Biochemical and Biophysical Research Communications 388 (2009) 395–399

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The glycolipid transfer protein interacts with the vesicle-associated membrane protein-associated protein VAP-A

Jessica Tuuf, Lina Wistbacka, Peter Mattjus *

Department of Biochemistry and Pharmacy, Åbo Akademi University, FI-20520 Turku, Finland

ARTICLE INFO

Article history:
Received 31 July 2009
Available online 8 August 2009

Keywords:
Glycolipid transfer protein
GLTP
Vesicle-associated membrane protein-associated proteins
VAP-A
FFAT
Glycosphingolipids
Synaptobrevin
Endoplasmic reticulum
Lipid transfer

ABSTRACT

The glycolipid transfer protein (GLTP) is a cytoplasmic protein with an ability to bind glycolipids and catalyze their *in vitro* transfer. In this study, we have found a FFAT-like motif in GLTP. The FFAT (two phenylalanines in an acidic tract) motif in lipid-binding proteins has previously been shown to interact with the VAPs (vesicle-associated membrane protein-associated proteins) in the endoplasmic reticulum. Here we used glutathione S-transferase pull-down experiments to confirm that GLTP and VAP-A interact. By displacing different amino acids in the motif we clearly show that the interaction is dependent on the FFAT-like motif in GLTP. The potential role of GLTP in the endoplasmic reticulum association is discussed.

Introduction

Lipid regulation and transport is of utmost importance in eukaryotic cells. Numerous key interplayers in the lipid metabolism have been identified in mammals; among them are the VAPs, vesicle-associated membrane protein-associated proteins (VAMP-associated proteins). VAP-33 was initially discovered in *Alypsia californicatia* and it was suggested that the protein plays a role in the exocytosis of neurotransmitters [1]. Since then various VAPs have been found in mammals. In humans, the different VAPs that have been reported include; VAP-A, VAP-B, VAP-C [2], and hVAP-33 [3]. The different isoforms are expressed ubiquitously [2,3] and it was later demonstrated that the VAP-A is residing in the endoplasmic reticulum (ER) and ER/Golgi intermediate compartment (ERGIC) [4]. The VAP structure consists of three different domains; an N-terminal domain with an immunoglobulin-like β-sheet, a central coiled–coiled domain and a transmembrane domain in the C-terminus [2,3].

Several different cytosolic lipid-binding proteins are able to associate with the VAPs and are consequently targeted to the ER. The common feature within these proteins is a short motif called FFAT (two phenylalanines (FF) in an acidic tract) [5]. The FFAT motif has been reported to bind to VAP-A through its N-terminal domain (MSP-domain) [6,7]. One protein that contains the FFAT motif and interacts with both VAP-A and VAP-B is the ceramide transport protein (CERT) [8]. The function of CERT is to transfer newly synthesized ceramide from the ER to the *trans*-Golgi, where it will be converted to sphingomyelin [9]. Another protein which is targeted to the ER via the VAPs is the oxysterol-binding protein (OSBP) [10], a protein which was demonstrated to be able to regulate sphingomyelin metabolism via the ceramide transport protein [11]. The glycolipid transfer protein (GLTP) is a cytosolic protein that is able to catalyze the intermembrane transfer of glycosphingolipids (GSL) between two membranes *in vitro* [12]. The GLTPs contain a unique structure composed of only α-helices and they are members of a new superfamily of lipid-binding proteins [13]. It has been shown that GLTPs can transfer several glycolipids, but it can not use phospholipids, sphingomyelin and neutral lipids as substrates [14]. We have previously demonstrated that GLTP is located in the cytosol in HeLa cells, and not within any organelles [15]. Furthermore, we showed that the level of glucosylceramide is increased when over-expressing GLTP, but remains unchanged when down-regulating GLTP [15]. In a recent review on the GLTPs and their membrane interactions, it was suggested that GLTP could be involved in glycolipid regulation and metabolism [14].

To further investigate whether GLTP could play a role in the lipid regulation we have examined the interaction of GLTP with VAP-A, an integral protein residing in the ER. We produced three

* Corresponding author. Address: Department of Biochemistry and Pharmacy, Åbo Akademi University, Tykistokatu 6A, III, FI-20520 Turku, Finland. Fax: +358 2 2154271.
E-mail address: Peter.Mattjus@abo.fi (P. Mattjus).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.08.023

mutants disrupting a FFAT-like motif in GLTP and examined their ability to bind to VAP-A. In this study, we show that VAP-A is indeed interacting with GLTP and that this association is dependent on the FFAT-like motif found in GLTP.

Materials and methods

Materials. The polyclonal antibody against human GLTP (antibody I) has previously been described [15]. The rabbit anti-β-actin was from Rockland (USA) and the secondary peroxidase conjugated antibodies; rabbit anti-goat and goat anti-rabbit were from Pierce Biotechnology (USA). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Avanti Polar Lipids (Alabaster, USA). The fluorescent lipids, BODIPY-C_{12}-glucosylceramide (BODIPY-GlcCer) and the quencher DiI-C_{18} were from Invitrogen (Carlsbad, USA).

Construction of the plasmids. The pGEX-3X-VAP-A-construct was a kind gift from Dr. Neale Ridgway (Dalhousie University, Canada) [10]. The pcDNA-GLTP(h)-vector [15] was modified by adding a histidine-tag (6-His) in the C-terminus of the gene using PCR. Site-directed mutagenesis in the FFAT motif of human GLTP was performed using the following oligonucleotides: D35A (5-CCTGC CGCCCTTCTTCGCATGCCTTGGGTCCCC-3), FFD-AAA (33–35) (5-CC TGCCGCCCGCCGCCGCATGCCTTTGGGTCC-3) and FF-AA (33–34) (5-CCTGCCGCCCGCCGCCGATTGCCTTGGGTCC-3). The point mutations were introduced using PCR according to the manual of Stratagene and all the constructs were checked by DNA sequencing.

Expression and purification of recombinant proteins in Escherichia coli. The expression and purification of the His-tagged bovine GLTP and the human GLTP in *E. coli* cells have previously been described [16,17]. The activity of the recombinant GLTPs was checked using a previously described resonance energy transfer (RET) assay [15]. BL-21 cells, containing the construct GST-VAP-A, were cultured at 29 °C until the OD A_{600} had reached 0.5. The fusion protein was then induced by adding 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated for an additional 3 h at 25 °C. The purification of GST-VAP-A and control GST-protein was performed according to GE Healthcare. The protein concentration was determined using the method of Lowry [18] and the samples were analyzed on SDS–PAGE.

Cell culture and transfection experiments. HeLa cervical carcinoma cells were cultured in Dulbecco's modified Eagle's medium, Sigma (St. Louis, USA) supplemented with penicillin/streptomycin and 10% fetal calf serum. 5×10^6 HeLa cells were transiently transfected using the electroporator Gene Pulser II RF Module, BIO-RAD (Hercules, USA). After 48 h, the cells were collected for further analysis.

Lysis of transfected cells for the GST pull-down assay. The transfected cells were washed twice with phosphate buffered saline (PBS), pH 7.4. The cells were then dissolved in 200 μl lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 0.5% Tween-20, 0.5 mM PMSF, 1× protease inhibitor cocktail (Sigma), 1 mM dithiotreithol, pH 8.0) and incubated for 40 min on ice. The supernatant was collected after centrifugation at 13,000 rpm, 30 min.

GST pull-down assay and Western blotting analysis. Different amounts of purified GST-VAP-A and GST were incubated for 1 h at room temperature (RT) with either purified hGLTP/bGLTP produced in bacteria or lysates from HeLa cells over-expressing hGLTP/mutant hGLTP constructs. Glutathione Sepharose 4B beads (GE Healthcare), in a slurry of 1:1, were added and incubated from 45 min up to over-night. The beads were collected by centrifugation and washed three times with either the lysis buffer, containing only 0.05% Tween or wash buffer (10 mM NaH_2PO_4, 150 mM NaCl, 5 mM KCl, 2 mM EDTA, 2 mM EGTA, pH 7.4). The samples were then denatured at 95 °C for 5 min in a Laemmli buffer. SDS–PAGE and Western blotting were used for analysis of the samples.

Resonance energy transfer assay and sample preparation. The RET method has previously been described [15]. The donor vesicles were composed of 0.5 mol% fluorescent probe (BODIPY-GlcCer), 3 mol% DiI-C_{18} and 96.5 mol% POPC and the acceptor vesicles of 100 mol% POPC. The lipids were dissolved in 10 mM sodium dihydrogen phosphate buffer (pH 7.4) and the vesicles were prepared by probe sonication. HeLa cells transiently transfected with human GLTP or its mutant constructs were harvested after 48 h and washed twice with PBS. The cells were dissolved in Hepes-buffer (15 mM KCl, 1.5 mM Mg-acetate, 10 mM Hepes (pH 7.5), 0.5 mM PMSF, 1× protease inhibitor cocktail (Sigma), 1 mM dithiotreithol) and incubated 10 min on ice. The cells were then homogenized using a 27-gauge needle and the homogenate was centrifuged at 13,000 rpm for 30 min. The supernatant was collected and glycerol was added to a final concentration of 10% to conserve the activity of the proteins. Total protein (700 μg) was used in the RET assay.

Results and discussion

The human GLTP contains a FFAT-like motif

It is crucial that the directing of various proteins to their specific cellular targets is well coordinated. The FFAT motif residing within some lipid-binding and lipid-sensing proteins has been shown to be a significant factor for guiding the proteins to the VAP-A protein in the ER [5]. Analysis of the GLTP amino acid sequence reveals that the GLTPs contain a motif, which closely resembles the consensus sequence of the FFAT motif (Fig. 1A and B). The FFAT-like motif in human GLTP contains two phenylalanines (FF) and an aspartate residue (D). In addition to these residues, a proline, cysteine, leucine and a glycine residue is found in the motif of GLTP. Loewen et al. proposed that the consensus sequence of the FFAT motif is EFFDAxE, but also suggested that some of the amino acids in the motif are not absolute and that different variations can be tolerated [5]. In addition, based on structural studies on the VAP-FFAT interaction, Kaiser et al. suggested that the consensus sequence is not necessary for the association [6]. As the GLTP sequence contains a motif with two phenylalanines and an aspartate we examined whether an interaction between VAP-A and the GLTPs would occur.

Interaction of GLTP with VAP-A using a GST pull-down assay

In order to analyze whether GLTP could interact with VAP-A, we initially performed a GST pull-down assay using recombinant GST-VAP-A and human/bovine GLTP. The results in Fig. 2A and B clearly show that when increasing the amount of GST-VAP in the assay, an increased binding of both hGLTP and bGLTP to the VAP-A could be observed (lanes 5–7). These results suggest that the detected interaction is direct and that no additional factors are needed for the association. In the control samples no GLTP could be detected (lanes 1–4). This implies that no unspecific binding of the GLTPs to either the beads or the GST-tag occurred under these conditions.

To further investigate whether GLTP could be recognized by VAP-A in a more physiological environment, HeLa cells were transfected with a plasmid expressing His-tagged human GLTP. The cells were lysed and the supernatant was used in a GST pull-down assay together with GST-VAP-A. Fig. 3A shows that hGLTP is binding to GST-VAP-A. The detection of GLTP is getting stronger with increasing amount of GST-VAP-A (lanes 5–7) and no unspecific binding can be detected in the control samples (lanes 1–4). Based on the results in Figs. 2 and 3A we conclude that GLTP and VAP-A interact. The fact that the FFAT-like motif resides on the surface of GLTP, based on structural evidence (PDB 2BV7) supports the idea that GLTP is able to interact with the VAPs.

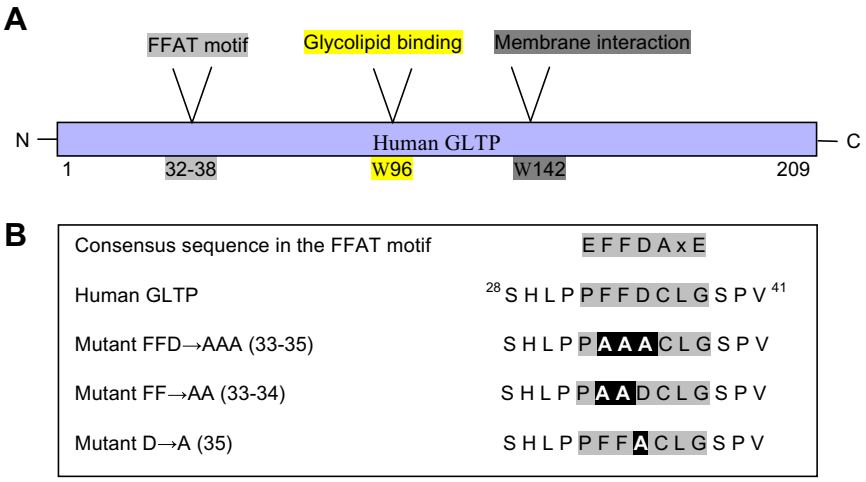

Fig. 1. A schematic presentation of human GLTP and the mutant constructs used in the study. (A) Important amino acids for the function of human GLTP are shown. The tryptophan at position 96 has previously been shown to be important for lipid binding and the tryptophan at position 142 for membrane interaction [16]. The FFAT-like motif in GLTP is located at position 32–38. (B) Construction of FFAT motif mutants of GLTP. The consensus sequence of the FFAT motif is highlighted as a gray box and the mutated amino acids as black boxes.

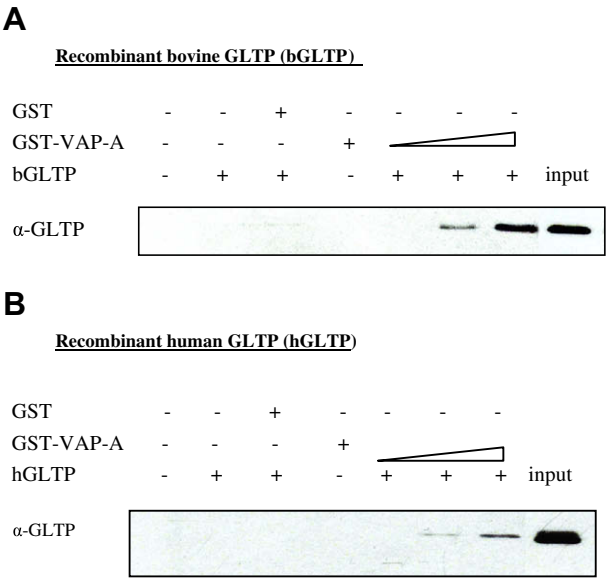

Fig. 2. GST pull-down assay using recombinant GST-VAP-A and GLTP. Different amounts of GST-VAP-A (0.1 μM, 1.0 μM and 2.5 μM) or GST (2.5 μM) were incubated with either recombinant (A) bovine or (B) human GLTP (1 μM). The pull-down samples were analyzed on SDS–PAGE and Western blotting using an α-GLTP antibody.

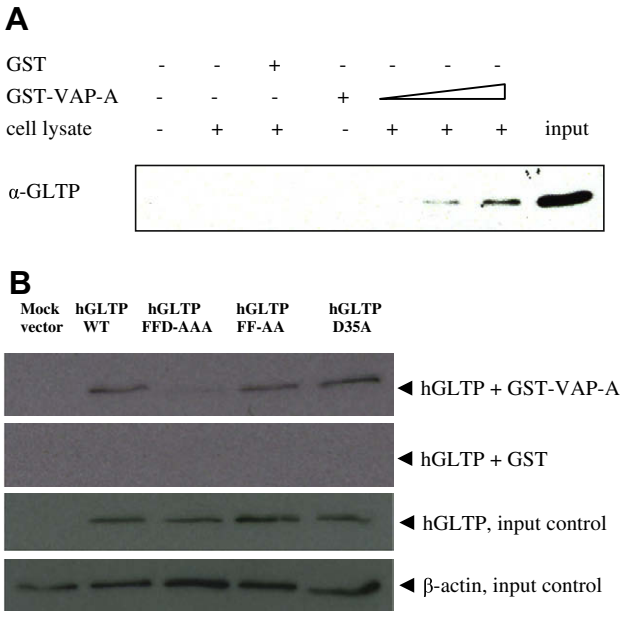

Fig. 3. Binding of VAP-A to wild type and mutant hGLTP using a GST pull-down assay. (A) Different amounts of GST-VAP-A (1.0 μM, 2.5 μM and 5.0 μM) or GST (5.0 μM) were incubated with equal amounts of cell lysate over-expressing wild type hGLTP. (B) Different GLTP mutants were over expressed in HeLa cells and analyzed for VAP-A binding ability. The pull-down samples were analyzed on SDS–PAGE and Western blotting using antibodies detecting hGLTP or β-actin.

The interaction between VAP-A and GLTP is dependent on the FFAT-like motif in GLTP

In order to examine whether the binding was due to the FFAT-like motif in GLTP we produced three FFAT motif mutants of human GLTP and analyzed their ability to associate with VAP-A (Fig. 1B). In Fig. 3B, we show that the FFAT-like motif in GLTP is crucial for the interaction between VAP-A and GLTP. The association is clearly destroyed by mutating the two phenylalanines (FF) and the aspartate (D) to alanines (A) (FFD-AAA). When we only changed the aspartate to alanine (D35A) or the two phenylalanines to alanines (FF-AA), a strong interaction could still be seen. No unspecific binding to GST was occurring. Previous studies have suggested that especially the aspartate residue is essential for the association [5,6]. However, we found an interaction between the two proteins though we changed the D35 in GLTP to A35 or when we mutated the two phenylalanine residues to alanines. The results in this study clearly show that there is a binding between the two proteins and that the binding is destroyed only when disrupting the FFD amino acids to three alanines. This further suggests that the FFAT consensus sequence is not conclusive, rather that other FFAT-like motifs are possible candidates for VAP-interaction.

The mutations in GLTP are not inactivating the proteins

To rule out the possibility that the lipid transfer activity of the GLTP mutants was lost by the amino acid substitutions, we performed activity measurements using a well characterized RET

assay. HeLa cells were transfected with hGLTP or mutant hGLTPs, lysed and the supernatant was collected. We then measured the transfer of BODIPY-labeled GlcCer from donor vesicles to acceptor vesicles, mediated by the supernatant fractions. In Fig. 4A, we show a representative experiment of recombinant bovine GLTP transferring BODIPY-GlcCer between the two vesicle populations. We then measured the glycolipid transfer activity in the supernatant fractions from HeLa cells over-expressing hGLTP or the mutant constructs (Fig. 4B and C). The results show that the GLTP mutants have a significant higher transfer activity than the control sample (HeLa cells over-expressing mock-vector). Taken together, we demonstrate that all of the mutants still possess glycolipid transfer capacity, and we suggest that the FFAT-like motif in GLTP does not appear to be directly involved in the intermembrane transfer activity of glycolipids.

Conclusions

VAP-A has previously been reported to bind to several different lipid-binding proteins [8,10]. GLTP has several homologs and orthologs and one of them is ACD11 from *Arabidopsis thaliana*, an accelerated cell death protein and a possible sphingosine transfer protein. It was recently reported that ACD11 is indeed interacting with VAP27-1, a homolog to mammalian VAP-A [19]. However, this protein does not contain the FFAT motif and is apparently interacting with VAP27-1 through another unknown motif.

The biological implication of the VAP-A–GLTP-interaction is still unclear. We previously showed that GLTP is in the cytosol and that it is not residing inside any other organelles [15]. The VAPs are integral ER-proteins with a cytoplasmic N-terminal domain and would therefore consequently target the GLTPs to the ER. One reason for us not finding GLTP together with the ER-fractions in our previous study could be that GLTP is recruited to the ER-membranes only under specific conditions *in vivo*, or that the interaction is weak. Furthermore, based on our previous results where we showed that the level of glucosylceramide is increased when over-expressing GLTP, but remained unchanged in GLTP-knockdown cells [15], we suggested that GLTP could act as an intracellular sensor of GlcCer in cells. As glucosylceramide is synthesized at the cis/medial-Golgi-apparatus [20], which lies in close proximity to the ER, one possible action of GLTP could be to sense the level of GlcCer at this location after it has been directed to the ER via VAP-A. Although it is clear that GLTP interacts with VAP-A in a FFAT motif-dependent manner, further investigations need to be performed in order to explain the *in vivo* function of GLTP.

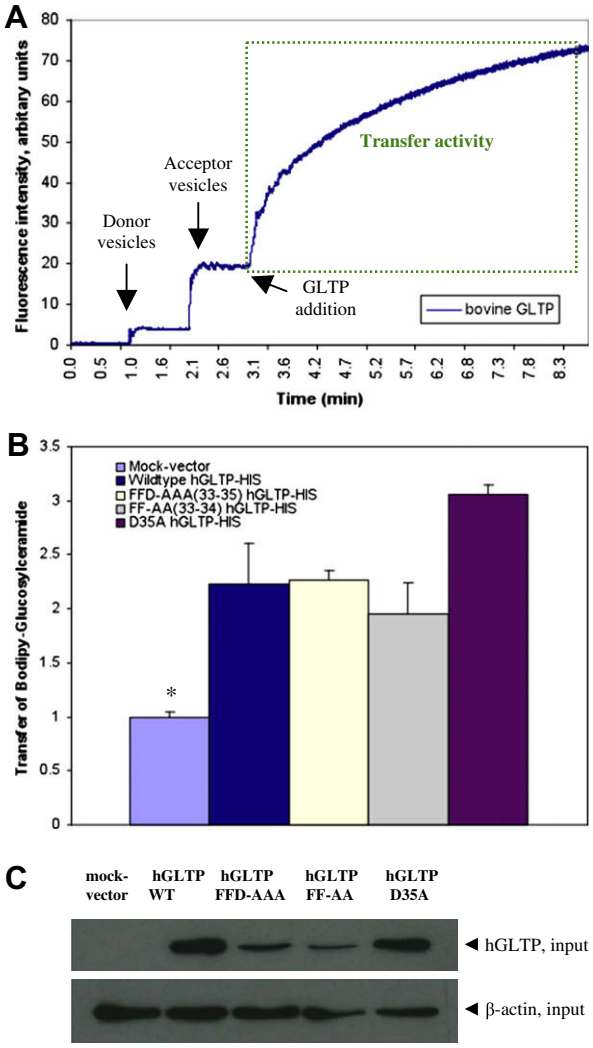

Fig. 4. Activity measurements of wild type and mutant hGLTP using a RET assay. (A) 2 μg of recombinant His-tagged bovine GLTP was used in a control experiment to demonstrate the RET assay viability. (B) The transfer of BODIPY-GlcCer mediated by the supernatant fractions was measured. Results are means ± SD of two independent experiments and the transfer is normalized to 1 for the control sample (mock-vector). The data in (B) are significantly different ($P < 0.05$). (C) Equal protein amounts of HeLa cell lysate were analyzed by Western blotting for presence of over expressed GLTP. As a loading control an antibody against β-actin was used.

Acknowledgments

We thank J. Peter Slotte for access to the tissue culture facilities and Neale Ridgway for providing us the VAP-A-plasmid. We also thank Henna Ohvo-Rekilä and Pia Roos-Mattjus for critical reading of the manuscript. This work was supported by the Academy of Finland, Sigrid Jusélius Foundation, Magnus Ehrnrooth Foundation, Oscar Öflund Foundation, Svenska Kulturfonden, K. Albin Johanssons Stiftelse, Åbo Akademi University and Medicinska Understödsföreningen Liv och Hälsa r.f.

References

[1] P.A. Skehel, K.C. Martin, E.R. Kandel, D. Bartsch, A VAMP-binding protein from Aplysia required for neurotransmitter release, Science 269 (1995) 1580–1583.
[2] Y. Nishimura, M. Hayashi, H. Inada, T. Tanaka, Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins, Biochem. Biophys. Res. Commun. 254 (1999) 21–26.
[3] M.L. Weir, A. Klip, W.S. Trimble, Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP, Biochem. J. 333 (1998) 247–251.
[4] M.L. Weir, H. Xie, A. Klip, W.S. Trimble, VAP-A binds promiscuously to both v- and tSNAREs, Biochem. Biophys. Res. Commun. 286 (2001) 616–621.
[5] C.J. Loewen, A. Roy, T.P. Levine, A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP, EMBO J. 22 (2003) 2025–2035.
[6] S.E. Kaiser, J.H. Brickner, A.R. Reilein, T.D. Fenn, P. Walter, A.T. Brunger, Structural basis of FFAT motif-mediated ER targeting, Structure 13 (2005) 1035–1045.
[7] C.J. Loewen, T.P. Levine, A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins, J. Biol. Chem. 280 (2005) 14097–14104.
[8] M. Kawano, K. Kumagai, M. Nishijima, K. Hanada, Efficient trafficking of ceramide from the endoplasmic reticulum to the Golgi apparatus requires a VAMP-associated protein-interacting FFAT motif of CERT, J. Biol. Chem. 281 (2006) 30279–30288.
[9] K. Hanada, K. Kumagai, S. Yasuda, Y. Miura, M. Kawano, M. Fukasawa, M. Nishijima, Molecular machinery for non-vesicular trafficking of ceramide, Nature 426 (2003) 803–809.
[10] J.P. Wyles, C.R. McMaster, N.D. Ridgway, Vesicle-associated membrane protein-associated protein-A (VAP-A) interacts with the oxysterol-binding protein to modify export from the endoplasmic reticulum, J. Biol. Chem. 277 (2002) 29908–29918.
[11] R.J. Perry, N.D. Ridgway, Oxysterol-binding protein and vesicle-associated membrane protein-associated protein are required for sterol-dependent activation of the ceramide transport protein, Mol. Biol. Cell 17 (2006) 2604–2616.
[12] P. Mattjus, H.M. Pike, J.G. Molotkovsky, R.E. Brown, Charged membrane surfaces impede the protein mediated transfer of glycosphingolipids between phospholipid bilayers, Biochemistry 39 (2000) 1067–1075.

[13] L. Malinina, M.L. Malakhova, A. Teplov, R.E. Brown, D.J. Patel, Structural basis for glycosphingolipid transfer specificity, Nature 430 (2004) 1048–1053.
[14] P. Mattjus, Glycolipid transfer proteins and membrane interaction, Biochim. Biophys. Acta 1788 (2009) 267–272.
[15] J. Tuuf, P. Mattjus, Human glycolipid transfer protein—intracellular localization and effects on the sphingolipid synthesis, Biochim. Biophys. Acta 1771 (2007) 1353–1363.
[16] G. West, M. Nylund, J.P. Slotte, P. Mattjus, Membrane interaction and activity of the glycolipid transfer protein, Biochim. Biophys. Acta 1758 (2006) 1732–1742.
[17] G. West, Y. Nymalm, T.T. Airenne, H. Kidron, P. Mattjus, T.T. Salminen, Crystallization and X-ray analysis of bovine glycolipid transfer protein, Acta Crystallogr. Sect. D, Biol. Crystallogr. 60 (2004) 703–705.
[18] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[19] N.H. Petersen, J. Joensen, L.V. McKinney, P. Brodersen, M. Petersen, D. Hofius, J. Mundy, Identification of proteins interacting with *Arabidopsis* ACD11, J. Plant Physiol. 166 (2009) 661–666.
[20] A.H. Futerman, R.E. Pagano, Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver, Biochem. J. 280 (1991) 295–302.