Biochimica et Biophysica Acta 1808 (2011) 229–235

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

The intermembrane ceramide transport catalyzed by CERT is sensitive to the lipid environment

Jessica Tuuf [a], Matti A. Kjellberg [a], Julian G. Molotkovsky [b], Kentaro Hanada [c], Peter Mattjus [a,*]

[a] Biochemistry, Department of Biosciences, Åbo Akademi University, Turku, Finland
[b] The Shemyakin-Ovchinnikov Institute for Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia
[c] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan

ARTICLE INFO

Article history:
Received 28 May 2010
Received in revised form 15 September 2010
Accepted 16 September 2010
Available online 25 September 2010

Keywords:
CERT
Ceramide
Resonance energy transfer
Substrate specificity
Phosphatidylinositol 4-monophosphate
Lipid transfer

ABSTRACT

The *in vitro* activity of the ceramide transporter, CERT has been studied using a fluorescence assay. CERT is responsible for the *in vivo* non-vesicular trafficking of ceramide between the endoplasmic reticulum and Golgi. In this study we have examined how the membrane environment surrounding the ceramide substrate, the membrane packing density and the membrane charge, are affecting the ceramide transfer activity. To examine this we have used an anthrylvinyl-labeled ceramide analogue. We found that if ceramide is in a tightly packed environment such as in sphingomyelin or dipalmitoylphosphatidylcholine containing membranes, the CERT transfer activity is markedly reduced. Ceramide in fluid membranes on the other hand are available for CERT mediated transfer. CERT also favors membranes that contain phosphatidylinositol 4-monophospate, due to its binding capacity of the pleckstrin homology domain towards phosphatidylinositol 4-monophospate. From this study we conclude that the membrane matrix surrounding ceramide, that is ceramide miscibility, is largely affecting the transfer activity of CERT.

1. Introduction

Ceramide is a precursor of several important lipids such as sphingomyelin (SM) and a large number of glycosphingolipids. Ceramide plays a key role as a lipid messenger in cell signaling, and it has also been proposed to be a regulator of cell apoptosis [1,2]. The synthesis and organization of lipids are precisely regulated, and these processes occur at the endoplasmic reticulum (ER) and the Golgi complex. Ceramide is synthesized at the ER and is later transported to other locations. It can either undergo vesicular trafficking to the cis-Golgi, where it is converted to glucosylceramide (GlcCer) or it can be transported to the trans-Golgi for conversion to sphingomyelin (SM). This latter step is mainly catalyzed by a lipid transfer protein called the ceramide transfer protein, CERT [3].

The ceramide transfer protein was found when non-vesicular transport of ceramide between ER and Golgi was studied. The protein was shown to be cytosolic and to consist of three domains; a lipid transfer domain, a pleckstrin homology (PH) domain and an ER-targeting domain [3]. The START (steroidogenic acute regulatory protein-related lipid transfer) domain, located at the C-terminus, binds ceramide and is catalyzing the interorganelle ceramide transfer. The N-terminal PH domain of CERT targets the protein to Golgi through recognition and binding to phosphatidylinositol 4-monophospate (PI4P) and is regulated by the phosphatidylinositol 4-kinase III beta [4]. The middle part of the CERT sequence contains a motif targeting the protein to the ER [5]. This motif is called FFAT (two phenylalanines in an acidic tract) and it was shown to enable CERT to interact with the VAPs, vesicle-associated membrane protein-associated proteins (VAMP-associated proteins) located on the cytosolic surface of the ER [5,6]. Several other important lipid transport proteins have been reported to bind to the VAPs through FFAT or FFAT like motifs [7,8].

The structure of the CERT START domain was recently resolved both in its apo-form as well as bound to ceramide having different acyl chains [9]. The cavity of CERT was shown to be amphiphilic with an important glutamate residue at position 446 (Glu-446) that is crucial for ceramide transfer. Moreover the domain contained two tryptophans (Trp-473 and/or Trp-562) exposed on the surface that were suggested to be important for membrane interaction. By using the surface plasma resonance (SPR) technique it was recently confirmed that Trp-473 is indeed necessary for membrane binding and, furthermore, the molecular mechanisms behind the inhibiting

Abbreviations: AV-Cer, *N*-[12-(9-anthryl)-11*E*-dodecenoyl]sphingosine; CERT, ceramide transfer protein; DiO-C_{16}, 3,3′-dihexadecyloxacarbocyanine perchlorate; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ER, endoplasmic reticulum; FFAT, two phenylalanines in an acidic tract; GLTP, glycolipid transfer protein; GlcCer, glucosylceramide; GPBP, Goodpasture antigen-binding protein; PH, pleckstrin homology; PI, phosphatidylinositol; PI4P, phosphatidylinositol 4-monophosphate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; P-SM, palmitoyl-sphingomyelin; RET, resonance energy transfer; SM, sphingomyelin; START, steroidogenic acute regulatory protein-related lipid transfer; VAP, VAMP-associated protein

* Corresponding author. Biochemistry, Department of Biosciences, Åbo Akademi University, FI-20520 Turku, Finland. Tel.: +358 2 2154745; fax: +358 2 2410014.
E-mail address: Peter.Mattjus@abo.fi (P. Mattjus).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.09.011

effect of the synthetic analogue HPA ((1*R*,3*R*)-N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)alkanamide) was explained [10,11].

Previously it has been shown that CERT is specific for ceramide substrates having acyl chains consisting of 14, 16, 18 and 24 carbon atoms [12]. In the same study it was also demonstrated that SM, sphingosine, cholesterol and phosphatidylcholine are not transferred by CERT. In this study we also confirmed that CERT is not able to move glycosphingolipids, such as galactosylceramide. So far no information exists whether other lipids surrounding ceramide in the membrane are affecting the ceramide transport mediated by CERT. Therefore, we investigate this using a fluorescent probe, anthrylvinyl-labeled ceramide as a ceramide analogue to measure the transfer of ceramide by CERT. This was performed by using a resonance energy transfer (RET) method that has successfully been used when analyzing another lipid transfer protein, the glycolipid transfer protein (GLTP) [13]. We now provide evidence that the ceramide transport is affected by several parameters affecting the surrounding matrix of the ceramide molecule. Furthermore, we show that the transport is sensitive to PI4P that appears to attract CERT and enhance the transfer.

2. Materials and methods

2.1. Materials

sn-1-Palmitoyl-2-oleoyl-phosphatidylcholine (POPC), *sn*-1,2-dipalmitoyl-phosphatidylcholine (DPPC), cholesterol, phosphatidylinositol 4-monophospate (PI4P), phosphatidylinositol (PI), palmitoylsphingomyelin (P-SM), *sn*-1,2-dioleoylphosphatidylcholine (DOPC) and C_8-ceramide were all from Avanti Polar Lipids (AL, USA). 3,3′-dihexadecyloxacarbocyanine perchlorate (DiO-C_{16}) was from Invitrogen (OR, USA), N-[12-(9-anthryl)-11*E*-dodecenoyl]sphingosine (AV-Cer) was synthesized as described in the next paragraph. Anthrylvinyl-labeled galactosylceramide (AV-GalCer) was synthesized as previously described [14].

2.2. Preparation of AV-Cer

12-(9-Anthryl)-11*E*-dodecenoic acid (11 mg, 29 μmol) [15], (benzotriazole-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP, 14 mg, 32 μmol) and 15 μl 1-methylimidazole were dissolved in 1 ml dry chloroform by vortexing; after 1-h incubation, a solution of D-sphingosine sulfate (from bovine brain sphingomyelin; Sigma, MO, USA.) (10 mg, 26.7 μmol) in 2 ml chloroform and 0.5 ml isopropanol was added, stirred for 6 h at 24 °C and left overnight. Chromatography of reaction products on a silica gel column in chloroform with 0→2% methanol gave 15 mg (the reaction yield was 86%) AV-Cer as yellowish amorphous powder, individual by thin-layer chromatography on Kieselgel 60 pre-coated plates (Merck, Germany), $R_f = 0.40$ in chloroform-methanol, 19:1 v/v, system, detection with phosphomolybdic acid, and with UV light. Its UV (λ_{max} 351, 368 and 387 nm, in ethanol) and fluorescence (λ_{em} 430, shoulder 412 nm, in ethanol) spectra are the same as of other anthrylvinyl probes, Fig. 1 [16]. The purity and identity of AV-Cer was positively verified by ESI-MS (HCT-Ultra ion trap mass spectrometer; Bruker Daltonics, Germany), Fig. 1.

2.3. Protein expression and purification of CERT and mutant CERT ΔST in *Escherichia coli*

The construction of the human CERT plasmids has previously been described [3]. His-tagged hCERT and CERTΔST were transformed in *E. coli* BL-21 (DE3) cells, and grown at 37 °C until A_{600} reached 0.6. The expression of the CERT proteins was induced by addition of isopropyl 1-thio-β-D-galactopyranoside and the bacteria were grown at 25 °C for an additional 4 h. The bacteria were lysed and purified on a TALON metal affinity resin (BD Biosciences Clontech) according to the manufacturer's instructions. The protein concentration was determined by the method of Lowry [17].

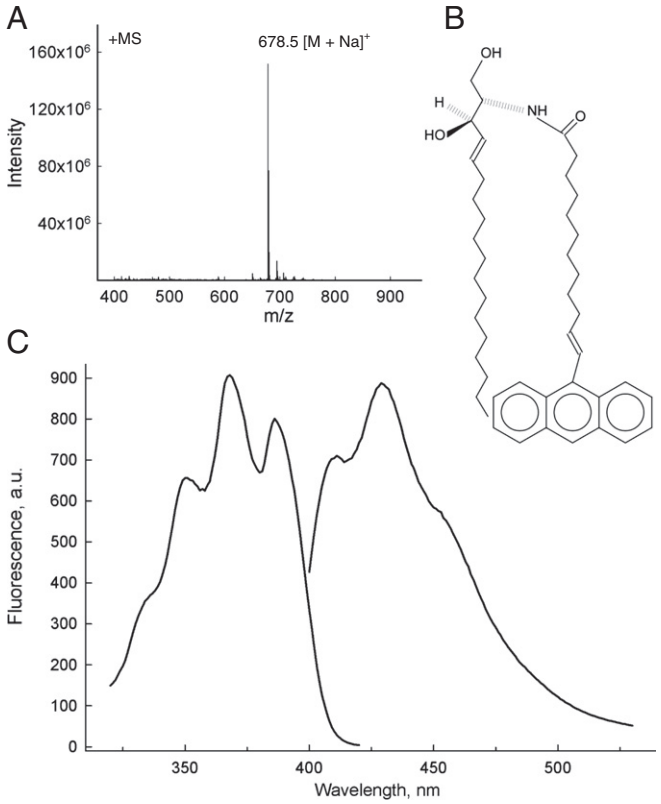

Fig. 1. AV-Ceramide characterization. The fluorescent probe anthrylvinyl-ceramide molecular weight 655.99 g/mol (A), with C_{18} sphingosine was used to monitor the CERT mediated ceramide transfer (B). Normalized excitation (emission, 430 nm) and emission (excitation, 370 nm) spectra of AV-Cer probe in ethanol (C). MS data, 654.5 $[M-H]^-$, 678.5 $[M + Na]^+$, 679.5 $[M + H + Na]^+$, 694.4 $[M + K]^+$.

2.4. Preparation of phospholipid vesicles by probe sonication

The donor vesicles had different compositions, containing the fluorophore holder AV-Cer, quencher DiO-C_{16} and a varying matrix lipid. The lipid mixtures were dried under nitrogen and dissolved in a 10-mM sodium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol, 140 mM NaCl and 1 mM EDTA. The suspension was sonicated using a Branson 250 titanium probe (micro tip with a diameter of 3 mm) sonifier and centrifuged for 15 min at 15000 g to remove multilamellar aggregates and titanium probe particles. The acceptor vesicles contained 10 times the molar concentration lipids compared to donor vesicles. The size of the vesicles has previously been determined to be about 45 nm in diameter using light scattering measurements [18], Malvern Zetasizer Nano-S ZEN1600, Malvern Instruments (Worcestershire, UK). The POPC acceptor vesicles were also prepared by sonication as described above for the sonicated donor vesicles.

2.5. Resonance energy transfer assay

The fluorescence method used for measuring the transfer of ceramide between two bilayer vesicle populations was adopted from another assay that measures glycolipid transfer, previously thoroughly described [13,18]. Probe sonicated vesicles consisting of 1% AV-Cer, 3% DiO-C_{16} (nontransferable quencher), and a varying lipid. The assay was started by injection of 10 μg of CERT. The assay as well as the vesicle preparation was done in a 10 mM sodium phosphate buffer

(pH 7.4) containing 1 mM dithiothreitol, 140 mM NaCl and 1 mM EDTA at 37 °C. The transfer rates in the first minute after CERT injection, initial transfer, could be calculated by comparing the increase in the fluorescence intensity (unquenching) and comparing this value to the total fluorescence intensity obtained when treating the sample with Triton X-100 (final concentration 1%) subtracted with the Triton X-100 blank. For a more detailed explanation of the calculations, see previously published data [19]. Data in Figs. 3, 4 and 5 are obtained from two different CERT expression and purification batches. Each vesicle composition is made in triplicate preparations and assayed at least in triplicates. The data points are average transfer rates calculated from both protein batches.

2.6. Fluorescence anisotropy of AV-Cer

Fluorescence anisotropy of anthrylvinyl-labeled ceramide was registered on a PTI QuantaMaster 1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) that operates in the T-format. Samples contained POPC with 1-mol% AV-Cer and 4- or 19-mol% C_8-ceramide. The melting temperature T_m for C_8-ceramide is 64.4 °C as determined by differential scanning calorimetry [20]. The samples of bath sonicated multilamellar vesicles were excited at 370 nm, and the emission was recorded at 430 nm, with all the slits set to 5 nm. The scan (from 40 to 90 °C) was done in a quartz cuvette under constant stirring, and the temperature was controlled by a Peltier element. The anisotropy was calculated with the PTI software package. Representative scans were chosen from multiple experiments.

3. Results

3.1. AV-Cer reports the melting of C_8-ceramide in POPC bilayers

CERT efficiently transfers ceramides with C_{14}, C_{16}, C_{18}, and C_{20} amide-linked acyl chains, but not longer acyl chains. CERT also facilitates the transfer of C_{16}-dihydro- and phytoceramide [12]. In addition, binding assays showed that CERT recognizes short chain fluorescent analogs such as C_5-BODIPY-ceramide and C_6-NBD-ceramide with a stoichiometry of 1:1 [12]. Because short chain fluorescent analogs of lipids have a high spontaneous transfer between membranes [21] they are unsuitable in protein mediated lipid transfer assays. Therefore, we have in this assay used an anthrylvinyl-labeled ceramide with a C_{12} acyl chain linked fluorophore.

To validate the AV-Cer probe, we show the anisotropy values of AV-Cer in C_8-ceramide/POPC probe-sonicated vesicles versus temperature (Fig. 2 upper panel). The T_m for the thermodynamic gel to liquid phase transition of C_8-ceramide corresponded well with the change in the anisotropy value (arrow in Fig. 2, upper panel). The change in the anisotropy was around 62–64 °C. We therefore conclude that, at low concentrations, such as those used under our experimental conditions, AV-Cer disperses with short ceramides and reports their melting.

We also used a CERT mutant that does not have the START domain, and clearly show that it does not have any transfer activity, compared to the wild-type trace that shows a normal transfer activity (Fig. 2, lower panel). This also shows that AV-Cer will remain in the donor vesicles and does not show any significant spontaneous transfer.

In another control experiment, we examined the ability of CERT to facilitate the movement of another sphingolipid, anthrylvinyl-labeled galactosylceramide, AV-GalCer. We show that CERT is not able to transfer AV-GalCer from donor membranes to acceptor membranes (Fig. 2, lower panel), as is anticipated based on previous studies [12]. Neither does another transfer protein, glycolipid transfer protein (GLTP), show any transfer activity for AV-Cer (data not shown). The mutant CERT ΔST (START domain deleted) is neither transferring AV-Cer nor AV-GalCer (data not shown). Based on these results we conclude that AV-Cer is a suitable probe for examining the *in vitro* CERT mediated transfer.

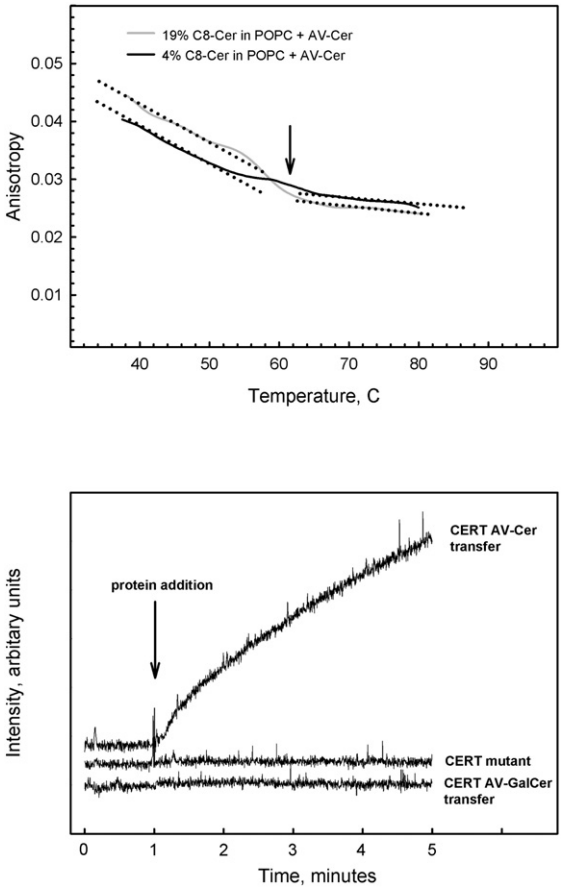

Fig. 2. Upper panel, fluorescence anisotropy of POPC:C_8-Cer:AV-Cer, 95:4:1 (black trace), or 80:19:1 (grey trace) between 40 and 90 °C. Excitation was detected at 370 nm, and emission was detected at 430 nm. The arrow indicates the melting temperature of 64 °C for C_8-Cer. Lower panel shows a typical CERT (10 μg) mediated transfer of AV-Cer, compared to the inactive CERT mutant, as well as the inability of CERT to transfer AV-GalCer. The arrow in the lower panel indicated the addition of CERT to the transfer assay containing donor and acceptor vesicles. Donor vesicles were composed of either AV-Cer or AV-GalCer with the DiO-C_{16} quencher in POPC. POPC acceptor vesicles were in 10-fold excess compared to the donor vesicles.

3.2. Transfer of AV-Cer from donor vesicles containing different compositions of DOPC; DPPC or SM

We have examined the CERT activity as a function of different matrices; in particular, the fluidity of the donor membranes was examined. We started with POPC (melting temperature −4 °C) membranes with AV-Cer and the quencher DiO-C_{16} with increasing DOPC (melting temperature −18 °C) to generate an environment with increasing fluidity. At the temperature of 37 °C, a 30–40% increase in the transfer of AV-Cer can be seen with the addition of 25% DOPC to the POPC donors vesicles (Fig. 3A). A further increase in the DOPC amount did not further change the rate of AV-Cer. When POPC is replaced with DOPC the matrix becomes fully unsaturated and AV-Cer will most likely be completely phase separated into AV-Cer rich domains, readily available for CERT. A successive increase in the DPPC (melting temperature 42 °C) content to POPC to generate a more rigid donor membrane resulted in a stepwise lowering of the AV-Cer transfer rate (Fig. 3B). CERT was not able to transfer any AV-Cer from donor membranes composed of only DPPC. Substituting DPPC for P-SM

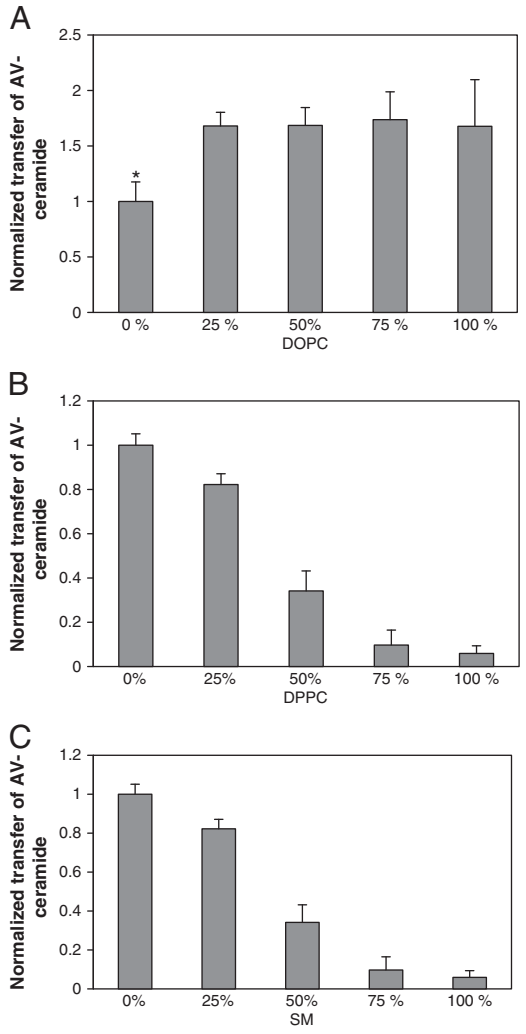

Fig. 3. CERT activity is dependent on the lipids surrounding the AV-Cer substrate. (A) Different amounts of DOPC (0, 25, 50, 75, 100 mol%) was included into POPC donor vesicles and the AV-Cer (1 mol%) transfer to POPC acceptor vesicles was examined. The level of transfer of AV-Cer for the 100% POPC (0% DOPC) mixture is significantly lower ($P<0.001$) than the other mixtures. (B) Donor vesicles containing different mol% of DPPC (0, 25, 50, 75, 100 mol%) in POPC. (C) Donor vesicles composed of different mol% of SM (0, 25, 50, 75, 100 mol%) in POPC. Results in A and B are means ± SD of two independent sets of CERT batches and in C of one batch. Transfer rates are averages of at least triplicate transfer measurements per batch. The transfer is normalized to 1 for the sample containing AV-Cer donor vesicles composed of only POPC. 10 μg of CERT was used in the assay.

(melting temperature 41 °C) showed a similar response in the CERT mediated AV-Cer transfer, and no AV-Cer was moved from 100% P-SM (Fig. 3C). AV-Cer is likely to be more soluble in the gel phase membranes and more dispersed and not as readily available for CERT.

3.3. Transfer of AV-Cer from donor vesicles containing different compositions of DOPC, DPPC and cholesterol

One function of cholesterol in tightly packed membranes is to maintain the fluidity, and in fluid membranes to minimize acyl chain trans-gauche configuration and thus make the membrane more rigid. Addition of cholesterol up to 20 mol% to POPC vesicles did not, to any significant portion, change the AV-Cer transfer rate (Fig. 4, blue diamonds). Cholesterol was not able to cause any greater transfer of AV-Cer from DPPC membranes (Fig. 4, red squares). In the POPC/DPPC 1:1 mixture cholesterol at 5 and 10 mol% was able to increase slightly the CERT mediated transfer of AV-Cer (Fig. 4, green triangles),

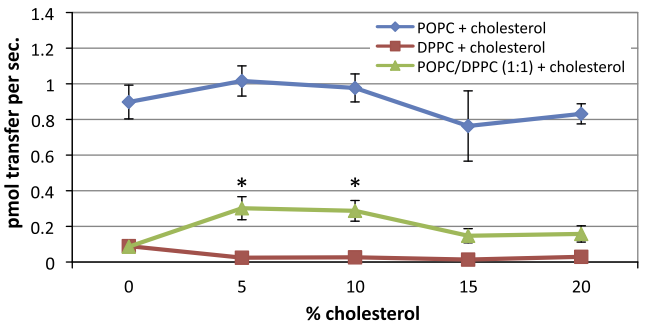

Fig. 4. CERT-mediated transfer of AV-Cer (1 mol%) from POPC (diamonds), DPPC (squares) or POPC/DPPC [1:1] (triangles) vesicles containing increasing amounts of cholesterol. Results for POPC and DPPC vesicles are means ± SD of two independent CERT purification batches and for POPC/DPPC (1:1) vesicles of one batch. Data are averages of at least triplicate samples per protein batch. 10 μg of CERT was used in the assay. The levels of transfer of AV-Cer for the 5% and 10% cholesterol in POPC/DPPC (1:1) mixtures (green) are significantly higher ($P<0.005$) than the 5% and 10% cholesterol in DPPC (red).

compared to the no-cholesterol sample. Higher cholesterol amounts on the other hand returned the transfer rate of AV-Cer to zero.

3.4. Effects of PI4P and PI on the transfer of AV-Cer

PI4P promotes the recruitment of different lipid transfer proteins OSBP1, CERT and FAPP2 through the PH domain, causing further changes in the lipid composition of these target membrane domains [22]. FAPP2 transfers GlcCer to the trans-Golgi network, whereas OSBP1 senses the levels of cholesterol in the trans-Golgi network membranes [23]. We analyzed the effect of PI4P present in membranes with regard to the CERT mediated transfer of AV-Cer. If PI4P is present in the acceptor membranes between 1 and 4 mol%, the transfer rate slows down to between 30% and 40%. With PI present, the rate is not slowed down to the same extent (Fig. 5A). If donors vesicles contain PI4P in 1 mol% it caused a much faster AV-Cer transfer rate compared to equivalent amounts of PI containing donor membranes (Fig. 5B). These experiments were done in the presence of 140 mM NaCl that should eliminate simple charge–charge interactions between CERT and the negatively charged lipids.

4. Discussion

CERT, a splicing variant of Goodpasture antigen-binding protein (GPBP), consists of three regions [24]. These are an N-terminal region with a PH domain that binds to PI4P, a middle region containing a FFAT motif that participates in the association with the ER, and in the C-terminal region the START domain that extracts ceramide from donor membranes and releases the bound ceramide to acceptor membranes [25,26]. The other splice variant, GPBP, a protein with an additional 26 amino acid serine-rich domain, is associated with collagen IV in patients with the autoimmune disease Goodpasture syndrome. The two splice forms carry out different functions in specific sub-cellular localizations [26].

It has previously been shown that CERT is not able to transfer SM, phosphatidylcholine and cholesterol [12], consequently we therefore assumed that CERT is only transferring ceramide from membranes and not other lipids. In this study, we used anthrylvinyl (AV) as the fluorescent label positioned on the amide linked acyl chain on the ceramide molecule to measure the CERT-mediated transfer. AV-labeled galactosylceramide has previously been utilized when examined the transfer activity of GLTP [18,27,28]. The anthrylvinyl moiety of AV-Cer reportedly localizes to the hydrophobic region of the

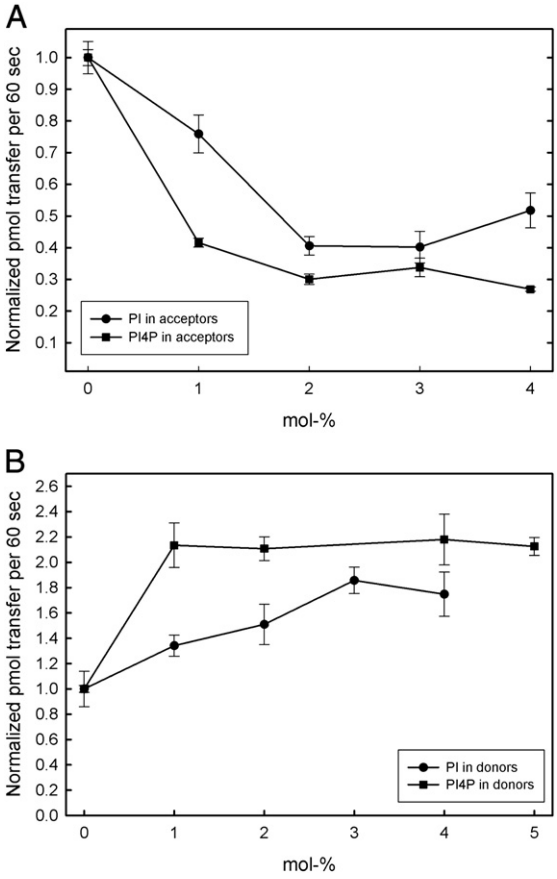

Fig. 5. CERT-mediated initial AV-Cer transfer rate from unilamellar vesicles. (A) Transfer of AV-Cer from POPC donor to POPC acceptor vesicles with increasing concentration of PI (circles) or PI4P (squares). (B) Transfer of AV-Cer from POPC donor vesicles with increasing concentration of PI (circles) or PI4P (squares) to POPC acceptor vesicles. 10 μg of CERT was used in the assay. Data in panel B are significantly different ($P<0.05$). Values are means ± SD of at least three measurements.

bilayer and thus produces minimal disturbance in the bilayer interfacial/polar region [29]. In addition, the anthrylvinyl fluorophore is nearly independent of the medium polarity [29]. The anthrylvinyl bearing acyl chains are hydrophobic in nature, and are not looping out to the interfacial region like, for instance, acyl chains labeled with the more polar fluorophore NBD [30–32].

It is known from previous studies that the membrane environment largely affects different lipid transfer proteins' ability to move their substrate from one membrane to another [33–38]. This is due to complex processes including the substrate miscibility and interaction with its neighboring lipids in the membrane as well as the ability of the proteins to scan the membrane surface for their substrates. Ceramide in natural membranes is likely to be influenced by its surrounding lipids, much to its relatively small abundance [39,40] and due to its small hydroxyl headgroup. The small headgroup results in a low hydration and allows the ceramide molecules to pack tightly. Another factor influencing the tight packing is the ability of ceramide to function both as a hydrogen bond donor and acceptor, a capability that ceramide shares with other sphingolipids [41,42].

A fluid membrane environment appears to allow CERT to extract ceramide from the membrane. This could be explained by the tendency of ceramide to fuse into ceramide enriched platforms, because of the small headgroup of ceramide and the ability to form extensive hydrogen bonding networks with each other [20,43,44]. A ceramide platform would be a suitable environment for CERT to bind and extract ceramide. The lower transfer rate of AV-Cer from the POPC containing mixture could be a result of an interaction of AV-Cer with POPC because of the saturated *sn-1* 16:0 chain. AV-Cer would not be fully phase separated. When the saturated chain is lost as a function of increasing the DOPC portion, AV-Cer will most likely be unable to as effectively interact with the unsaturated bulkier 18:1 chain in position *sn-1* of DOPC and become fully separated into AV-Cer rich domains, readily available for CERT. On the other hand, with increasing content of gel phase lipids such as with the addition of DPPC and P-SM, ceramide would become unavailable for CERT, presumably becoming dispersed and in part miscible in the gel-phase [20]. It is tempting to speculate that the fluidity of the membrane could also play a role in regulating the CERT activity *in vivo*.

We also observed that when cholesterol between 5 and 10 mol% was added to an equimolar mixture of POPC and DPPC, the transfer of AV-Cer appeared to be higher than the case of no cholesterol, and for mixtures above 10 mol% cholesterol. The addition of 5–10 mol% cholesterol to a fluid and gel coexistence phase mixture such as POPC:DPPC most likely generates a cholesterol/DPPC phase with liquid ordered phase-like properties [43]. In this phase the acyl chains are not in a complete all-trans state, such as in the gel phase [44,45] but rather increases the trans-gauge isomerization of the DPPC acyl chains. It was previously shown extensively that ceramide is more soluble in cholesterol-rich membranes than in cholesterol-poor membranes. At low cholesterol amounts ceramide segregates into gel domains that disappear upon increasing cholesterol levels [46]. It is possible that the cholesterol generated phase dispersed in the fluid and gel phase co-existence membrane allows ceramide to localize to the phase boundaries where it can be accessed by CERT. In the no cholesterol mixture, the boundaries between the fluid POPC and gel DPPC are fewer and not as suitable environment for CERT to access ceramide, and ceramide is likely dispersed in the DPPC phase [47]. Above 10 mol% cholesterol, liquid ordered phase is starting to form [48–52] resulting in large liquid ordered phase areas where ceramide would be miscible [20], and the membrane again becomes unsuitable for CERT to access its substrate. The accumulation of cholesterol at the phase boundaries (between DPPC and POPC) contributes to a reduction of the interfacial line tension between regions of different composition [53–55]. The origin of the reduced interfacial tension is hypothesized based on simulation work to be a consequence of the cholesterol molecules orienting their smooth alpha-face toward tighter packed molecules (DPPC) leaving the rough beta-face to interact with the more fluid molecules (POPC) [56,57]. If AV-Cer also localizes to these boundary regions the amount of cholesterol would also affect the ceramide lateral arrangement and consequently CERT activity. We acknowledge that the difference is small but could be explained by the effects that cholesterol has on the line tension as well as how ceramide is solubilized in different cholesterol-rich membranes.

The production of PI4P in Golgi is stimulated by an active ADP ribosylation factor, ARF [58,59]. As with phosphatidylinositol phosphates in general, PI4P acts as a meeting point for the recruitment of specific cytosolic proteins that contribute to the local specialization of their surrounding membrane environment. We have shown here for the first time that the presence of PI4P in the membrane largely affects CERT transfer activity specifically attracting CERT to the membrane interface. The PH domain of CERT specifically binds to PI4P among various recently tested phosphoinositides [3]; however, the PH domain of a mutant CERT (G67E) did not recognize PI4P [3,25]. The results in this study give valuable information about the effects of the matrix surrounding the ceramide molecule. Differences in the environment around ceramide will affect the ability of CERT to transfer ceramide. We speculate that, when PI4P is in the acceptor membranes, equivalent to the natural environment in the Golgi membrane, it causes a stronger binding of CERT at the acceptor surfaces due to the affinity towards PI4P compared to the PI containing membranes. The off rate of apo-CERT is slow from the acceptor surface, and it is not allowed to go back to the donor

membrane and bind ceramide with the same rate as for the membranes that contain PI. When CERT with a ceramide molecule bound arrives at PI4P-containing membranes, it appears to be arrested at the surface. We can only speculate that perhaps a PI4P-containing membrane interface such as in Golgi could function as a mechanism to keep CERT on the Golgi surface, and at a certain threshold of ceramide level CERT is releasing its ceramide and is allowed to go to the ER surface after more. On the other hand, in order for CERT to bind to the ER membrane *in vivo*, VAP-A is needed that allows CERT to bind via its FFAT motif. If the donor membrane contains PI4P, more CERT is attracted to the membrane and consequently faster transfer rates were observed, compared to PI containing membranes.

In conclusion, we demonstrate that the membrane matrix surrounding ceramide largely affects the transfer activity of CERT. The complete transfer process is a complex event including the protein scanning the membrane surface for its target lipid, the lipid binding event and subsequently CERT leaving the membrane. For the transfer to be complete, the lipid loaded protein needs to encounter a membrane surface and unload its cargo [19,37]. These intricate steps are regulated by a large number of factors that we are only beginning to understand.

Acknowledgments

We thank Henna Ohvo-Rekilä and Pia Roos-Mattjus for critical reading of the manuscript and J. Peter Slotte for helpful discussions. This work was in part supported (for PM) by the Academy of Finland, ISB Graduate School, Sigrid Jusélius Foundation, Magnus Ehrnrooth Foundation, Oscar Öflund Foundation, and Medicinska Understödsföreningen Liv och Hälsa r.f. Also the work was in part supported (for JM) by Russian Foundation for Basic Research, grant 09-04-00313.

References

[1] L.M. Obeid, C.M. Linardic, L.A. Karolak, Y.A. Hannun, Programmed cell death induced by ceramide, Science 259 (1993) 1769–1771.
[2] D.K. Perry, Y.A. Hannun, The role of ceramide in cell signalling, Biochim. Biophys. Acta 1436 (1998) 233–243.
[3] 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.
[4] B. Toth, A. Balla, H. Ma, Z.A. Knight, K.M. Shokat, T. Balla, Phosphatidylinositol 4-kinase III beta regulates the transport of ceramide between the endoplasmic eeticulum and Golgi, J. Biol. Chem. 281 (2006) 36369–36377.
[5] 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.
[6] 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.
[7] 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.
[8] J. Tuuf, L. Wistbacka, P. Mattjus, The glycolipid transfer protein interacts with the vesicle-associated membrane protein-associated protein VAP-A, Biochem. Biophys. Res. Commun. 388 (2009) 395–399.
[9] N. Kudo, K. Kumagai, N. Tomishige, T. Yamaji, S. Wakatsuki, M. Nishijima, K. Hanada, R. Kato, Structural basis for specific lipid recognition by CERT responsible for nonvesicular trafficking of ceramide, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 488–493.
[10] N. Kudo, K. Kumagai, R. Matsubara, S. Kobayashi, K. Hanada, S. Wakatsuki, R. Kato, Crystal structures of the CERT START domain with inhibitors provide insights into the mechanism of ceramide transfer, J. Mol. Biol. 396 (2010) 245–251.
[11] T. Sugiki, H. Takahashi, M. Nagasu, K. Hanada, I. Shimada, Real-time assay method of lipid extraction activity, Anal. Biochem. 399 (2010) 162–167.
[12] K. Kumagai, S. Yasuda, K. Okemoto, M. Nishijima, S. Kobayashi, K. Hanada, CERT mediates intermembrane transfer of various molecular species of ceramides, J. Biol. Chem. 280 (2005) 6488–6495.
[13] P. Mattjus, J.G. Molotkovsky, J.M. Smaby, R.E. Brown, A fluorescence resonance energy transfer approach for monitoring protein-mediated glycolipid transfer between vesicle membranes, Anal. Biochem. 268 (1999) 297–304.
[14] J.G. Molotkovsky, I.I. Mikhalyov, A.B. Imbs, L.D. Bergelson, Synthesis and characterization of new fluorescent glycolipid probes. Molecular organisation of glycolipids in mixed-composition lipid bilayers, Chem. Phys. Lipids 58 (1991) 199–212.
[15] J.G. Molotkovsky, P.I. Dmitriev, L.F. Nikulina, L.D. Bergelson, Synthesis of new fluorescence labeled phosphatidylcholines, Bioorg. Khim. (Russ.) 5 (1979) 588–594 (Engl. Transl. 437-442).
[16] L.B. Johansson, J.G. Molotkovsky, L.D. Bergelson, Fluorescence properties of anthrylvinyl lipid probes, Chem. Phys. Lipids 53 (1990) 185–189.
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurment with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[18] M. Nylund, C. Fortelius, E.K. Palonen, J.G. Molotkovsky, P. Mattjus, Membrane curvature effects on glycolipid transfer protein activity, Langmuir 23 (2007) 11726–11733.
[19] P. Mattjus, Glycolipid transfer proteins and membrane interaction, Biochim. Biophys. Acta 1788 (2009) 267–272.
[20] B. Westerlund, P.M. Grandell, Y.J. Isaksson, J.P. Slotte, Ceramide acyl chain length markedly influences miscibility with palmitoyl sphingomyelin in bilayer membranes, Eur. Biophys. J. 39 (2010) 1117–1128.
[21] J. Bai, R.E. Pagano, Measurment of spontaneous transfer and transbilayer movement of BODIPY-labeled lipids in lipid vesicles, Biochemistry 36 (1997) 8840–8848.
[22] M.A. De Matteis, A. Di Campli, G. D'Angelo, Lipid-transfer proteins in membrane trafficking at the Golgi complex, Biochim. Biophys. Acta 1771 (2007) 761–768.
[23] M.A. De Matteis, A. Luini, Exiting the Golgi complex, Nat. Rev. Mol. Cell Biol. 9 (2008) 273–284.
[24] A. Raya, F. Revert-Ros, P. Martinez-Martinez, S. Navarro, E. Rosello, B. Vieites, F. Granero, J. Forteza, J. Saus, Goodpasture antigen-binding protein, the kinase that phosphorylates the goodpasture antigen, is an alternatively spliced variant implicated in autoimmune pathogenesis, J. Biol. Chem. 275 (2000) 40392–40399.
[25] T. Yamaji, K. Kumagai, N. Tomishige, K. Hanada, Two sphingolipid transfer proteins, CERT and FAPP2: their roles in sphingolipid metabolism, IUBMB Life 60 (2008) 511–518.
[26] C. Mencarelli, M. Losen, C. Hammels, J. De Vry, M.K. Hesselink, H.W. Steinbusch, M.H. De Baets, P. Martinez-Martinez, The ceramide transporter (CERT) and the Goodpasture antigen binding protein (GPBP). One protein - one function? J. Neurochem. 113 (2010) 1369–1386.
[27] M. Nylund, P. Mattjus, Protein mediated glycolipid transfer is inhibited FROM sphingomyelin membranes but enhanced TO sphingomyelin containing raft like membranes, Biochim. Biophys. Acta 1669 (2005) 87–94.
[28] M. Nylund, M.A. Kjellberg, J.G. Molotkovsky, H.S. Byun, R. Bittman, P. Mattjus, Molecular features of phospholipids that affect glycolipid transfer protein-mediated galactosylceramide transfer between vesicles, Biochim. Biophys. Acta 1758 (2006) 807–812.
[29] L.D. Bergelson, J.G. Molotkovsky, Y.M. Manevich, Lipid-specific fluorescent probes in studies of biological membranes, Chem. Phys. Lipids 37 (1985) 165–195.
[30] A. Chattopadhyay, E. London, Parallax method for direct measurement of membrane penetration depth utilizing fluorescence quenching by spin-labeled phospholipids, Biochemistry 26 (1987) 39–45.
[31] D. Huster, P. Muller, K. Arnold, A. Herrmann, Dynamics of lipid chain attached fluorophore 7-nitrobenz-2-oxa-1, 3-diazol-4-yl (NBD) in negatively charged membranes determined by NMR spectroscopy, Eur. Biophys. J. 32 (2003) 47–54.
[32] D. Huster, P. Muller, K. Arnold, A. Herrmann, Dynamics of membrane penetration of the fluorescent 7-nitrobenz-2-oxa-1, 3-diazol-4-yl (NBD) group attached to an acyl chain of phosphatidylcholine, Biophys. J. 80 (2001) 822–831.
[33] A.R. Tall, Plasma lipid transfer proteins, Annu. Rev. Biochem. 64 (1995) 235–257.
[34] U. Seedorf, P. Ellinghaus, J.R. Nofer, Sterol carrier protein-2, Biochim. Biophys. Acta 1486 (2000) 45–54.
[35] X.C. Jiang, H.W. Zhou, Plasma lipid transfer proteins, Curr. Opin. Lipidol. 17 (2006) 302–308.
[36] K.W. Wirtz, Phospholipid transfer proteins in perspective, FEBS Lett. 580 (2006) 5436–5441.
[37] R.E. Brown, P. Mattjus, Glycolipid transfer protein—review, Biochim. Biophys. Acta 1771 (2007) 746–776.
[38] B.D. Sha, M. Luo, PI transfer protein: the specific recognition of phospholipids and its functions, Biochim. Biophys. Acta 1441 (1999) 268–277.
[39] A.d.E. Cremesti, F.M. Goni, R.N. Kolesnick, Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? FEBS Lett. 531 (2002) 47–53.
[40] B. Stancevic, R. Kolesnick, Ceramide-rich platforms in transmembrane signaling, FEBS Lett. 584 (2010) 1728–1740.
[41] J.M. Boggs, Intermolecular hydrogen bonding between lipids: influence on organization and function of lipids in membranes, Can. J. Biochem. 58 (1980) 755–770.
[42] J.M. Boggs, Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function, Biochim. Biophys. Acta 906 (1987) 353–404.
[43] J.H. Ipsen, G. Karlström, O.G. Mouritsen, H. Wennerström, M.J. Zuckermann, Phase equilibria in the phosphatidylcholine-cholesterol system, Biochim. Biophys. Acta 905 (1987) 162–172.
[44] M.R. Vist, J.H. Davis, Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: 2H nuclear magnetic resonance and differential scanning calorimetry, Biochemistry 29 (1990) 451–464.
[45] J.L. Thewalt, M. Bloom, Phosphatidylcholine: cholesterol phase diagrams, Biophys. J. 63 (1992) 1176–1181.

[46] B.M. Castro, L.C. Silva, A. Fedorov, R.F. de Almeida, M. Prieto, Cholesterol-rich fluid membranes solubilize ceramide domains: implications for the structure and dynamics of mammalian intracellular and plasma membranes, J. Biol. Chem. 284 (2009) 22978–22987.
[47] S.N. Pinto, L.C. Silva, R.F. de Almeida, M. Prieto, Membrane domain formation, interdigitation, and morphological alterations induced by the very long chain asymmetric C24:1 ceramide, Biophys. J. 95 (2008) 2867–2879.
[48] S.L. Veatch, S.L. Keller, Organization in lipid membranes containing cholesterol, Phys. Rev. Lett. 89 (2002) 2681011–2681014.
[49] S.L. Veatch, S.L. Keller, Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol, Biophys. J. 85 (2003) 3074–3083.
[50] A.R. Honerkamp-Smith, S.L. Veatch, S.L. Keller, An introduction to critical points for biophysicists; observations of compositional heterogeneity in lipid membranes, Biochim. Biophys. Acta 1788 (2009) 53–63.
[51] J. Juhasz, F.J. Sharom, J.H. Davis, Quantitative characterization of coexisting phases in DOPC/DPPC/cholesterol mixtures: comparing confocal fluorescence microscopy and deuterium nuclear magnetic resonance, Biochim. Biophys. Acta 1788 (2009) 2541–2552.
[52] J.H. Davis, J.J. Clair, J. Juhasz, Phase equilibria in DOPC/DPPC-d62/cholesterol mixtures, Biophys. J. 96 (2009) 521–539.
[53] D.J. Benvegnu, H.M. McConnell, Line tension between liquid domains in lipid monolayers, J. Phys. Chem. B 96 (1992) 6820–6824.
[54] B.L. Stottrup, A.H. Nguyen, E. Tuzel, Taking another look with fluorescence microscopy: image processing techniques in Langmuir monolayers for the twenty-first century, Biochim. Biophys. Acta 1798 (2010) 1289–1300.
[55] A.R. Honerkamp-Smith, P. Cicuta, M.D. Collins, S.L. Veatch, M. den Nijs, M. Schick, S.L. Keller, Line tensions, correlation lengths, and critical exponents in lipid membranes near critical points, Biophys. J. 95 (2008) 236–246.
[56] S.A. Pandit, E. Jakobsson, H.L. Scott, Simulation of the early stages of nano-domain formation in mixed bilayers of sphingomyelin, cholesterol, and dioleylphosphatidylcholine, Biophys. J. 87 (2004) 3312–3322.
[57] S. Pöyry, T. Róg, M. Karttunen, I. Vattulainen, Significance of cholesterol methyl groups, J. Phys. Chem. B 112 (2008) 2922–2929.
[58] H. Inoue, P.A. Randazzo, Arf GAPs and their interacting proteins, Traffic 8 (2007) 1465–1475.
[59] R.A. Kahn, Toward a model for Arf GTPases as regulators of traffic at the Golgi, FEBS Lett. 583 (2009) 3872–3879.