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

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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

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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.