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The Arf GTPase-activating protein SMAP1 promotes transferrin receptor endocytosis and interacts with SMAP2

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ABSTRACT

Arf GTPase-activating proteins (Arf GAP) play important roles in the formation of the membrane vesicles that traffic between subcellular membranous organelles. The small Arf GTPase-activating protein (SMAP) subfamily of Arf GAPs has two members, SMAP1 and SMAP2, in mammals. The present study investigated whether these two proteins may have an overlapping function in addition to their previously reported distinct functions. Results showed that the presence of either SMAP1 or SMAP2 was sufficient for endocytosis of the transferrin receptor, and that transferrin incorporation was impaired only by the absence of both SMAP1 and SMAP2. This suggests the involvement of both SMAP1 and SMAP2 in transferrin endocytosis. Results also demonstrated a physical association between SMAP1 and SMAP2, which might serve as a basis for a functional interaction, and identified the intramolecular domains responsible for this association.

1. Introduction

Arf proteins are GTPases of small molecular mass activated by Arf GTPase-activating proteins (Arf GAP). Arf and Arf GAP are gene families in mammals, composed of 6 and 31 human genes, respectively, and both are pivotal players in membrane trafficking, contributing to the homeostasis of subcellular membrane organelles [1–5]. A GTP-bound, active form of Arf recruits adaptor molecules necessary for vesicle formation. By contrast, Arf GAP functions not only as a signal terminator of active Arf but also as an effector of vesicle formation by interacting with various molecules [6–8]. The small Arf GTPase-activating protein (SMAP) subfamily, the focus of this study, is one of several Arf GAP families. SMAP has two members, SMAP1 and SMAP2 [9].

SMAP1 and SMAP2 are considered to exert distinct functions in membrane trafficking. For example, SMAP1 and SMAP2 prefer Arf6 and Arf1 as substrates, respectively [10,11]. Thus, SMAP1 is involved in the Arf6-dependent endocytosis of the transferrin receptor and E-cadherin [10,12], whereas SMAP2 is involved in the Arf1-dependent membrane trafficking between early endosomes and the trans-*Golgi* network (TGN) [11,13]. In addition, a gene targeting study revealed that *Smap1* deficiency predisposes mice to myelodysplastic syndrome due to impaired sorting of endocytosed c-Kit from multivesicular bodies to lysosomes [14]. Activated c-Kit thus escapes degradation in lysosomes and keeps on eliciting growth signals in hematopoietic progenitors. By contrast, *Smap2* deficiency in mice causes male infertility due to abnormal acrosome formation during spermiogenesis: vesicle budding from the TGN is perturbed so that the fusion of vesicles and subsequent formation of acrosomes do not proceed properly [15]. Thus, there exist distinct trafficking pathways that are dependent solely on either SMAP1 or SMAP2 function.

SMAP1 and SMAP2 have common features. The two proteins exhibit homology with an overall 47% amino acid (aa) identity and harbor an Arf GAP domain with 83% aa identity [11]. Both SMAP1 and SMAP2 bind to clathrin heavy chain molecules and are thus involved in the formation of clathrin-coated membrane vesicles. We previously identified the aa motif outside of the Arf

Abbreviations: aa, amino acid; bsa, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; HA, hemagglutinin; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline; TGN, trans-*Golgi* network.

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GAP domain that is responsible for clathrin binding [10,11]. In addition to this motif, multiple aa sequences are shared by the two SMAPs, suggesting a possible overlapping function in addition to the known distinct functions. In the present study, we tested this possibility by assessing the involvement of both SMAP1 and SMAP2 in the endocytosis of the transferrin receptor and the physical association between the two molecules.

2. Materials and methods

2.1. Plasmids

The Myc-tagged SMAP1 and hemagglutinin (HA)-tagged SMAP2 expression vectors were as follows: pcDNA3-Myc-SMAP1 (aa 1–440), pcDNA3-Myc-SMAP1 (aa 166–349), pcDNA3-Myc-SMAP1 (aa 350–440), pcDNA3-HA-SMAP2 (aa 1–428), pcDNA3-HA-SMAP2 (aa 163–338), and pcDNA3-HA-SMAP2 (aa 339–428). Each DNA fragment, encoding either the intact full-length protein or a protein fragment, was inserted into the *EcoR*I/*Xho*I sites of the pcDNA3 vector. pEGFP (enhanced green fluorescent protein)-SMAP2 (aa 339–377) and pEGFP-SMAP2 (aa 378–428) encode EGFP-SMAP2 fusion proteins. A DNA fragment encoding a portion of SMAP2 was inserted into the *BamH*I/*EcoR*I sites of pEGFP.

2.2. Cell culture

HEK293T and COS7 cells were propagated in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. PLAT-E cells were propagated in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml blasticidin, and 1 μg/ml puromycin. The cells were maintained in 5% CO_2 in air at 37 °C. The procedure for preparing mouse embryo fibroblasts (MEF) was described previously [13,14]. Briefly, male and female mice with the *Smap1*(f/f);*Smap2*(+/−) genotype were mated, and El5.5 embryos were collected from pregnant mice. The skin was isolated, minced, and digested in 0.05% (w/v) trypsin and 0.2% (w/v) EDTA in phosphate-buffered saline (PBS). The dispersed cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1× MEM non-essential amino acid solution, and 10 mM Hepes–KOH, pH 7.4. After 3 days of culture, MEFs were transfected with the pMX-puro-SV40 expression vector. Opti-MEM (1 ml) containing 2.5 μg of linearized plasmid DNA and 35 μl of Lipofectamine LTX (Invitrogen) was added to the MEFs in a 6 cm diameter dish. After 72 h of incubation, the medium was changed to that containing 5 μg/ml puromycin. The cells were passaged and cultured for two additional weeks to establish immortalized cell lines.

2.3. DNA transfection and retrovirus infection

In the case of 293T cells, 1 ml of Opti-MEM containing 5 μg of DNA (expression vector) and 14 μl of Lipofectamine LTX was added to the cells in a 6 cm diameter dish. In the case of COS7 cells, 500 μl of Opti-MEM containing 2 μg of DNA (expression vector) and 9 μl of Lipofectamine LTX was added to the cells in a 6-well plate. The procedure to prepare retrovirus stock was as follows: PLAT-E cells seeded in a 10 cm diameter dish received 500 μl of Opti-MEM containing 10 μg of pMX-Cre-IRES-GFP and 20 μl of Fugene HD. After 24 h of incubation, the medium was changed to that used for the MEFs. After 24 additional hours, the virus-containing supernatant was collected. MEFs were infected in a 6 cm diameter dish with 2 ml of retrovirus solution diluted in an equal amount of complete DMEM containing 0.5 μg/ml polybrene (Sigma).

2.4. Endocytosis assay

MEFs were cultured on a cover glass placed in a 6-well plate and starved in serum-free medium for 2 h. Then, the cells received either 40 μg/ml Alexa Fluor 555-conjugated transferrin

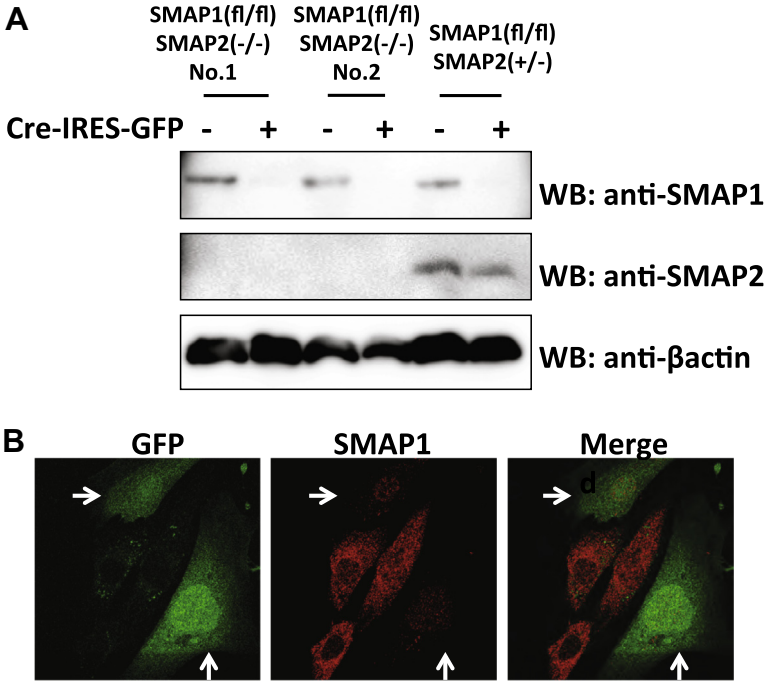

Fig. 1. Introduction of Cre-recombinase into MEFs by retrovirus infection. (A) MEFs of the indicated genotypes were infected with retrovirus encoding Cre as well as GFP. After 24 h of incubation, protein lysates were prepared and processed for immunoblotting with anti-SMAP1 and anti-SMAP2 antibodies. (B) Retrovirus-infected MEFs were cultured further for 2 weeks, fixed, and processed for immunofluorescence detection of endogenous SMAP1. Cells indicated by arrows are GFP-positive and SMAP1-negative, whereas the other cells are GFP-negative and SMAP1-positive.

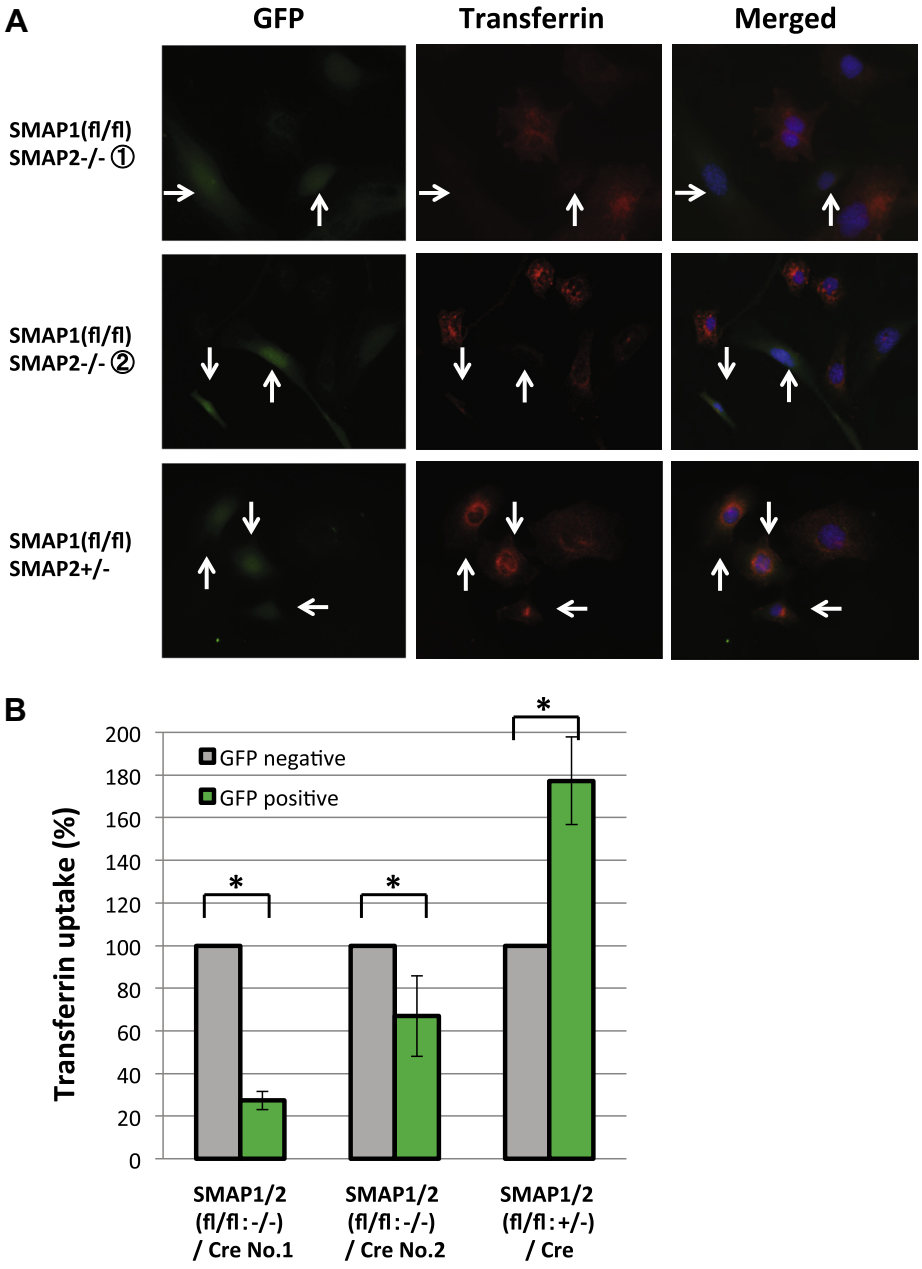

Fig. 2. Effect of SMAP1/SMAP2 deficiency on transferrin endocytosis. MEFs of the indicated genotypes were infected with pMX-Cre-IRES-GFP retrovirus and cultured for 2 weeks. The cells were starved for 2 h in serum-free medium, incubated with dye-conjugated transferrin for 15 min, and fixed. The cells were photographed for GFP- as well as transferrin-derived fluorescence. Arrows in (A) indicate the GFP-positive cells. In (B), fluorescence intensities are presented for the GFP-negative and -positive cell populations.

(Invitrogen), 50 μg/ml Alexa Fluor 555-conjugated bovine serum albumin (BSA) (Invitrogen) or 100 μg/ml Alexa Fluor 555-conjugated dextran (Invitrogen), and were incubated at 37 °C for 15 min (transferrin) or 30 min (BSA, dextran). The cells were rinsed twice in cold PBS, fixed in 3.7% (w/v) formaldehyde in PBS for 15 min, immersed in mounting buffer (Vector Laboratories), and observed/photographed with a BZ-9000 microscope (Keyence) or a LSM510 laser scan confocal microscope (Zeiss). Fluorescence intensity was measured using the Zeiss LSM Image Browser software. The average intensity from 50 GFP-positive cells was divided by that from the same number of GFP-negative cells, and this ratio was used as the efficiency of substrate incorporation into the cells.

2.5. Immunohistochemistry, immunoprecipitation, and immunoblotting

The cells were fixed in 3.7% (w/v) formaldehyde in PBS for 15 min and permeabilized with 0.1% (v/v) Triton X-100 in 1% (w/v) BSA-containing PBS for 15 min. The primary antibodies were anti-HA rat monoclonal antibody (mAb) (3F10, Roche) or anti-Myc mouse mAb (9E10, Sigma). The secondary antibodies were Alexa 568-conjugated anti-rat IgG or Alexa 488-conjugated anti-mouse IgG (Invitrogen). The cells were lysed in ice-cold, low-salt buffer (10 mM Hepes–KOH, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% NP40, and 1X protease inhibitors) for 10 min and the lysates were sonicated for 10 s. After centrifugation, the supernatant was collected

as total cellular lysate. Immunoprecipitation and immunoblotting were performed as described previously [14].

3. Results

3.1. Establishment of SMAP1/SMAP2-deficient MEFs

To test the function of *Smap1* and *Smap2* separately, we prepared MEFs from several *Smap* genotypes. Using the *Smap1*- and *Smap2*-targeted mice reported previously [14,15], we mated male and female mice of the *Smap1*(f/f);*Smap2*(+/−) genotype (note that *Smap2*(f/f) was not available). Embryos were collected from pregnant female mice and MEFs were prepared. Two immortalized *Smap1*(f/f);*Smap2*(−/−) lines and one *Smap1*(f/f);*Smap2*(+/−) line were established. These lines were infected with pMX-Cre-IRES-GFP retrovirus. After 24 h, cell lysates were prepared and processed for immunoblot analysis (Fig. 1A). Endogenous SMAP1 was detected prior to virus infection but not in virus-infected MEFs of each genotype, indicating efficient deletion of the *floxed-Smap1* gene by Cre-recombinase. Endogenous SMAP2 was detected in *Smap2*(+/−) MEFs but not in *Smap2*(−/−) MEFs.

The virus-infected MEFs were cultured for two additional weeks. During this incubation period, we noticed that the growth rate of GFP-positive cells was substantially slower than that of GFP-negative cells (confirmed by time lapse video observation of living cells). The percentage of GFP-positive cells, which was approximately 95% immediately after virus infection, gradually decreased to about 30% in 2 weeks. When these MEFs were stained by immunofluorescence (Fig. 1B), the GFP-positive cells were found to still be negative for endogenous SMAP1, but substantial numbers of GFP-negative, SMAP1-positive cells could be detected as well. This mixed culture of SMAP1-negative and -positive cells is a useful experimental tool for evaluating SMAP function on a single cell basis.

3.2. Effect of SMAP1/SMAP2 deficiency on the endocytosis of various molecules

The transferrin receptor is constitutively endocytosed via Arf6-regulated, clathrin-coated vesicles [16,17]. To evaluate the function of SMAP1 and SMAP2 in this process, retrovirus-infected MEFs were incubated with dye-conjugated transferrin. After 15 min of incubation, cells were fixed and photographed to assess GFP- and transferrin-derived fluorescence (Fig. 2A). Assuming the fluorescence intensity from the incorporated transferrin in the GFP-negative cells to be 100%, the relative value in the GFP-positive cells was calculated (Fig. 2B). In control *Smap2*(+/−) MEFs, the lack of SMAP1 increased transferrin incorporation by 1.8-fold. By contrast, in *Smap2*(−/−) MEFs, the lack of SMAP1 decreased transferrin incorporation by 80% in one line and by 35% in another line. Therefore, it is likely that either SMAP1 or SMAP2 is necessary for efficient incorporation, and that the absence of both SMAP1 and SMAP2 impairs transferrin incorporation.

BSA, when bound to its cell surface receptor, gp60, is endocytosed via caveolae, which are also dependent on an Arf function [18]. Retrovirus-infected MEFs were incubated with dye-conjugated BSA for 30 min. Incorporation of BSA was evaluated (Supplementary Fig. 1A and B). The lack of SMAP1 did not affect the extent of BSA incorporation regardless of the *Smap2* genotype of the MEF lines. Thus, neither SMAP1 nor SMAP2 is involved in caveolae-mediated endocytosis. Similarly, experiments testing the incorporation of dextran, a cargo endocytosed through a GPI-anchored protein-enriched early endocytic compartment [19], failed to reveal any involvement of the SMAP family (data not shown).

3.3. Physical association of SMAP1 and SMAP2

We previously conducted yeast two-hybrid assays using a fragment of SMAP2 (aa 163–428) as bait, and identified clathrin assembly protein (CALM) and the sumoylation-related factors Ubc9, PIAS1, and PIAS3 as SMAP2-interacting molecules [20]. Among the interactors, there was a cDNA encoding a fragment of SMAP1 (unpublished observation). Since this indicates that SMAP1 and SMAP2 can interact directly, we validated this observation by immunoprecipitation and immunofluorescence.

Protein lysates were prepared from *Smap2*(+/+) and (−/−) MEFs, and immunoprecipitated with anti-SMAP2 antibody (Fig. 3A). Immunoblotting of the precipitates with anti-SMAP1 revealed co-precipitation of SMAP1 and SMAP2 in *Smap2*(+/+) MEFs, indicating association of the two endogenous proteins. We then performed immunoprecipitation using an overexpression system. HEK293T cells were transfected with Myc-SMAP1 and HA-SMAP2, and

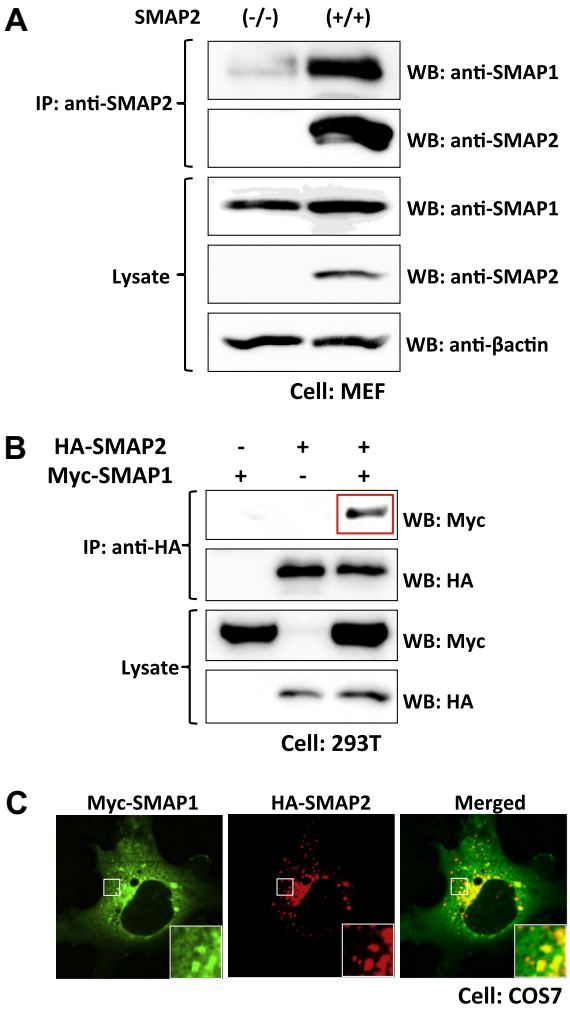

Fig. 3. Interaction of SMAP1 and SMAP2 detected by co-immunoprecipitation and colocalization inside the cell. (A) Protein lysates were prepared from MEFs of the *Smap2*(−/−) and *Smap2*(+/+) genotypes, and processed for immunoprecipitation by anti-SMAP2 antibody. The precipitates were immunoblotted with the indicated antibodies. In parallel, protein lysates (without immunoprecipitation) were probed with the same antibodies. (B) HEK293T cells were transfected with the indicated expression vectors. In the upper two lanes, protein lysates were processed for immunoprecipitation followed by immunoblotting using the indicated antibodies. In the lower two lanes, protein lysates were directly processed for immunoblotting with the indicated antibodies. (C) COS7 cells were transfected with Myc-SMAP1 and HA-SMAP2 and processed for immunofluorescence detection.

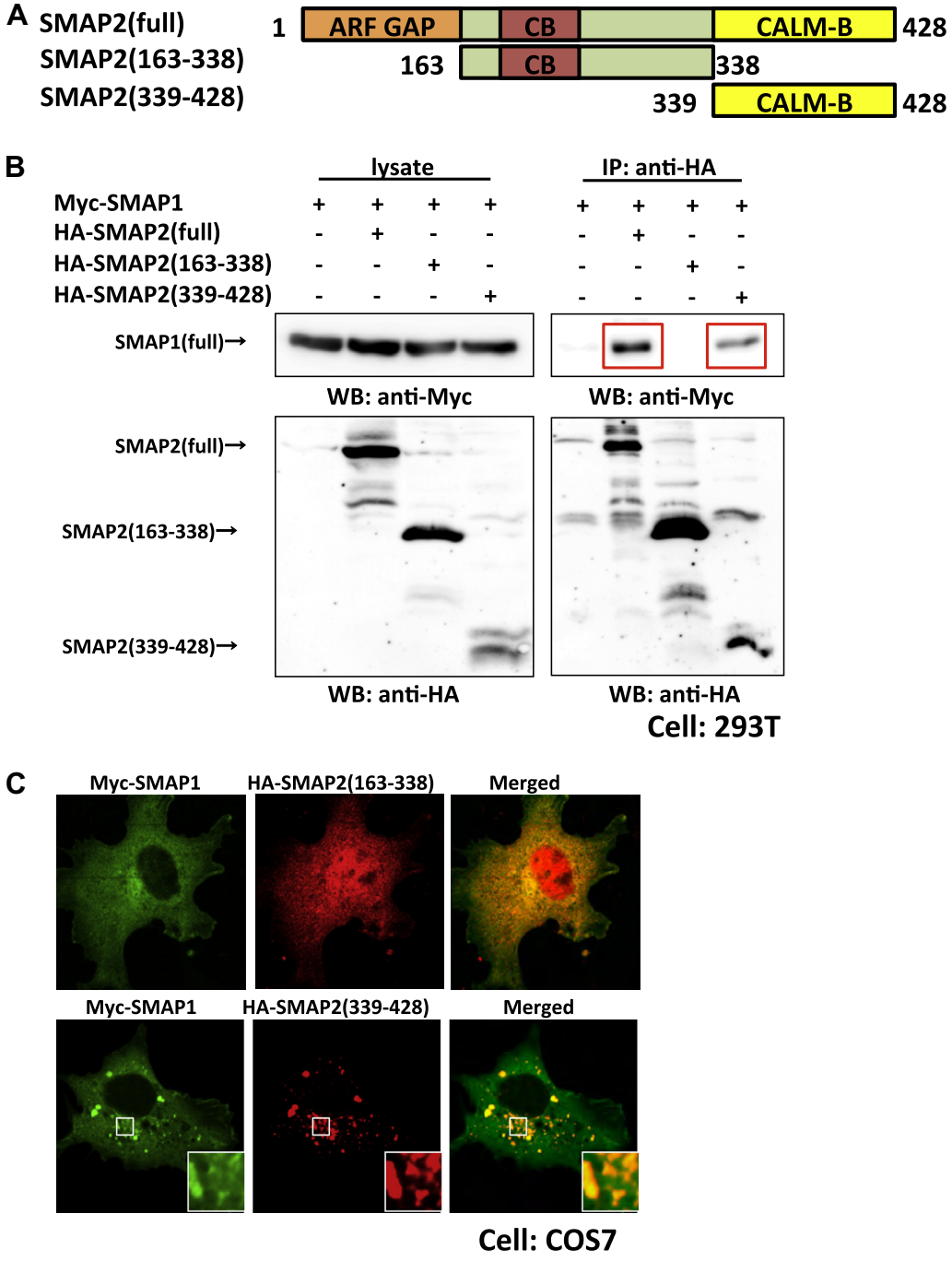

Fig. 4. Identification of the SMAP1-interacting region within SMAP2. (A) Intact SMAP2 and fragments. Numbers indicate amino acid residues. CB, clathrin-binding motif; CALM-B, CALM-binding region. (B) HEK293T cells were transfected with the indicated expression plasmid. In the left panels, protein lysates were directly probed with the indicated antibodies. In the right panels, protein lysates were immunoprecipitated by anti-HA first and then immunoblotted using the indicated antibody. (C) COS7 cells were transfected with the indicated expression plasmids and processed for double immunofluorescence detection of Myc-SMAP1 and HA-SMAP2.

protein lysates were prepared. As seen in Fig. 3B, Myc-SMAP2 was detected in the anti-HA precipitate.

In our previous study of protein overexpression [9–11], SMAP1 by itself was distributed along the cell contour and also showed diffuse cytoplasmic staining, whereas SMAP2 by itself was detected on early endosomes as multiple dots and on the TGN as a juxta-nuclear crescent staining pattern. It must be pointed out that SMAP2, if expressed in an excessive amount, tends to form aggregates. Thus, we utilized aggregate formation as an indicator of SMAP2-interacting molecules. COS7 cells were transfected with expression vectors and processed for double immunofluorescence staining (Fig. 3C). A substantial fraction of SMAP1 and apparently almost all SMAP2 appeared to be co-localized as aggregates, suggesting a physical (although artificial) association of SMAP1 and SMAP2.

3.4. Identification of SMAP1–SMAP2-interacting domains

Using the overexpression system, we tried to identify regions within SMAP1 and SMAP2 responsible for the association between the two proteins. First, we used intact SMAP1 and SMAP2 fragments (Fig. 4A). Co-immunoprecipitation with anti-Myc antibody

and subsequent immunoblotting detected the SMAP2 339–428 fragment but not the SMAP2 163–338 fragment (Fig. 4B). Similarly, aggregate formation with SMAP1 was observed with SMAP2 339–428 but not SMAP2 163–338 (Fig. 4C). This result indicates that a region responsible for the SMAP1 interaction resides within the 339–428 region of SMAP2, a previously assigned CALM-binding domain.

Second, intact SMAP2 and fragments of SMAP1 were used (Supplementary Fig. 2A). In the anti-HA precipitate, both SMAP1 166–349 and SMAP1 350–440 were detected (Supplementary Fig. 2B), and both fragments of SMAP1 formed cytoplasmic aggregates with SMAP2 (Supplementary Fig. 2C). Thus, it is likely that either SMAP1 166–349 or SMAP1 350–440 can interact with SMAP2.

4. Discussion

We previously reported that overexpression of SMAP1 (but not of SMAP2) in tissue culture cells impaired the clathrin-dependent incorporation of the transferrin receptor [10]. This observation supports the involvement of SMAP1 in the endocytosis of the transferrin receptor. In the present study, we used *Smap2*(−/−) MEFs where transferrin endocytosis was still functional. Interestingly, we could show a substantial reduction of transferrin incorporation upon abrogation of SMAP1 expression. Thus, the present study provides the first evidence of a positive role for SMAP1 in transferrin endocytosis. By contrast, we previously employed *Smap1*(−/−) MEFs and found no impairment of transferrin incorporation. Depletion of SMAP2 in *Smap1*-deficient cells then reduced transferrin incorporation, implying a positive role for SMAP2 [14]. Considering the present and previous findings collectively, both endogenous SMAP1 and SMAP2 appear to function positively in transferrin receptor endocytosis.

ArfGAP1 has long been thought to be involved in the trafficking of COPI-coated vesicles between the Golgi apparatus and the endoplasmic reticulum [21–23]. Recently, in addition to this Arf1-dependent process, ArfGAP1 has been shown to play roles in the Arf6-dependent endocytosis of the transferrin receptor through interaction with the AP-2 adaptor molecule [24]. Endocytosis of the transferrin receptor is, in general, highly dependent on clathrin-coated vesicles and proceeds in a ligand-independent fashion [25,26]. Still, efficiency of this endocytosis is significantly modulated by kinases such as Akt2 and Jak3 [27]. In addition, the existence of another pathway for transferrin internalization that is dependent on ligand-binding has been reported [28]. Thus, it may not be surprising to find that multiple kinds of Arf GAP molecules are involved in transferrin endocytosis, including SMAPs.

In our previous study, we reported that overexpression of SMAP1 in tissue culture cells did not interfere with the incorporation of BSA, suggesting no involvement of SMAP1 in caveolin-dependent endocytosis [12]. In the present study, we demonstrated that the abrogation of SMAP1 did not reduce the incorporation of BSA either, even in a *Smap2*-deficient background. Therefore, the SMAP family appears to be an authentic player in the formation of clathrin-coated vesicles but not in that of caveolin-coated vesicles.

In this study, we also demonstrated a physical association between SMAP1 and SMAP2, and could identify domains that are responsible for this association. In yeast two-hybrid assays using a fragment of SMAP2 as bait, we isolated clones encoding SMAP1 and SMAP2 as candidate SMAP2-interacting molecules (unpublished observations). Therefore, the SMAP1-SMAP2 (and SMAP2-SMAP2) association observed in the present study likely reflects the direct binding of the two molecules. The possibility of a SMAP1-SMAP1 interaction remains to be examined.

We mentioned above the involvement of multiple Arf GAPs in transferrin receptor endocytosis. Thus, it would be interesting to see if SMAP molecules function as monomers or dimers, and, if the latter, whether dimer formation might confer complexity to SMAP function. Indeed, GAPs are known that form dimers and whose GAP activity is modulated by dimer formation [29–31].

In summary, while distinct functions unique to SMAP1 or SMAP2 had been recognized so far, the present study identified a function common to the two molecules, thereby expanding our understanding of the SMAP family and broadening the scope of future SMAP research.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.108.

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