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FEBS Letters 586 (2012) 3024–3029

FEBS Letters

journal homepage: www.FEBSLetters.org

Acyl-CoA binding domain containing 3 (ACBD3) recruits the protein phosphatase PPM1L to ER–Golgi membrane contact sites

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ARTICLE INFO

Article history:
Received 16 April 2012
Revised 20 June 2012
Accepted 25 June 2012
Available online 10 July 2012

Edited by Sandro Sonnino

Keywords:
PPM1L
ACBD3
CERT
GOLD domain

ABSTRACT

The metal-dependent protein phosphatase family (PPM) governs a number of signaling pathways. PPM1L, originally identified as a negative regulator of stress-activated protein kinase signaling, was recently shown to be involved in the regulation of ceramide trafficking at ER–Golgi membrane contact sites. Here, we identified acyl-CoA binding domain containing 3 (ACBD3) as an interacting partner of PPM1L. We showed that this association, which recruits PPM1L to ER–Golgi membrane contact sites, is mediated by a GOLD (Golgi dynamics) domain in ACBD3. These results suggested that ACBD3 plays a pivotal role in ceramide transport regulation at the ER–Golgi interface.

Structured summary of protein interactions:
ACBD3 and **PPM1L** colocalize by fluorescence microscopy (View interaction)
FYCO1 physically interacts with **PPM1L** by pull down (View interaction)
SEC14L2 physically interacts with **PPM1L** by pull down (View interaction)
ACBD3 physically interacts with **PPM1L** by pull down (View interaction)
SEC14L1 physically interacts with **PPM1L** by pull down (View interaction)
PPM1L physically interacts with **ACBD3** by two hybrid (View interaction)

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1. Introduction

The metal-dependent protein phosphatase family (PPM, formerly called protein phosphatase 2C) is one of two major protein serine/threonine phosphatase families found in eukaryotes [1]. In contrast to members of the phosphoprotein phosphatase family (PPP), which function as oligomeric complexes, PPM members do not have regulatory subunits but contain unique domains that may regulate activity or determine substrate specificity or subcellular localization [2,3]. PPM contains a large family of highly conserved protein phosphatases, with 17 distinct genes in the human genome. Although PPM family members are involved in a number of cellular functions, including cell proliferation, survival, differentiation, and apoptosis, their major function appears to be regulation of the stress response [2–4].

PPM1L (formerly PP2Cε) was originally identified as a negative regulator of stress-activated protein kinase signaling pathways. In these settings, PPM1L represses the activity of TGFβ-activated kinase 1 (TAK1) and apoptosis regulating kinase 1 (ASK1), two mitogen-activated protein kinase kinase kinases [5,6]. Recently, we demonstrated that PPM1L is an endoplasmic reticulum (ER)-resident transmembrane protein involved in the regulation of ceramide trafficking at specialized regions of membrane apposition termed ER–Golgi membrane contact sites [7]. We found that vesicle-associated membrane protein-associated protein A (VAPA) is a binding partner of PPM1L and that VAPA-dependent dephosphorylation of the ceramide transport protein CERT by PPM1L is required for the stimulation of ceramide transport. However, the mechanism by which PPM1L is recruited to ER–Golgi membrane contact sites was unclear. In this study, we identified the Golgi resident protein, acyl-CoA binding domain containing 3 (ACBD3, also known as GCP60 and PAP7), as a novel binding partner of PPM1L and suggested that ACBD3 recruits PPM1L to ER–Golgi membrane contact sites.

Abbreviations: PPM1L, metal-dependent protein phosphatase 1L; ACBD3, acyl-CoA binding domain containing 3; ER, endoplasmic reticulum; GOLD, Golgi dynamics; FYCO1, FYVE and coiled-coil domain-containing protein 1

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http://dx.doi.org/10.1016/j.febslet.2012.06.050

2. Materials and methods

2.1. Materials

Modifying enzymes for DNA manipulation were obtained from New England Biolabs (Beverly, MA). Glutathione-Sepharose beads, polyvinylidene difluoride (PVDF) membrane, and ECL kits were obtained from GE Healthcare (Buckinghamshire, UK). Anti-HA antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG antibody was obtained from Sigma (Saint Louis, MO). Anti-GST antibody was obtained from Cell Signaling Technology (Danvers, MA). Anti-protein disulfide isomerase (PDI) was obtained from BD (Franklin Lakes, NJ). Alexa Flour 488-anti rabbit IgG and Alexa Flour 568 anti-mouse IgG antibodies were obtained from Invitrogen (Rockville, MD). All other reagents were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Yeast two-hybrid screening

Yeast two-hybrid screening was carried out as described previously [7]. To generate a diploid strain expressing bait and prey proteins, an *Saccharomyces cerevisiae* Y187 strain containing pACT2-ACBD3 was mated to an AH109 strain containing one of the following: pGBK-PPM1L (WT), pGBK-PPM1L (D302A), pGBK-PPM1A, pGBK-PPM1B, pGBK-ILKAP, or pGBK-PPM1J. The diploid cells were streaked onto SD/Trp, Leu (−) and SD/Trp, Leu, His (−) plates and incubated at 30 °C for 3 days.

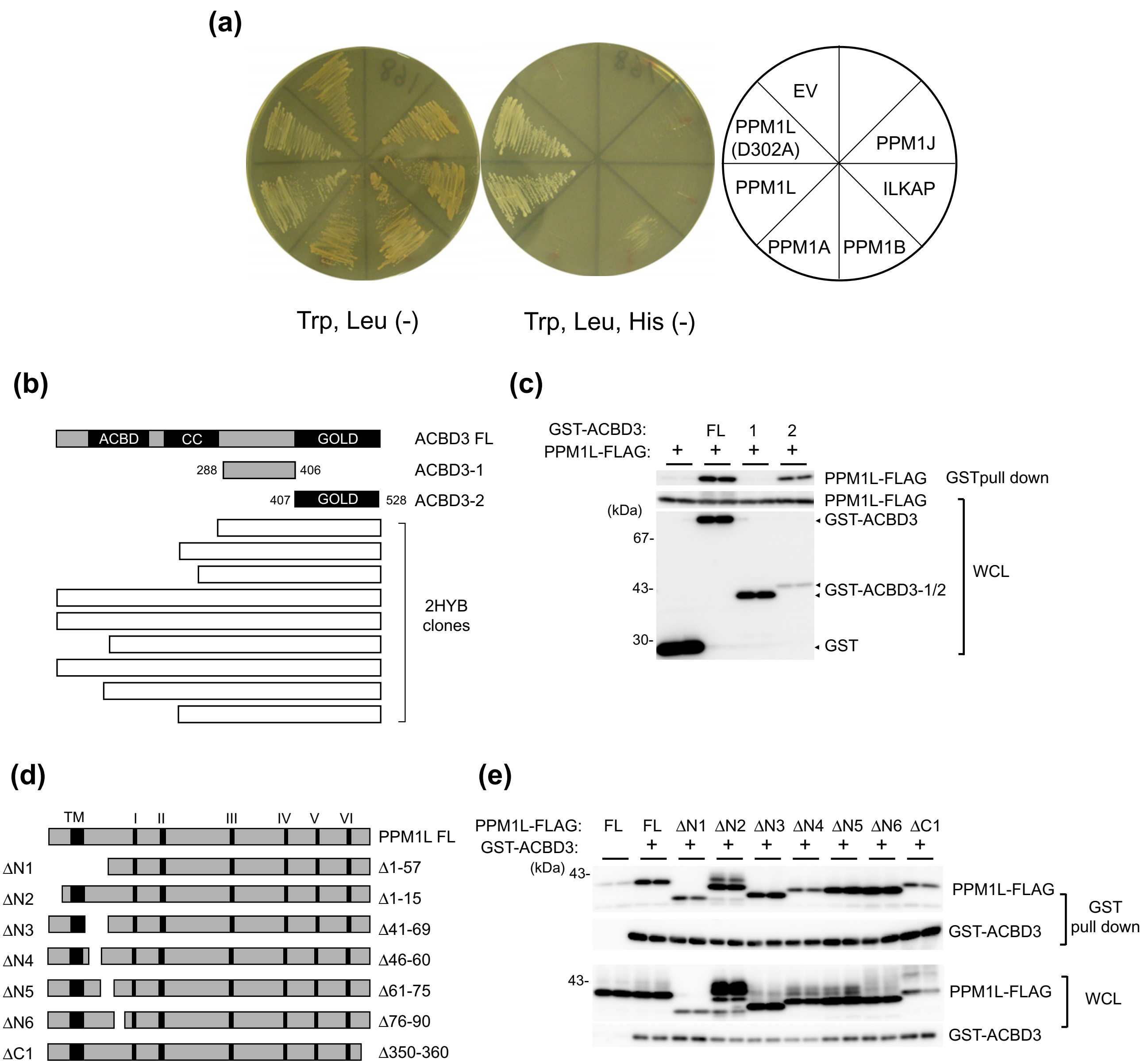

Fig. 1. ACBD3 specifically interacts with PPM1L. (a) Diploid strains expressing bait and prey proteins were generated by mating an *S. cerevisiae* Y187 strain expressing the GAL4 activation domain fused with ACBD3 and an AH109 strain expressing the GAL4 DNA binding domain fused with PPM1L (WT), PPM1L (D302A), PPM1A, PPM1B, ILKAP, or PPM1J. Diploid cells were streaked onto SD/Trp, Leu (−) and SD/Trp, Leu, His (−) plates (left and middle panels, respectively). The location of transformants on the plates is shown on the right. (b) Schematic representation of full length ACBD3 and its mutants (ACBD3-1 and ACBD3-2). Acyl-CoA binding (ACBD), coiled-coil (CC), and GOLD domains are shown. Regions contained in the 2HYB clones are also indicated. (c) HEK293 cells were co-transfected with an expression plasmid encoding PPM1L-FLAG and either full length GST-ACBD3, ACBD3-1, or ACBD3-2, followed by GST-pull down analysis. WCL; whole cell lysate. FL; full length GST-ACBD3. 1; ACBD3-1. 2; ACBD3-2. (d) Schematic representation of full length PPM1L and its mutants. The transmembrane domain (TM) and six motif (I–VI) that are conserved in all PPM family members are shown. (e) HEK293 cells were co-transfected with an expression plasmid encoding GST-ACBD3 and either wild type or deletion mutants of PPM1L, followed by GST-pull down analysis.

2.3. DNA cloning and deletion mutants

Full length ACBD3 and the GOLD domains of FYCO1 (1362–1480aa), SEC14L1 (523–674aa), and SEC14L2 (277–383aa) were obtained from a human cDNA library. Deletion mutants of PPM1L and ACBD3 were generated by PCR.

2.4. Cell culture, transfection, and GST-pull down assays

HEK293 and HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum. Transfections were performed using modified calcium phosphate and linear polyethylenimine methods for HEK293 and HeLa cells, respectively [8,9]. After transfection, cells were cultured for 48 h before harvest. The cells were washed twice with phosphate-buffered saline (PBS) and lysed with ice-cold lysis buffer containing 50 mM Tris–HCl pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM Na orthovanadate, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM Na pyrophosphate, and Complete Protease Inhibitor Cocktail (Roche). For GST-pull downs, 200 μg of cell lysate was incubated for 0.5 h with 5 μL glutathione-Sepharose beads. The beads were washed 5 times with lysis buffer, and then bound proteins were subjected to 10% (w/v) SDS–PAGE and transferred to PVDF membranes. Membranes were incubated with primary antibody for 1 h at 25 °C, followed by incubation with HRP-conjugated secondary antibody for 1 h at 25 °C. Finally, signal was detected by chemiluminescence and quantified using an imaging analyzer (LAS4000 mini; GE Healthcare).

2.5. Immunofluorescence staining and imaging

Cells were seeded on coverslips coated with poly-D-lysine, fixed with 3% (w/v) paraformaldehyde in PBS for 10 min, and permeabilized with PBS containing 0.05% (v/v) Triton X-100 for 30 min at 25 °C. Then, coverslips were blocked by incubation with 3% (w/v) BSA in PBS for 30 min, followed by 1 h incubation with primary antibody. Next, cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG for 1 h. The immunostained samples were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized by confocal microscopy (LSM 5; Carl Zeiss, Oberkochen, Germany).

3. Results

3.1. ACBD3 specifically interacts with PPM1L

We carried out a yeast two-hybrid screen to identify the substrate(s) or protein(s) responsible for regulating PPM1L activity. The coding region of full length PPM1L [D302A], a mutant deficient in phosphatase activity, was fused to the GAL4 DNA binding domain (DBD) and used as bait in a screen of 1×10^6 clones obtained from a human brain cDNA library. This process identified acyl-CoA binding domain containing 3 (ACBD3) as a PPM1L interacting partner. Furthermore, in this screen ACBD3 interacted with PPM1L, but not with GAL4 DBD, PPM1A, PPM1B, ILKAP, or PPM1J, suggesting that the interaction between ACBD3 and PPM1L was specific (Fig. 1a). To examine whether this interaction also occurred in mammalian cells, a GST fusion protein of ACBD3 was co-expressed with PPM1L-FLAG in HEK293 cells. Indeed, ACBD3 interacted with PPM1L, as determined by a GST-pull down assay on cell lysates.

We noticed that all of the clones obtained in two-hybrid screening possessed the C-terminal region of ACBD3, *i.e.*, GOLD domain. Therefore, to determine the interacting region of ACBD3 with PPM1L, we generated GST fusion proteins with different C-terminal regions (Fig. 1b). A GST-pull down assay showed that GST-ACBD3-2, containing the GOLD domain, but not GST-ACBD3-1, which included the other C-terminal portion, interacted with PPM1L (Fig. 1c). These data indicated that the GOLD domain was responsible for interactions with PPM1L.

To identify the complementary region of PPM1L that is required for interactions with ACBD3, deletion mutants of PPM1L were expressed and interactions with GST-ACBD3 were examined (Fig. 1d). All deletion mutants tested bound to ACBD3, suggesting that the catalytic domain of PPM1L interacted with the ACBD3

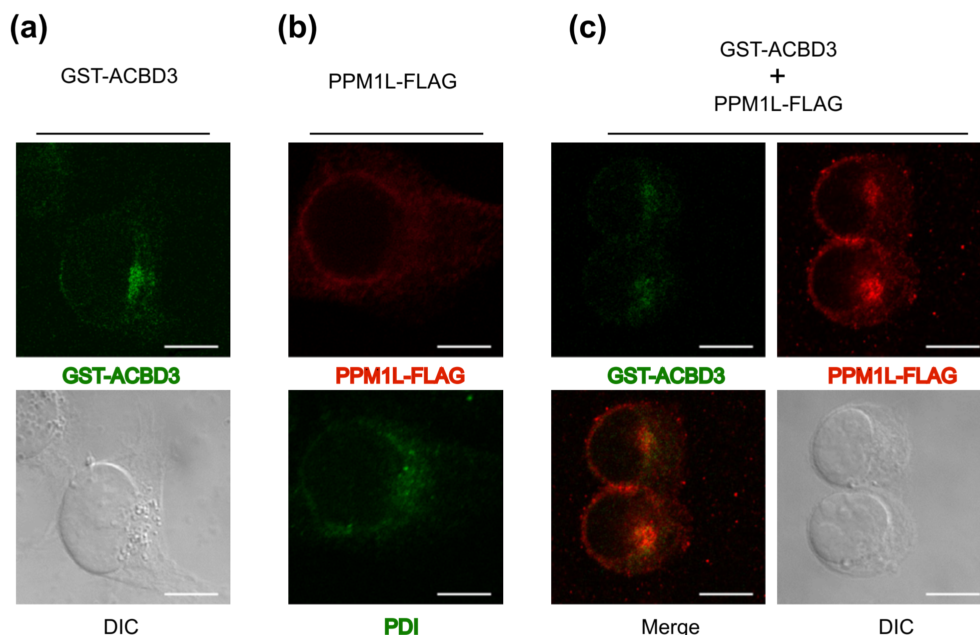

Fig. 2. ACBD3 recruits PPM1L to the Golgi complex. HeLa cells were transiently transfected with expression plasmid(s) for GST-ACBD3 and/or PPM1L-FLAG. 48 h after transfection, the cells were fixed, permeabilized, and incubated with primary antibodies against (a) GST-ACBD3, (b) PPM1L-FLAG, and (c) both proteins. Immunoreactivity was detected with Alexa Fluor 568-anti-mouse IgG (red) or Alexa Fluor 488-anti-rabbit IgG (green). DIC; differential interference contrast image. PDI; protein disulfide isomerase. Scale bars, 10 μm.

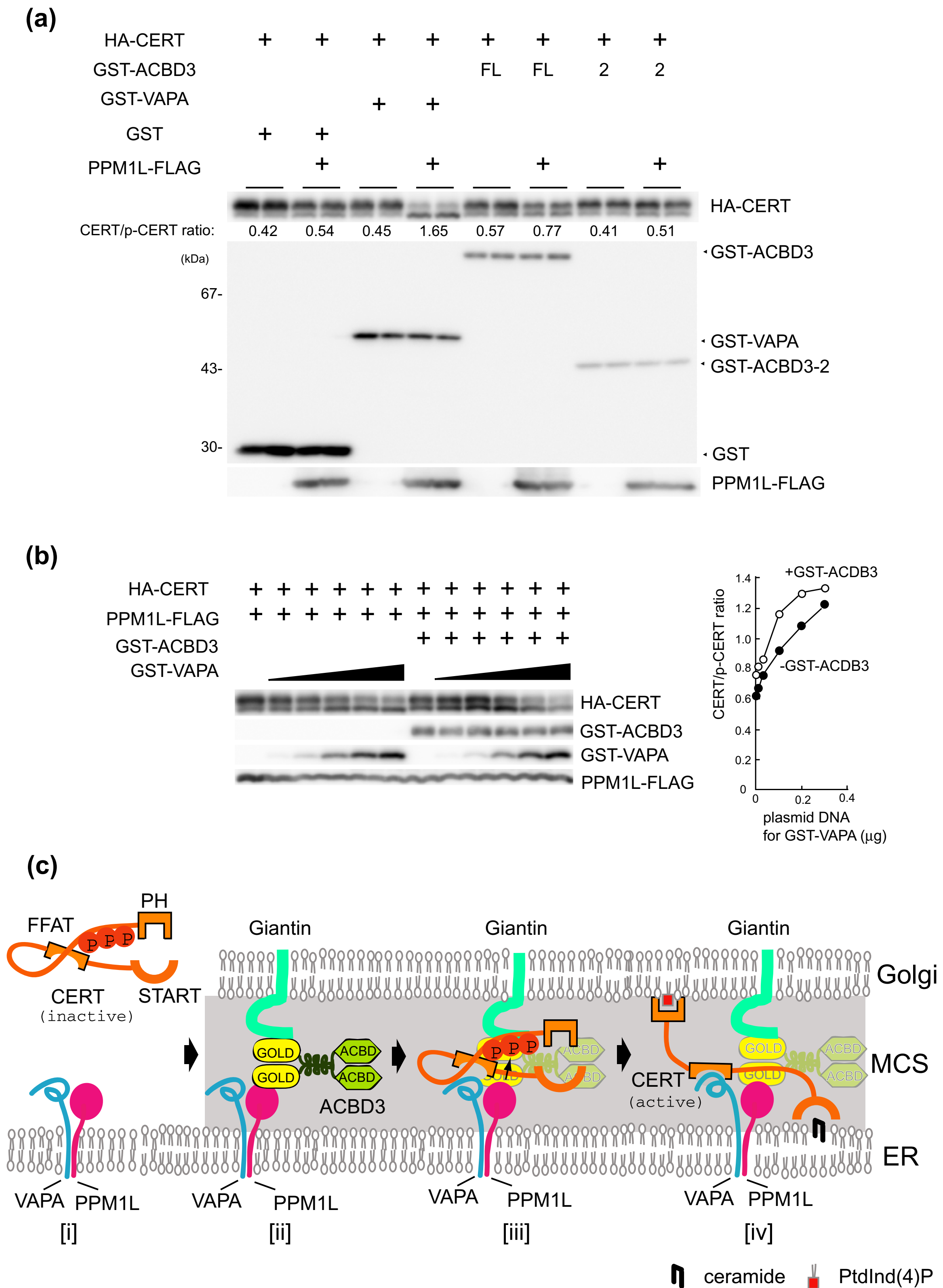

Fig. 3. ACBD3 promotes dephosphorylation of the ceramide transport protein CERT. (a) HEK293 cells were transiently transfected with the indicated combinations of expression plasmid(s) encoding GST-ACBD3, GST-ACBD3-2, GST-VAPA, GST alone, PPM1L-FLAG, and HA-CERT. Cells were lysed after 48 h and lysates were subjected to immunoblotting with anti-HA, anti-GST, or anti-FLAG antibodies. FL; full length ACBD3. 2; ACBD3-2. The ratios of CERT(lower band)/p-CERT(upper band) are indicated below the upper panel to assess for the dephosphorylation of CERT. (b) HEK293 cells were transiently transfected with the indicated combinations of expression plasmid(s) with increasing amount of expression plasmid of GST-VAPA (0.01–0.3 μg). Cells were lysed after 48 h and the lysates were subjected to immunoblotting with anti-HA, anti-GST, or anti-FLAG antibody. The ratios of CERT/p-CERT are indicated in the right panel. (c) Proposed model for ACBD3 regulation of PPM1L localization to the membrane contact site (MCS). CERT normally exists in a hyperphosphorylated form residing in the cytosol. In this conformation, the function of the PH and START domains is fully repressed and the binding activity of the FFAT motif is partially inhibited [i]. ACBD3 interacts with giantin and recruits PPM1L to the MCS [ii]. Although ACDB3 is depicted as a homo-dimer [10], a single GOLD domain may simultaneously interact with both PPM1L and giantin. CERT weakly interacts with VAPA and the Ser/Thr residues within the SR motif are dephosphorylated by PPM1L [iii]. Upon dephosphorylation, CERT assumes an active conformation in which the PH, FFAT, and START domains are fully activated [iv].

GOLD domain (Fig. 1e). Some of the mutants (*e.g.*, ΔN2, ΔN4 and ΔN5) were accompanied by species with relatively high molecular weight when expressed in HEK293 cells. However, their interaction with ACBD3 appeared to be substantially weaker than that of the species with the original molecular weight, suggesting that only intact form of PPM1L could interact efficiently with ACBD3. Although identity of these molecules is uncertain, they may be the products of some kind of post-translational modification such as ubiquitination, which may lead to the degradation of ΔN2, ΔN4, and ΔN5, ectopically expressed in the cells.

3.2. Binding of ACBD3 alters subcellular distribution of PPM1L

Next, we examined whether co-expression of ACBD3 and PPM1L affected their subcellular distribution. As previously reported, single expression of GST-ACBD3 or PPM1L-FLAG in HeLa cells resulted in perinuclear localization (Golgi complex) and diffuse reticular staining that overlapped with PDI expression (ER), respectively [7,10] (Fig. 2a and b). However, when these proteins were co-expressed, PPM1L-FLAG localized along with GST-ACBD3 to a perinuclear location (Fig. 2c). These results suggested that PPM1L was recruited to the Golgi apparatus through interactions with ACBD3.

3.3. ACBD3 promotes dephosphorylation of the ceramide transporter CERT

Although we previously demonstrated that VAPA-dependent dephosphorylation of the ceramide transport protein CERT by PPM1L was required for ceramide transport from the ER to the Golgi [7], the mechanism by which PPM1L is translocated to the ER–Golgi membrane contact site, where CERT is dephosphorylated, was unclear. The results presented here implicate ACBD3 in the recruitment of PPM1L to the ER–Golgi membrane contact site. Accordingly, we examined the effect of over-expression of ACBD3 on CERT phosphorylation. While over-expression of either PPM1L-FLAG or GST-ACBD3 had little effect on CERT phosphorylation, co-expression of these proteins enhanced the dephosphorylation of the substrate (Fig. 3a). Furthermore, co-expression of GST-ACBD3-2, a mutant that contains only the GOLD domain, with PPM1L-FLAG did not affect CERT phosphorylation. To examine whether PPM1L recruitment to the Golgi is responsible for VAPA-dependent CERT dephosphorylation, we evaluated the effect of co-expression of GST-ACBD3 with PPM1L-FLAG and GST-VAPA on dephosphorylation of CERT. As shown in Fig. 3b, expression of GST-ACBD3 further enhanced the VAPA-mediated CERT dephosphorylation by PPM1L. These results are consistent with the observations from Fig. 3a and suggest that ACBD3 and VAPA act coordinately in CERT dephosphorylation by PPM1L in different ways.

3.4. GOLD domains in other proteins may also have PPM1L-binding activities

Finally, we examined whether GOLD domains in other proteins also interacted with PPM1L. cDNA encoding the GOLD domains from FYVE and coiled-coil domain-containing protein 1 (FYCO1), SEC14L1, and SEC14L2 were cloned from a human cDNA library to produce GST fusion proteins. GST-pull down assays indicated that these GOLD domains interacted with PPM1L, raising the possibility that FYCO1, SEC14L1, and SEC14L2 may also associate with PPM1L (Fig. 4).

4. Discussion

ACBD3 (also known as Golgi complex-associated protein of 60 kDa) is a Golgi resident protein comprising a putative acyl-CoA binding domain, a coiled-coil domain, and a GOLD (Golgi

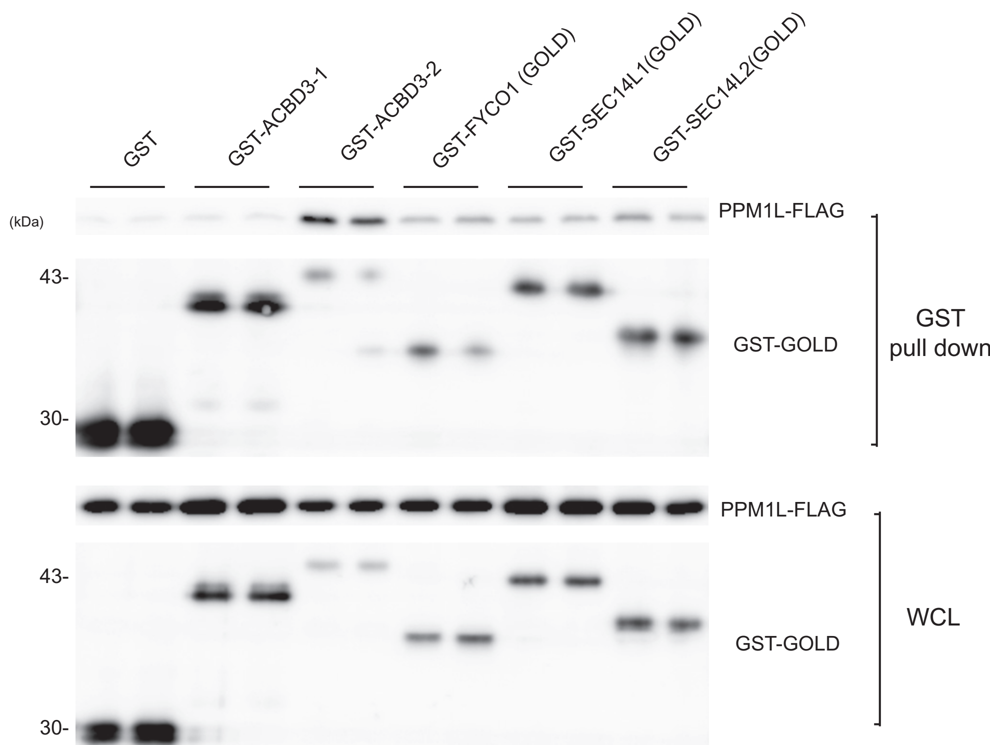

Fig. 4. PPM1L binds GOLD domains derived from different proteins. PPM1L-FLAG was co-expressed in HEK293 cells with either GST-ACBD3-1, GST-ACBD3-2, GST-FYCO1(GOLD), GST-SEC14L1(GOLD), or GST-SECL2(GOLD). Then, cells were lysed and GST-pull down analysis was performed. WCL; whole cell lysate.

dynamics) domain. This protein is thought to be involved in maintaining Golgi structure and regulating protein transport between the ER and Golgi [10]. In this study, we identified ACBD3 as a novel binding partner of PPM1L and found that the GOLD domain of ACBD3 was responsible for interactions with PPM1L.

We concluded that ACBD3 recruited PPM1L to the ER–Golgi membrane contact site, where PPM1L dephosphorylates CERT in a VAPA-dependent manner. This conclusion was based on the observation that co-expression of PPM1L with ACBD3 induced a redistribution of the former protein to a perinuclear location along with ACBD3. Furthermore, co-expression of ACBD3 and PPM1L, but not PPM1L alone, enhanced the dephosphorylation of CERT. By contrast, co-expression of PPM1L with ACBD3-2, a deletion mutant containing only the GOLD domain, tightly interacted with PPM1L but did not affect CERT phosphorylation. Because the ACBD3-2 mutant has been shown not to localize to the Golgi [10] and therefore does not seem to recruit PPM1L to ER–Golgi membrane contact sites, these results suggested that the recruitment of PPM1L to these sites through association with full length ACBD3 contributes to the dephosphorylation of CERT (Fig. 3c). Meanwhile, the effect of co-expression of ACBD3 on the dephosphorylation of CERT appeared to be smaller than that of VAPA (Fig. 3a, lanes 11, 12 vs. 7, 8 and Fig. 3b). These results suggested that, while VAPA has an essential role in dephosphorylation of CERT, the role of ACBD3 in this process is rather supportive. Further studies are required to elucidate whether ACBD3 is indeed involved in the enhanced sphingomyelin synthesis [7].

The GOLD domain is a protein motif that may have important roles in mediating Golgi dynamics and secretion. Predicted to mediate diverse protein interactions [11], this domain is conserved in proteins from yeast to mammals and is classified into six subfamilies [12–15]. With the exception of the p24 family, the GOLD domain is always found in combination with lipid-, sterol-, or fatty acid-binding protein domains, *e.g.,* PH, Sec14p, and FYVE, as well as oxysterol- and acyl-CoA-binding domains, suggesting that GOLD domain-containing proteins may be membrane-associated. In this study, we demonstrated that PPM1L interacts with the GOLD domains of proteins from different subfamilies. FYCO1 was identified as a binding partner of autophagic marker, LC3 [13]. According to the proposed model [13], FYCO1, which preferentially resides on perinuclear ER membranes, binds to microtubule plus end-directed motors in response to starvation and redistributes preautophagosomal membranous compartments to sites of autophagosome formation throughout the cytosol. Although the role of the GOLD domain in this process is unclear, PPM1L may be involved in regulating FYCO1 through GOLD domain-dependent protein dephosphorylation.

Acknowledgements

This work was supported in part by a Grant-in-aid for Scientific Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

[1] Shi, Y. (2009) Serine/threonine phosphatases: mechanism through structure. Cell 139, 468–484.
[2] Lammers, T. and Lavi, S. (2007) Role of type 2C protein phosphatases in growth regulation and in cellular stress signaling. Crit. Rev. Biochem. Mol. Biol. 42, 437–461.
[3] Tamura, S., Toriumi, S., Saito, J., Awano, K., Kudo, T.A. and Kobayashi, T. (2006) PP2C family members play key roles in regulation of cell survival and apoptosis. Cancer Sci. 97, 563–567.
[4] Tamura, S., Hanada, M., Ohnishi, M., Katsura, K., Sasaki, M. and Kobayashi, T. (2002) Regulation of stress-activated protein kinase signaling pathways by protein phosphatases. Eur. J. Biochem. 269, 1060–1066.
[5] Li, M.G., Katsura, K., Nomiyama, H., Komaki, K., Ninomiya-Tsuji, J., Matsumoto, K., Kobayashi, T. and Tamura, S. (2003) Regulation of the interleukin-1-induced signaling pathways by a novel member of the protein phosphatase 2C family (PP2Cepsilon). J. Biol. Chem. 278, 12013–12021.
[6] Saito, J., Toriumi, S., Awano, K., Ichijo, H., Sasaki, K., Kobayashi, T. and Tamura, S. (2007) Regulation of apoptosis signal-regulating kinase 1 by protein phosphatase 2Cepsilon. Biochem. J. 405, 591–596.
[7] Saito, S., Matsui, H., Kawano, M., Kumagai, K., Tomishige, N., Hanada, K., Echigo, S., Tamura, S. and Kobayashi, T. (2008) Protein phosphatase 2Cepsilon is an endoplasmic reticulum integral membrane protein that dephosphorylates the ceramide transport protein CERT to enhance its association with organelle membranes. J. Biol. Chem. 283, 6584–6593.
[8] Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7, 2745–2752.
[9] Reed, S.E., Staley, E.M., Mayginnes, J.P., Pintel, D.J. and Tullis, G.E. (2006) Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. J. Virol. Methods 138, 85–98.
[10] Sohda, M., Misumi, Y., Yamamoto, A., Yano, A., Nakamura, N. and Ikehara, Y. (2001) Identification and characterization of a novel Golgi protein, GCP60, that interacts with the integral membrane protein giantin. J. Biol. Chem. 276, 45298–45306.
[11] Anantharaman, V. and Aravind, L. (2002). The GOLD domain, a novel protein module involved in Golgi function and secretion. Genome Biol. 3, research0023.
[12] Johnson, K.G. and Kornfeld, K. (2010) The CRAL/TRIO and GOLD domain protein TAP-1 regulates RAF-1 activation. Dev. Biol. 341, 464–471.
[13] Pankiv, S., Alemu, E.A., Brech, A., Bruun, J.A., Lamark, T., Overvatn, A., Bjorkoy, G. and Johansen, T. (2010) FYCO1 is a Rab7 effector that binds to LC3 and PI3P to mediate microtubule plus end-directed vesicle transport. J. Cell Biol. 188, 253–269.
[14] Ribeiro, F.M., Ferreira, L.T., Marion, S., Fontes, S., Gomez, M., Ferguson, S.S., Prado, M.A. and Prado, V.F. (2007) SEC14-like protein 1 interacts with cholinergic transporters. Neurochem. Int. 50, 356–364.
[15] Zingg, J.M., Kempna, P., Paris, M., Reiter, E., Villacorta, L., Cipollone, R., Munteanu, A., De Pascale, C., Menini, S., Cueff, A., Arock, M., Azzi, A. and Ricciarelli, R. (2008) Characterization of three human sec14p-like proteins: alpha-tocopherol transport activity and expression pattern in tissues. Biochimie 90, 1703–1715.