receptor binds to SHIP2 through dimerization of both SAM domains [29].

To get more insight into the role of Arap3 as a downstream PI3K effector protein, we looked for new interaction partners of Arap3. Here, we describe the identification of the lipid phosphatase SHIP2 as a binding partner of Arap3 and show that the interaction is mediated by heterodimerization of their SAM domains. We show that the SAM domains are both necessary and sufficient for this interaction and that the two domains have a high affinity for one another. Since Arap3 is a protein regulated by PI(3,4,5)P_3, the substrate for SHIP2, we propose that the SAM domain of Arap3 can function to recruit a negative regulator of PI3K signaling into the effector complex.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal antibodies recognizing the FLAG-M2 epitope and the penta-His epitope were obtained from Sigma and Qiagen, respectively. Rabbit polyclonal anti-SHIP2 antibody and sheep anti-Arap3 antibody were described before [3,15]. Where indicated, cells were stimulated with 20 ng/ml EGF (ICN Biomedicals Inc.), 1 μg/ml insulin (Sigma) and 10 μM LY294002 (Sigma).

2.2. Plasmids and constructs

GFP-ΔSAMArap3 (residues 71–1544) was made using mutagenesis PCR with GFP-Arap3 [3] as a template. FlagHis-tagged Arap3 was created using Gateway Technology (Invitrogen). FlagHis-ΔSAMArap3 (residues 71–1544) was also made using mutagenesis with FlagHis-Arap3 as a template. His-tagged SHIP2 and His-t-SHIP2 have been described before [20]. His-SHIP2-ΔSAM (residues 1–1192) was made using mutagenesis PCR. The GST-tagged SAM domains of Arap3 (residues 1–75) and SHIP2 (residues 1192–1258) were made by inserting *Sal*I/*Not*I-digested PCR products into *Xho*I/*Not*I-digested pGEX4T3 vector (Pharmacia). HA-RapV12 and HA-RapGAP were described previously [30,31].

2.3. Yeast two-hybrid screen

Four different Arap3 truncation constructs (residues 1–607, 608–1089, 1089–1544 and 1–1089) were PCR-amplified, cloned into a plasmid derived from pBTM116 using Gateway Technology and sequence verified. Yeast two-hybrid screening was carried out by Hybrigenics S.A. (Paris, France) as previously described [32].

2.4. Cell culture and transfections

HEK293T, HeLa and MEF cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and glutamine. Cells were transfected using FuGENE6 transfection reagent according to manufacturer's instructions (Roche). Typically, for a 10-cm culture dish, 2 μg DNA was used per construct. Where indicated, cells were serum starved overnight in DMEM with supplements but without FBS.

2.5. Co-immunoprecipitations

Cells were washed twice in ice-cold PBS, lysed in lysis buffer (1% Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl and protease inhibitors) and lysates were centrifuged at 14000 rpm at 4 °C for 8 min. After centrifugation, samples

Table 1
Proteins identified as Arap3 binding partners in a yeast two-hybrid screen

Protein	Interacts with	Identified with	Function
SHIP2*	SAM domain	Arap3_1 and Arap3_4	Inositol 5′ phosphatase—down regulation of insulin signaling, regulation of actin cytoskeleton
CIN85	SH3 domain (SH3B)	Arap3_1 and _4	Adaptor protein—endocytosis
CMS	SH3 domain (SH3B)	Arap3_1 and _4	Adaptor protein—endocytosis
TNKS2*	Ankyrin repeats	Arap3_1 and _4	ADP-ribose polymerase—regulation of telomere length
ANKS1*	SAM domain	Arap3_1 and _4	Unknown
αPix/ARHGEF6	SH3 and part of RhoGEF domain (DH)	Arap3_1	Guanine nucleotide exchange factor for Rac and Cdc42—regulation of actin cytoskeleton
GGA3	Alpha-adaptin C2 domain	Arap3_3	Adaptor protein—trafficking between the trans-Golgi network and the lysosome
AP3 μ subunit	C-terminus	Arap3_3	Member of the clathrin-associated adaptor complex, involved in vesicle budding and protein sorting
Par-6 beta	PDZ domain and C-terminus	Arap3_1	Asymmetrical cell division and cell polarization
SAMHD1*	SAM and HD domain	Arap3_1	Phosphodiesterase

Several binding partners of Arap3 identified in a yeast two-hybrid screen are listed. The interacting regions are indicated in the second column and previously reported functions of the identified proteins are mentioned. Proteins indicated with an* contain a SAM domain. Full names of all abbreviated proteins: SH2 domain containing inositol phosphatase 2 (SHIP2); Cbl interacting protein of 85 kDa (CIN85) [13]; Cas ligand with multiple SH3 domains (CMS); Tankyrase 2 (TNKS2); ankyrin repeat and SAM domain containing 1(ANKS1); Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 (ARHGEF6); Partitioning defective 6 homolog beta (Par-6 beta); Golgi-associated, gamma adaptin ear containing ARF binding protein 3 (GGA3); SAM domain and HD domain 1 (SAMHD1).

were taken to analyze total cell lysate, the rest was incubated with protein agarose beads and either non-immune serum or the appropriate antibody for 2 h at 4 °C. After incubation, precipitates were washed 3 times with lysis buffer before dissolving bound proteins in Laemmli sample buffer. Protein samples were separated by SDS–PAGE and transferred to polyvinylidene difluoride membrane (PVDF, Immobilon). Western blot analysis was performed under standard conditions using the indicated antibodies. Membranes were probed with fluorophore-conjugated secondary antibodies and analyzed using the Odyssey Infra-red imaging system and software according to the manufacturer (LI-COR) or with horseradish peroxidase-coupled secondary antibodies and standard enhanced chemiluminescence (Amersham).

2.6. GST pull down assays

Cells were lysed and centrifuged as described. Glutathione–agarose beads were washed twice in lysis buffer and incubated with equal amounts of GST, GST-SAM-Arap3 or GST-SAM-SHIP2 for 30 min at 4 °C. Beads were washed three times with lysis buffer and incubated with lysate for 1 h at 4 °C. After incubation, beads were washed three times with lysis buffer before dissolving bound protein in Laemmli sample buffer. Bound proteins were analyzed as described above.

2.7. Protein purification, gel filtration and ITC

The SAM domains of Arap3 and SHIP2 were expressed from pGEX-4T3 (Pharmacia) as GST-fusion proteins in BL21 cells. Bacteria were grown at 37 °C and 170 rpm in Standard I medium (Merck). Protein expression was induced after an OD_{600} of 0.8 was reached and the bacteria were cultured overnight at room temperature, collected by centrifugation, re-suspended in 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5% glycerol, 5 mM DTE and 5 mM EDTA and lysed by sonication. Insoluble material was removed by centrifugation at 30,000×*g* and the soluble fraction was loaded onto a 20-ml Glutathione-column (Pharmacia). The column was washed with at least 5 volumes of 50 mM Tris–HCl pH 7.5, 400 mM NaCl, 5% glycerol and 5 mM DTE and 2 volumes of 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$ 5% glycerol and 5 mM DTE (buffer T). The column was loaded with 200 Units Thrombin (Serva) in buffer T, incubated overnight at 4 °C and eluted with buffer T. Protein containing fractions were concentrated using a Millipore concentrator unit (cut off 5 kDa) to a concentration of approximately 200 g/l.

Gel filtration experiments were carried out on a Sephacryl 100 (26/60) column (Pharmacia) equilibrated with 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2.5% glycerol and 5 mM DTE.

For ITC experiments, the buffer was exchanged to 100 mM K-phosphate, 50 mM NaCl and 5 mM DTE by gel filtration. Prior to loading onto the column, the protein solution was diluted in an equal volume of phosphate buffer and calcium phosphate was removed by centrifugation. ITC experiments were carried out at 25 °C using a VP-ITC instrument (MicroCal, USA).

3. Results

3.1. Identification of a SAM domain-mediated interaction between Arap3 and SHIP2

To identify putative regulators of Arap3, we performed a yeast two-hybrid screen of a human placenta cDNA library using four different truncation mutants of Arap3 as baits (Fig. 1A). Table 1 shows a list of ten proteins that were identified with the various Arap3 constructs. Interestingly, several of these proteins possess a SAM domain as is present in Arap3. For two of these proteins, ANKS1 and SHIP2, the fragments recovered from the yeast two-hybrid screen included the SAM domain, suggesting that these interactions were mediated by the dimerization of the SAM domains. As the inositol 5′-phosphatase SHIP2 is a known regulator of the PI3K pathway, we focused on the characterization of the interaction between Arap3 and SHIP2 (Fig. 1B). To validate the result from the yeast two-hybrid screen, we performed a co-immunoprecipitation experiment with over-expressed Arap3 and SHIP2 in HEK293T cells (Fig. 2A). Indeed, Arap3 is able to pull down full-length SHIP2 in vivo.

To verify that the endogenous proteins are in the same complex, we performed co-immunoprecipitations in HeLa cells with either anti-Arap3 or anti-SHIP2 antibody (Fig. 2B and C, respectively) and in 293T cells with anti-SHIP2 antibody (Fig. 2D). Although there is some unspecific binding of SHIP2 in the control samples where non-immune serum was used, the amount of co-precipitated protein is far higher in the lanes where anti-Arap3 antibody was used to precipitate the complex (Fig. 2B). Interestingly, while the Arap3 antibody recognizes a double band in both whole cell lysate and after immunoprecipitation with the same antibody (Fig. 2B), only the slower migrating protein is recovered with SHIP2 (Fig. 2C–D). Although a doublet has been

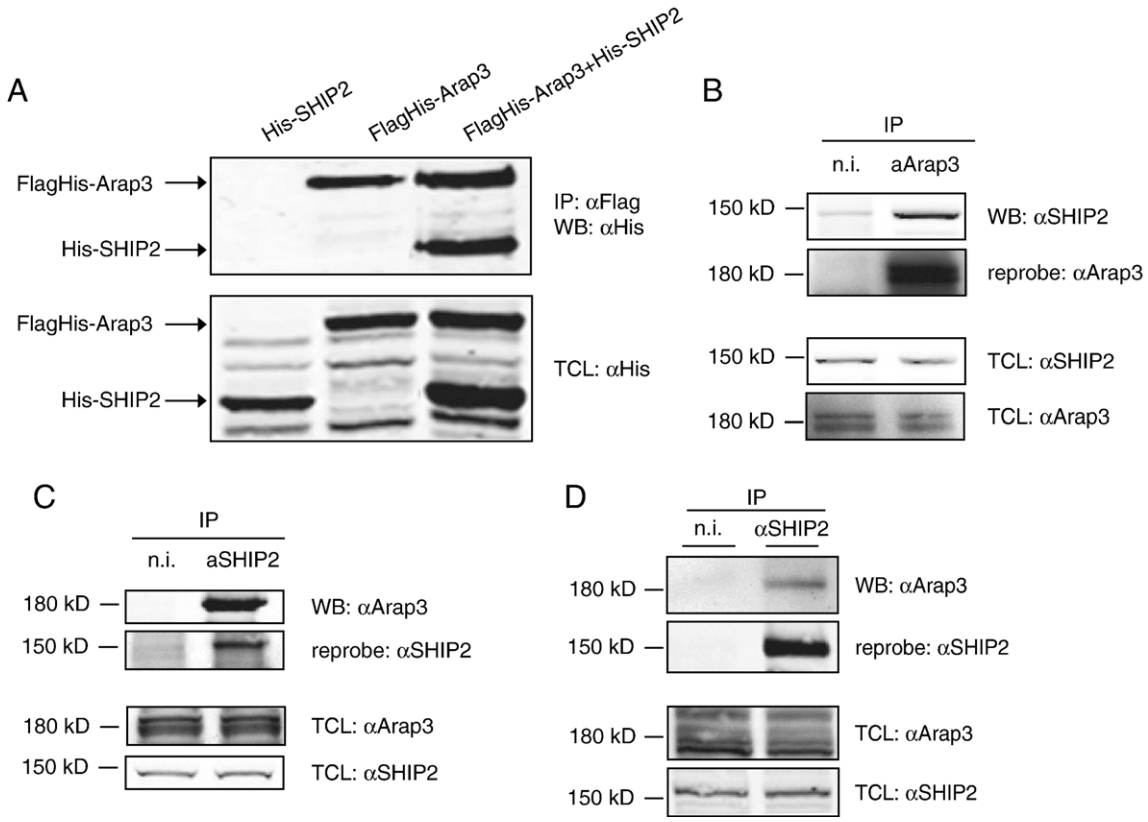

Fig. 2. Arap3 binds SHIP2 in vivo. (A) HEK293T cells were transiently transfected with the indicated constructs. Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates (IP) were analyzed for the presence of both over-expressed His-SHIP2 and FlagHis-Arap3. Total cell lysates (TCL) show total levels of transfected proteins. HeLa cell lysates were immunoprecipitated using a SHIP2 antibody or non-immune serum (n.i.) and probed for the presence of endogenous Arap3 (B) or immunoprecipitated with an Arap3 antibody and probed for the presence of endogenous SHIP2 (C). Membranes were reprobed with anti-Arap3 (B) or anti-SHIP2 (C), lower panels. (D) 293T cell lysate was immunoprecipitated using a SHIP2 antibody or non-immune serum and probed for the presence of endogenous Arap3. Blots shown are representatives of at least 3 identical experiments.

observed in other cell lines expressing Arap3 (SK, personal communication), the nature of these different bands is still unclear.

3.2. The SAM domains are both necessary and sufficient to mediate heterodimerization of Arap3 and SHIP2

As the yeast two-hybrid screen identified interacting fragments of Arap3 and SHIP2 both containing a SAM domain, we made deletion mutants of both proteins in this region (Table 1 and Fig. 1). When comparing these mutants in co-immunoprecipitation experiments, we observed that only the full-length proteins were capable of binding, confirming that the presence of both SAM domains is needed for the interaction (Fig. 3A).

We next investigated whether the SAM domains are sufficient to mediate the interaction. We made GST-fusion proteins of both SAM domains and performed in vitro GST pull down assays. As shown in Fig. 3B, the SAM domain of Arap3 indeed pulls down full-length SHIP2, but not a mutant of SHIP2 that lacks the proline-rich and SAM domain-containing C-terminus (t-SHIP2 [20]). Similarly, the SAM domain of SHIP2 only interacts with full-length Arap3, and not with the mutant lacking the SAM domain (Fig. 3C). As it is known that SAM domains can mediate the formation of both homo- and heterodimers, we wanted to determine the specificity of the SAM domains of both proteins for each other. As shown in Fig. 3B and C, neither isolated SAM domain interacted with its full-length protein, showing specificity of the SAM domains for heterodimerization.

3.3. SAM domains show specificity for a heterodimeric interaction

To further test the specificity of the interaction, we performed a GST pull down assay with the SAM domain of Arap3 in both wild type and SHIP2 knock-out mouse embryonic fibroblasts (MEFs) [24]. As shown in Fig. 3D, the SAM domain of Arap3 is sufficient to pull endogenous SHIP2 from WT MEFs. From these experiments, we conclude that the SAM domains are both required and sufficient to mediate the formation of a heterodimer between Arap3 and SHIP2.

To further analyze the properties of the heterodimeric interaction, we performed gel filtration experiments with the purified SAM domains alone or both (Fig. 4A). On a Sephacryl 100 column, we observed that the Arap3 SAM domain had a slightly longer retention time than the SAM domain of SHIP2, which could be due to differences in protein charge since the theoretical p*I* of the SAM domains is 7.1 and 4.3 for SHIP2 and Arap3, respectively. However, when both domains were combined on the

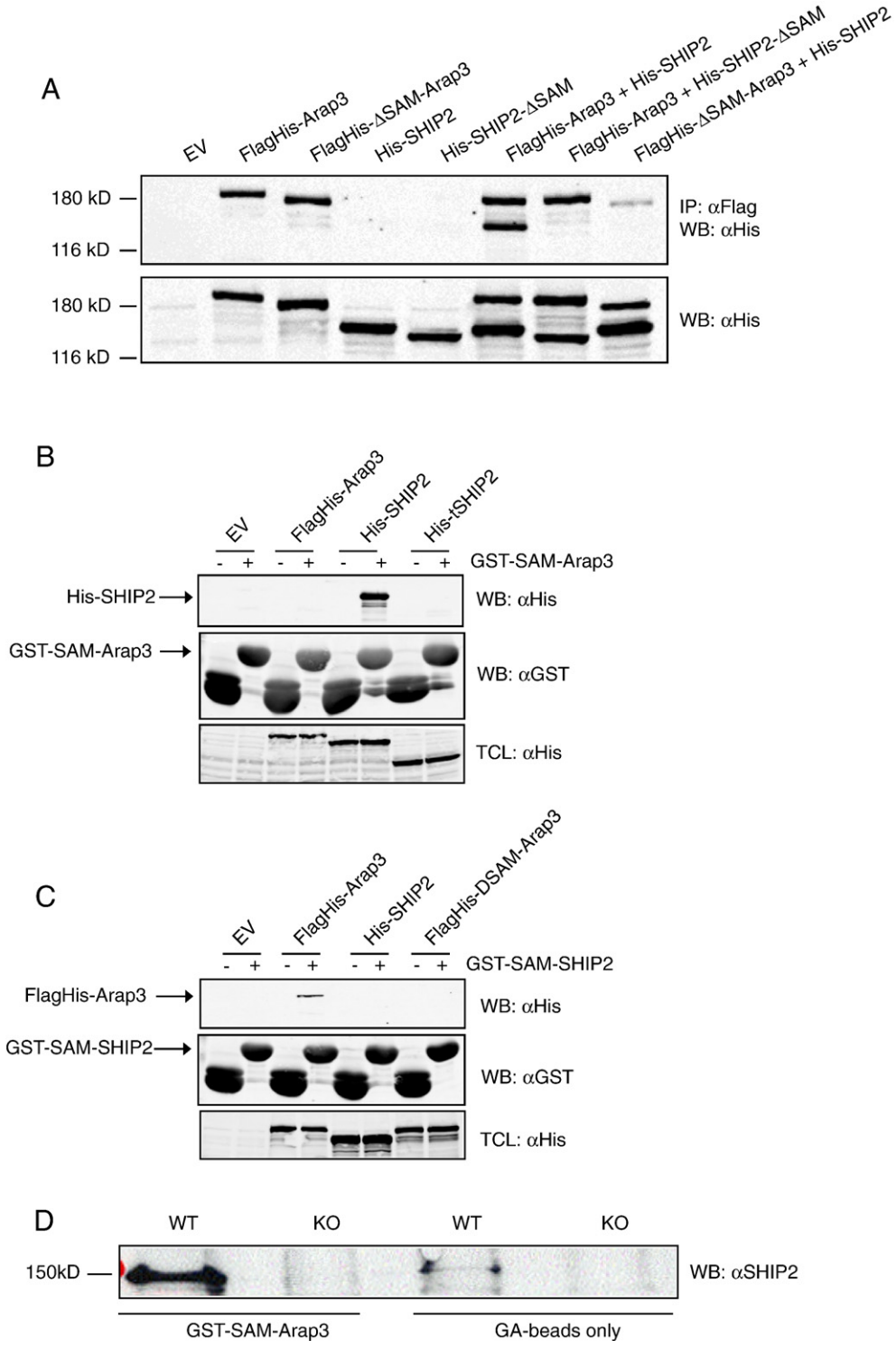

Fig. 3. The SAM domains are both necessary and sufficient to mediate the interaction between Arap3 and SHIP2. (A) HEK293T cells were transiently transfected with the indicated constructs or empty vector (EV). Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates were analyzed for the presence of both over-expressed His-SHIP2 and FlagHis-Arap3. HEK293T cells were transiently transfected with the indicated constructs. GST pull downs were performed with either (B) GST or GST-SAM-Arap3 or (C) GST or GST-SAM-SHIP2. Membranes were probed for presence of His-SHIP2 or FlagHis-Arap3 and with anti-GST antibody to show equal loading of GST proteins. (D) Lysates of either WT MEF cells or SHIP2−/− MEF cells were used in a GST pull down assay using GST-SAM-Arap3 (first two lanes) or GA (glutathione agarose) beads alone (last two lanes). Binding of SHIP2 was detected using anti-SHIP2 antibody. Blots shown are representatives of at least 3 identical experiments.

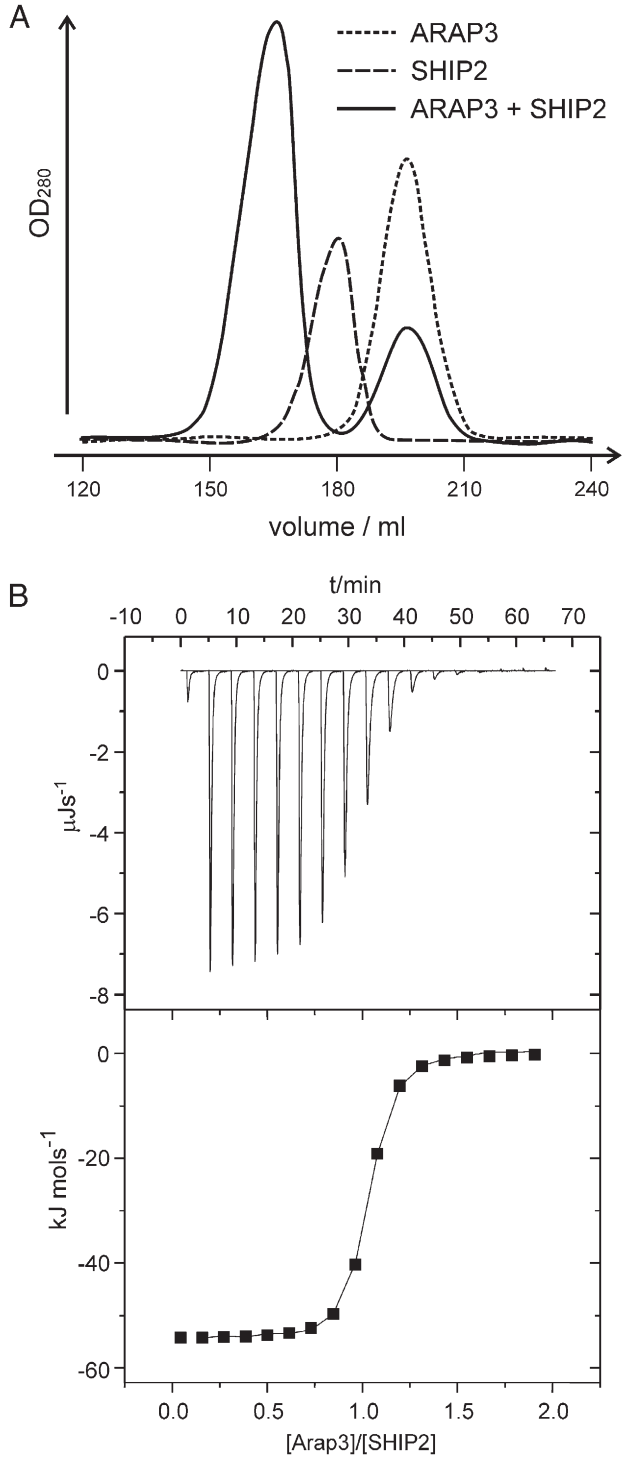

Fig. 4. The SAM domains show specificity for a heterodimeric interaction. (A) 20 mg of the SAM domain of SHIP2 (dashed line), 35 mg of the SAM domain of Arap3 (dotted line) or a mixture of 20 mg of the SAM domain of SHIP2 and 35 mg of that of Arap3 (continuous line) were subjected to gel filtration. (B) Arap3 SAM domain (744 µM) was placed in the syringe of the ITC apparatus and titrated into a solution of the SAM domain of SHIP2 (23 µM) at a temperature of 25 °C. The release of heat was measured as changes in heating power over time (upper panel.) The lower panel shows the released heat per injection normalized to the amount of added protein plotted versus the ratio of concentration of Arap3 SAM and SHIP2 SAM domain.

column, the retention time was decreased further, indicating an increase in size due to the formation of a dimer.

To determine the affinity of the interaction, isothermal titration calorimetry (ITC) was used. Upon titration of the Arap3 SAM domain into a solution of the SHIP2 SAM domain, the two SAM domains dimerized with an enthalpy change (ΔH) of 54 kJ/mol and an affinity (K_d) of 100 nM. Also, the ITC measurements indicate that the interaction indeed occurs at a 1:1 stoichiometry (Fig. 4B). ITC carried out with titration of SHIP2 into a solution of Arap3 SAM domain gave the same enthalpy change and affinity data (not shown). We thus conclude that Arap3 and SHIP2 interact as a dimer with an affinity that is physiologically relevant.

3.4. Presence of the Arap3 SAM domain does not affect SHIP2 phosphatase activity

As both proteins are involved in the PI3K pathway, we next investigated whether dimerization of its SAM domain would modulate the catalytic activity of SHIP2. To this end, His-SHIP2 was purified from COS-7 cells and PI(3,4,5)P_3 5′-phosphatase activity was measured in an in vitro phosphatase assay in the presence or absence of an excess (5 µM) of purified Arap3 or SHIP2 SAM domain [24]. The SHIP2 PI(3,4,5)P_3 5′-phosphatase activity was comparable in all conditions (data not shown). We therefore conclude that binding of Arap3 to SHIP2 does not affect SHIP2 activity in vitro.

3.5. The interaction between Arap3 and SHIP2 is not regulated by Rap1 or PI3K

Since Arap3 localization is regulated by both Rap1 and PI3K [4], we investigated whether the interaction with SHIP2 is modulated by either of these. We performed a co-immunoprecipitation with over-expressed proteins in either the presence or absence of RapV12, a constitutively active mutant of Rap1 [31], or of Rap1GAP, the GTPase activating protein specific for Rap, to reduce the amount of GTP-bound Rap1 [30]. We found that modulation of Rap1 activity did not affect the interaction between Arap3 and SHIP2 (Fig. 5A). We next investigated whether active PI3K is required for the interaction between endogenous proteins in HeLa cells and 293T cells. However, neither activation of PI3K by growth factor stimulation nor inhibition of PI3K by LY294002 affected the interaction (Fig. 5B and C). We therefore conclude that the interaction appears to be constitutive and is not modulated by the activation of Arap3 by PI3K or Rap1.

3.6. Arap3 is part of a multimeric protein complex

Previously it was shown that Arap3 is present in a multimeric protein complex with the SH3 domain-containing protein CIN85 that binds Arap3 via a specific proline-arginine motif [13]. Our screen also identified the CIN85-related protein, CMS. To investigate whether Arap3, SHIP2 and CIN85 or CMS can form a multimeric protein complex, we performed a co-immunoprecipitation experiment between CIN85 or CMS and SHIP2, either in the presence or absence of Arap3. As shown in Fig. 5D, Arap3 is indeed co-immunoprecipitated with both CIN85 and CMS. In

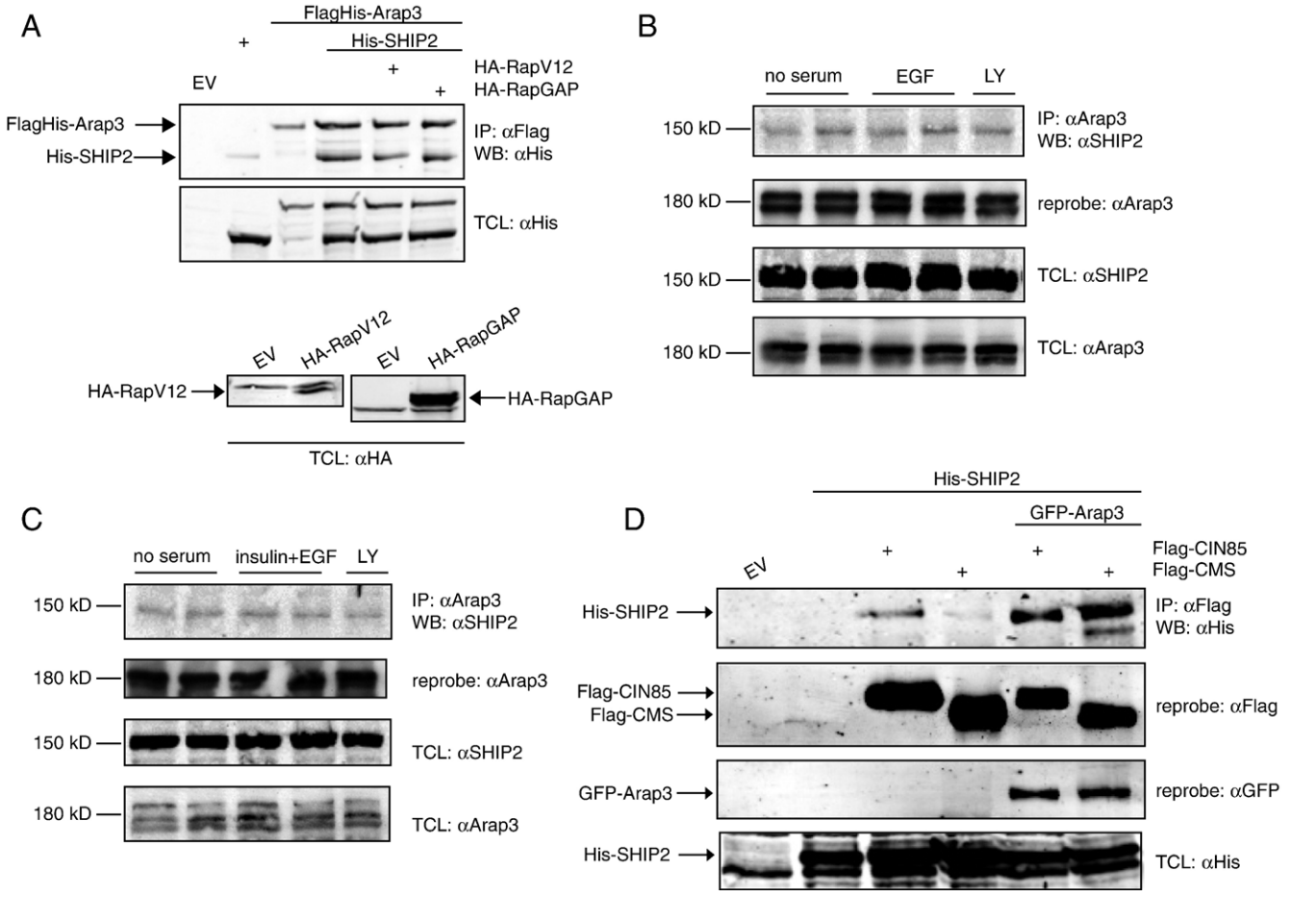

Fig. 5. The interaction between Arap3 and SHIP2 does not depend on the presence or absence of active Rap1 or PI3K. (A) HEK293T cells were transiently transfected with the indicated constructs. Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates were analyzed for the presence of both over-expressed His-SHIP2 and FlagHis-Arap3. Total cell lysates were probed with an anti-HA antibody to detect HA-RapV12 and HA-RapGAP. HeLa cells (B) and 293T cells (C) were grown to confluency and serum starved overnight. Starved cells were either left untreated (in duplo) or stimulated for 30 min with the PI3K inhibitor LY294002 or for 10 min with EGF (in duplo) (B) or insulin and EGF (in duplo) (C) as indicated. The lysates were immunoprecipitated using an Arap3 antibody and probed for the presence of endogenous SHIP2. Membranes were also reprobed with Arap3 antibody. Blots shown are representatives of at least 3 identical experiments. (D) HEK293T cells were transiently transfected with the indicated constructs. Immunoprecipitations were performed with an anti-Flag antibody. Immunoprecipitates were analyzed for the presence of both over-expressed His-SHIP2 and GFP-Arap3.

addition, SHIP2 is also co-immunoprecipitated with Arap3 and both CIN85 and CMS. In the absence of co-transfected Arap3, SHIP2 is still co-immunoprecipitated with CIN85 and CMS, albeit to a much reduced level. This residual co-immunoprecipitation is presumably due to the presence of endogenous Arap3. From these results, we conclude that SHIP2, Arap3 and CIN85/CMS form a multimeric protein complex.

4. Discussion

In this paper, we show a direct interaction between the PI3K effector Arap3 and the inositol 5′-phosphatase SHIP2. We identified SHIP2 as an Arap3 binding partner in a yeast two-hybrid screen and confirmed the interaction by co-immunoprecipitation of the endogenous proteins. Furthermore, by mutational analysis and ITC experiments, we demonstrate that the interaction is mediated by heterodimerization of the SAM domains present in both proteins. The interaction appears to be constitutive as it is not affected by regulators of Arap3, i.e. Rap1 and PI3K. Furthermore, we show that Arap3 and SHIP2 together can form multimeric protein complexes with the SH3 domain-containing adaptor proteins CIN85 and CMS.

The relevance of this finding comes from our previous observation that Arap3 is regulated by PI3K signaling, whereas SHIP2 is a negative regulator of PI3K signaling. PI3K phosphorylates $PI(4,5)P_2$ to create $PI(3,4,5,)P_3$ [33] and SHIP2 is a negative regulator of the PI3K pathway [17] that dephosphorylates $PI(3,4,5)P_3$ lipids to $PI(3,4)P_2$ [15]. Importantly, as shown previously by us, Arap3 binds $PI(3,4,5,)P_3$ stronger than it binds $PI(3,4)P_2$ [3]. Since binding of Arap3 to $PI(3,4,5)P_3$ is required for efficient membrane localization of Arap3, dephosphorylation of $PI(3,4,5,)P_3$ by SHIP2 implies a reduced affinity of Arap3 for the plasma membrane. We therefore conclude that Arap3 forms a complex with a negative regulator of its signaling pathway.

Previously, we have shown that one of the biological effects of Arap3 is to inhibit PDGF-induced lamellipodia formation [4]. We have investigated whether deletion of the SAM domain has any affect on this process. However, both wild-type Arap3 and a

mutant of Arap3 lacking the SAM domain have a similar inhibitory effect on PDGF-induced lamellipodia formation (data not shown). Furthermore, both wild-type SHIP2 and the mutant of SHIP2 lacking the SAM domain have a similar inhibitory effect on lamellipodia formation, presumably due to a general inhibition of PI3K signaling (data not shown). We therefore concluded that currently no biological systems are present to test our model that SHIP2 negatively regulates Arap3. Alternatively, since PI3K signaling has a strong spatial element, it may well be that the presence of SHIP2 in the Arap3 complex is important to restrict the distribution of $PI(3,4,5)P_3$ to local environments.

SAM domains are conserved modular domains that are widespread and common in nature. With a wide capacity to mediate interactions in signaling pathways [9], they can mediate protein–protein interactions and also regulate protein–lipid and protein–RNA binding. SAM domains mediate many forms of protein–protein interactions by homo-, hetero- or oligomerization with target proteins [7]. Interestingly, some protein families have differential conservation of the SAM domain, as is the case for SHIP1 and SHIP2. As SHIP1, that does not contain a SAM domain, is mainly expressed in hematopoietic cells and SHIP2 is more ubiquitously expressed [34], this may indicate that SHIP2 has acquired additional functions in these cells and the presence of the SAM domain is required to mediate these functions by recruiting new interaction partners.

For instance, both Arap3 and SHIP2 have binding partners involved in endocytosis. It was shown before that SHIP2 binds the E3 ligase Cbl and Cbl-associated protein (CAP) and SHIP2 is therefore suggested to have a role in endocytosis [29,35]. Furthermore, it was reported that Arap3 binds the adaptor protein CIN85 [13] and our screen identified the CIN85-related protein CMS as an Arap3 binding partner as well (Table 1). These two adaptor proteins both function in Cbl-mediated endocytosis [14]. We have found that SHIP2, Arap3 and either CIN85 or CMS are present in a complex, demonstrating that different binding surfaces on Arap3 are used for these interactions. This indicates that both Arap3 and SHIP2 (through its SAM and SH2 domain) can function as scaffold proteins, perhaps binding proteins that depend on their enzymatic activities. For instance, one of the other proteins identified in the yeast two-hybrid screen, ARHGEF6, or alpha-pix, is regulated by PI3K as well and is a GEF for Rac and Cdc42 [36]. As it is often seen that the GTP levels of Rac and Cdc42 are inversely regulated with Rho, it is quite interesting that Arap3 complexes with a Rac GEF.

It was also proposed before that SHIP2 is involved in the regulation of the actin cytoskeleton and cell adhesion, like Arap3, and that it interacts with multiple proteins in the cytoskeleton network [5,6,24,25,28]. It will therefore be interesting to see which of these proteins are found in the same complex together, and what exactly is the role of all these different interactions in the complex signaling pathways that eventually lead to cell adhesion.

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