A search for ceramide binding proteins using bifunctional lipid analogues yields CERT-related protein StarD7

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Running title: Ceramide binding site in PC transfer protein StarD7

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Abbreviations: CERT, ceramide transfer protein; DAG, diacylglycerol; ER, endoplasmic reticulum; MS, mass spectrometry; PC, phosphatidylcholine, PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; SMS, sphingomyelin synthase; SMSr, sphingomyelin synthase-related protein; START domain, steroidogenic acute regulatory transfer domain
ABSTRACT

Ceramides are central intermediates of sphingolipid metabolism with dual roles as mediators of cellular stress signalling and mitochondrial apoptosis. How ceramides exert their cytotoxic effects is unclear and their poor solubility in water hampers a search for specific protein interaction partners. Here, we report the application of a photoactivatable and clickable ceramide analogue, pacCer, to identify ceramide binding proteins and unravel the structural basis by which these proteins recognize ceramide. Besides capturing ceramide transfer protein CERT from a complex proteome, our approach yielded CERT-related protein StarD7 as novel ceramide binding protein. Previous work revealed that StarD7 is required for efficient mitochondrial import of phosphatidylcholine (PC) and serves a critical role in mitochondria function and morphology. Combining site-directed mutagenesis and photoaffinity labelling experiments, we demonstrate that the START domain of StarD7 harbours a common binding site for PC and ceramide. While StarD7 lacks robust ceramide transfer activity in vitro, we find that its ability to shuttle PC between model membranes is specifically affected by ceramides. Besides demonstrating the suitability of pacCer as a tool to hunt for ceramide binding proteins, our data point at StarD7 as a candidate effector protein by which ceramides may exert part of their mitochondria-mediated cytotoxic effects.

Key words: ceramide, click chemistry, lipid transfer protein, mitochondria, phosphatidylcholine, photoaffinity labelling
INTRODUCTION

Sphingolipids are abundant components of eukaryotic membranes that participate in a wide array of cellular processes by modulating vital physical membrane properties and as signalling molecules in responses to physiological cues and stresses (1–4). Notably ceramides, the central intermediates of sphingolipid metabolism, receive considerable attention as key mediators of anti-proliferative cellular responses including apoptosis, autophagy, cell cycle arrest and senescence (5–7). Various stress stimuli, such as tumor necrosis factor α (TNFα), ionizing radiation and chemotherapeutic drugs, trigger ceramide accumulation through activation of sphingomyelinases, stimulation of de novo ceramide synthesis, or both (8–10). Interventions that suppress ceramide accumulation render cells resistant to these stress-inducing agents while their biological effects can be partially mimicked by addition of exogenous ceramides (11).

Despite numerous reports on cellular processes controlled by ceramides, the mechanisms by which ceramides exert their signalling functions are unclear. It has been suggested that ceramides can generate or stabilize lipid microdomains that function as platforms for the recruitment of other signalling molecules (12, 13). Ceramides can also form stable channels in planar membranes, and it has been suggested that the pore-forming activity of ceramides promotes the cytosolic release of cytochrome c and other apoptogenic proteins during the execution phase of mitochondrial apoptosis (14, 15). An alternative mechanism of ceramide signalling is through direct interaction with target proteins. However, only few specific ceramide binding proteins have been described to date. These include the ceramide transfer protein CERT (16), the kinase suppressor of Ras (17), protein kinase c-Raf (18), cathepsin D (19), and protein phosphatase 2A inhibitor SET (PP2A; (20)). Identification of additional ceramide binding proteins is desirable, as this would likely lead to further mechanistic insights into ceramide-mediated signalling pathways and expand opportunities for exploiting their therapeutic potential.

Several proteome-wide methods have been developed to detect specific lipid–protein interactions, which include the application of protein microarrays in a screen for novel phosphoinositide binding proteins (21). In an inverted setup, lipid strips have been used to obtain lipid-binding fingerprints for a large number of proteins with predicted lipid binding domains (22). Column-based affinity purification strategies with lipids immobilized onto magnetic beads have also been utilized (23, 24). However, a major drawback of screens using lipids immobilized on solid supports is that such lipids are not presented in their natural state. Moreover, interactions where the lipid has to enter a deep hydrophobic binding pocket within the protein are likely to be missed. In recent years, bifunctional lipid analogs have emerged as promising new tools to circumvent some of these disadvantages,
enabling a global profiling of lipid-protein interactions in living cells (25–28). Bifunctional lipids possess a small diazirine group to allow photo-crosslinking with their protein interaction partners and a terminal alkyne or clickable group for functionalization. Biotinylation of crosslinked lipid-protein complexes enables their affinity purification and identification while their subcellular location can be visualized by click reaction with a fluorophore (29). A recent study combined the advantages of bifunctional and coumarin caged lipids to facilitate identification of protein binding partners of the signaling lipids sphingosine and diacylglycerol (30).

In the present study, we report the synthesis and application of a bifunctional ceramide analogue, pacCer, to search for novel ceramide binding proteins. Besides proteins involved in DNA damage response pathways, protein ubiquitination, membrane trafficking and signal transduction, our approach yielded CERT and the CERT-related PC transfer protein StarD7. Using molecular modelling in combination with photoaffinity labelling and lipid transfer assays, we demonstrate that StarD7 harbours a lipid-binding pocket with dual specificity for ceramide and PC, and pinpoint structural determinants of lipid recognition. As StarD7 is required for normal respiratory activity and cristae structure of mitochondria (31, 32), its ability to bind ceramides may be relevant to the mechanism by which ceramides mediate their cytotoxic effects.

MATERIALS AND METHODS

Reagents and antibodies – 1-palmitoyl-2-oleoyl-sn-glycerol (DAG, 16:0/18:1; cat. no. 800815), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-PC (POPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanol-amine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-lactosyl (lactosyl-PE), C18-ceramide (d18:1/18:0, cat. no. 860518) and C16:0-ceramide (d18:1/16:0; cat. no. 860516) were obtained from Avanti Polar Lipids. D-erythro-sphingosine was obtained from Enzo Biochem. Alexa Fluor647-N3 and biotin-N3 were from Thermo Fischer Scientific. Other fine chemicals were from Sigma Aldrich. Antibodies used were: rabbit polyclonal anti-StarD7 (cat. no. 15689-1-AP, 1:1000; Proteintech), mouse monoclonal anti-PARP-1 (cat. no. sc8007, 1:1000; Santa Cruz), mouse monoclonal anti-mitochondrial surface protein p60 (cat. no. MAB1273, 1:1000; Millipore), affinity-purified rabbit polyclonal anti-SMSr antibody (((33), 1:000), mouse monoclonal anti-biotin antibody conjugated to horse radish peroxidase (cat. no. 200-032-211, 1:1000; Jackson ImmunoResearch), goat anti-mouse and goat anti-rabbit IgG conjugated to horse radish peroxidase (cat. no. 31430 and 31460, respectively, 1:1000; Thermo Fischer Scientific).

Synthesis of bifunctional lipid analogs – A 15 carbon-long fatty acid containing a photo-activatable diazirine and clickable alkyne group, pacFA, was synthesized in 3 steps from commercially available
educts as described in (25); see Supplemental Information for further details). Next, pacFA was coupled to D-erythro-sphingosine (Enzo Biochem) using a combination of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and hydroxybenzotriazole (HOBT) as condensing reagents, yielding the photo-activatable and clickable C15-ceramide analogue, pacCer (85% overall yield). pacPC was synthesized starting from 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (Avanti Polar Lipids) and pacFA under the action of N,N-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) with satisfactory yield (39%). pacDAG was synthesized in 3 steps starting from 1-oleoyl-sn-glycerol (Santa Cruz Biotechnology). First, the primary HO-group was protected with the triphenylmethyl protecting group (trityl-chloride/pyridine; 92% overall yield). The glycerol obtained was coupled with the pacFA using EDCI/DMAP activation (58% overall yield). The final deprotection step was achieved using trifluoroacetic acid (TFAA) to generate pacDAG (28% overall yield). pacPE was synthesized in 3 steps starting from 1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids). First, the amino-group was protected with the tert-butoxycarbonyl protecting group (di-tert-butyldicarbonate/triethylamine; 98% overall yield). The ethanolamine obtained was coupled with pacFA using EDCI/DMAP activation in a good yield (52%). The final deprotection step was achieved with TFAA to generate pacPE (35%, overall yield). pacSM was synthesized starting from sphingosylphosphorylcholine (lyso-SM d18:1, Avanti Polar Lipids) and pacFA under the action of EDCI/HOBT (78% overall yield). pacGlcCer was synthesized from 1-β-D-glucosylsphingosine (Matreya) and pacFA in the presence of triphenylphosphine and dithiopyridine, essentially as described by (34); 62% overall yield. The synthesis of C1-deoxy-pacCer, C3-deoxy-pacCer and C3-deoxy-N-methyl-pacCer is described in Supplemental Information. All synthetic compounds were purified by thin layer chromatography to a high degree (purity >98%) and their structures were confirmed by 1H and 13C NMR and electrospray-ionisation mass spectrometry (ESI MS).

**DNA constructs** – Bacterial maltose binding protein (MBP) expression construct pMAL-c5X was obtained from New England Biolabs. A DNA insert encoding the START domain of human CERT was amplified from cDNA (kindly provided by K. Hanada) and cloned into the BamHI and NotI restriction sites of bacterial expression vector pET24a(+). DNA inserts encoding full-length human StarD7 (StarD7 isoform-I) and StarD7 lacking the N-terminal mitochondrial targeting sequence (StarD7 isoform-II; Horibata and Sugimoto, 2010) were PCR amplified from IMAGE clone 3842611 and cloned into the SalI and XhoI restriction sites of pET24a(+). DNA inserts encoding full-length human StarD2 and StarD10 were PCR amplified from IMAGE clones 4575824 and 4301295, respectively, and cloned into the EcoRI and XhoI restriction sites of pET24a(+). Single amino acid substitutions were introduced by site-directed mutagenesis according to the QuickChangeII™ manual (Agilent Technologies) with modifications. All expression constructs were verified by DNA sequencing.
Production of recombinant protein – E. coli BL21(DH3) pLysS cells transformed with the expression construct were grown in LB medium supplemented with 0.1 mM isopropyl-D-thiogalactoside for 2 h at 30°C. MBP was purified from cell lysates in-batch using amylose resin (New England BioLabs) according to the manufacturer’s instructions. Poly-His-tagged proteins were purified by Ni²⁺-NTA affinity (Qiagen) using an in-batch protocol, eluted in 50 mM Tris/HCl (pH 7.4), 300 mM NaCl, 300 mM imidazole, 2.5 mM β-mercaptoethanol and protease inhibitor cocktail (150 nM aprotinin, 1 µM leupeptin, 1.5 µM pepstatin, 7.5 µM antipain, 1mM benzamidine), supplemented with 10% glycerol (vol), aliquoted, and stored at -80°C until further use. Protein concentrations were determined by SDS-PAGE and Coomassie staining, using BSA as reference protein. StarD7 isoform-I was expressed and purified as described in (25), and used in experiments shown in Fig. 3. All other experiments were performed with StarD7 isoform-II.

Cell culture, RNAi and generation of StarD7°/° cells – Human cervical carcinoma HeLa (ATCC-CCL2), chinese hamster ovary CHO-K1 (ATCC-CCL-61) and mouse melanoma GM95 cells (kindly provided by Hein Sprong, University of Utrecht, the Netherlands) were grown in Dulbecco's Modified Eagle's Medium supplemented with 4.5 g/l glucose, 10% FCS and GlutaMAX™ (Invitrogen) at 37°C with 5% CO₂. To knock out StarD7 in HeLa cells, we obtained a mix of three different CRSPR/Cas9 plasmids and the corresponding HDR plasmids from Santa Cruz (sc-405820). StarD7-specific gRNA sequences: A/sense: 5'-ATCCAACTAACACAGTAGCG-3'; B/sense: 5'-GTCACCTCGTACTGGTAA-3'; C/sense: 5'-ACCCACCTTTACCAGTACCG-3'. HeLa cells were co-transfected with both plasmid mixes using Effectene (Qiagen) and grown for 48 h without selection. Next, cells were grown for two weeks under selection pressure with 2 µg/ml puromycin. Individual drug-resistant clones were picked and analysed for StarD7 expression by immunoblot analysis using anti-StarD7 antibody. Two independent StarD7°/° cell lines, StarD7-KO#1 and StarD7-KO#2, were used for subsequent RNAi experiments. To this end, cells were transfected with siRNA (Qiagen) using Oligofectamine reagent (Invitrogen) as described previously (35). siRNA target sequences used were: siNS (nonsense), 5'-AAUUCUCCGAACGUGACGU-3' and siSMSr, 5'-CAAGAAGCGGAUUUCUUGC-3'. Both adherent and non-adherent cells were harvested 72 h post-transfection, washed twice in ice-cold 0.25 M sucrose and homogenized in ice-cold IM buffer (5 mM Hepes-KOH, pH 7.0, 250 mM mannitol, 0.5 mM EGTA) supplemented with 0.1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail (150 nM aprotinin, 1 µM leupeptin, 1.5 µM pepstatin, 7.5 µM antipain, 1mM benzamidine). To this end, cells were flushed through a Balch homogenizer 20-30 times using a 2 ml syringe. Cell homogenates were centrifuged twice at 600 gmax for 5 min at 4°C to remove nuclei. Protein concentration of post-nuclear supernatants was determined by Bradford assay (Biorad). Post-nuclear supernatants were normalized for total protein content prior to immunoblot analysis.
Preparation of liposomes – Liposomes used in photoaffinity experiments with cytosolic fractions were prepared in PBS (1.4 M NaCl, 27 mM KCl, 18 mM KH₂PO₄, 126 mM Na₂HPO₄) from a mixture of egg-PC and pacLipid (95/5, mol%). Liposomes used in photoaffinity experiments with purified recombinant proteins were prepared from a defined lipid mixture (DOPC/DOPE/pacLipid, 80/20/1 mol%) in CHCl₃/MeOH (9/1, v/v). For competition assays, 0.5 or 0.25 mol% pacCer was used and C16:0 ceramide was added in 10 to 40-fold molar excess at the expense of DOPC and DOPE, keeping the DOPC/DOPE ratio constant. In brief, 10 µmol of total lipid was dried in a Rotavap and the resulting lipid film was resuspended in 1 ml Buffer L (50mM Tris-HCl, pH 7.4, 50mM NaCl) by vigorous vortexing and sonication, yielding a 10 mM lipid suspension. Liposomes with an average diameter of ~100 nm were obtained by sequential extrusion of the lipid suspension through 0.4-micron, 0.2-micron and 0.1-micron track-etched polycarbonate membranes (Whatman-Nuclepore) using a mini-extruder (Avanti Polar Lipids). Acceptor liposomes used in lipid transfer assays were prepared in Buffer L using a mixture of DOPG and DOPE (80/20 mol%). Donor liposomes were prepared using a mixture of DOPG, DOPE, lactosyl-PE and either C16:0-ceramide or DOPC (65/16/10/10, mol%). Donor liposomes used in competition assays were prepared using a mixture of DOPG, DOPE, lactosyl-PE, DOPC and C16:0-ceramide or DAG (69/17/10/5/10 mol%). All liposomes were stored under N₂ at 4°C and used within 2-3 days after preparation.

Photoaffinity labelling of cytosolic fractions – Five 15 cm dishes each of GM95 and HeLa cells and two 15 cm dishes of CHO cells were resuspended in 2 ml of ice-cold Lysis buffer (50mM Tris, pH 6.8; 1 mM EDTA, 0.3 M sucrose, 1 mM PMSF and 1x protease inhibitor cocktail). Cells were homogenized by passing them through a 26G1/2 needle using a 1 ml syringe. The suspension was centrifuged at 100.000 g for 1 h at 4°C to remove cell debris, nuclei and membranes. Protein concentration in the obtained cytosol was determined by Bradford assay and adjusted to 1.2 mg/ml with PBS. 55 µl of cytosol was mixed with 55 µl liposome suspension containing 5 mol% of pacLipid and incubated for 30 min at room temperature with gentle shaking. Subsequently, the samples were placed on ice and irradiated for 60 sec using a 1000 W Mercury lamp equipped with a dichroic mirror and a 345 nm bandpass filter (Newport) at 30 cm distance. Protein was recovered by chloroform-methanol precipitation and the air-dried protein pellet was dissolved in 20 µl of 1% SDS in PBS with vigorous shaking for 10 min at 70°C. Click reactions were performed by adding 8 µl of a freshly prepared ‘click’ mix (40 µl of 25 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 40 µl of 2.5 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA), 40 µl of 25 mM CuSO₄ and 40 µl of 25 mM biotin-N₃) per sample followed by incubation at room temperature for 2 h while shaking. After addition of 0.25 volume of 5x Sample Buffer (0.3 M Tris/HCl (pH 6.8), 10% SDS, 50% glycerol, 0.025% bromphenol blue, 10% β-mercaptoethanol), samples were boiled for 5 min at 95°C, subjected to SDS-PAGE and immunoblotting or Coomassie staining.
For the identification of ceramide binding proteins, a cytosolic fraction was prepared from twenty 15 cm dishes of GM95 cells and diluted to a protein concentration of 1.4 mg/ml in PBS. 450 µl of cytosol was mixed with 450 µl of a liposome suspension containing 5 mol% of pacCer or pacGlcCer and incubated for 30 min at room temperature with gentle shaking. Samples were split into four portions of 200 µl, UV irradiated and subjected to chloroform-methanol precipitation as above. Protein pellets were combined, resuspended in 400 µl 1% SDS in PBS and solubilized for 10 min at 70°C. Click reactions were performed by adding 80 µl of freshly prepared ‘click’ mix containing biotin-N₃, as above. Samples were split in two portions of 240 µl, subjected to chloroform-methanol precipitation twice. Protein pellets were resuspended in 200 µl of 1% SDS in PBS, solubilized by vigorous shaking for 10 min at 70°C and then diluted 5-fold in PBS. After centrifugation at 20,000 g for 1 min at room temperature, supernatants were collected and combined. 1600 µl of supernatant was mixed with 50 µl of a 50% slurry of NeutrAvidin beads (NeutrAvidin™ Agarose Resin, Thermo Scientific) equilibrated in 0.2% SDS in PBS and incubated at room temperature with rotation. The beads were collected by centrifugation (100 g, 1 min, room temperature), washed three times with 1 ml 0.1% SDS in PBS and three times with 1 ml of PBS. To elute bound proteins, the beads were incubated in SDS-PAGE sample buffer for 5 min at 95°C. The eluates were analysed by SDS-PAGE and Coomassie staining. Lanes of interest were cut into 10 equal sections, cut from the gel and protein in each section was subjected to trypsin digestion and peptides were analysed by LC-MS/MS using an LTQ-Orbitrap mass spectrometer (ThermoScientific) connected to an Agilent 1200 series nano LC system as described in (25). The following criteria were applied to select for pacCer-modified cytosolic proteins: (i) no spectral counts in the controls (no pacLipid/+UV, pacCer/-UV) and ≥3 spectral counts in the pacCer photoaffinity-labeled samples (pacCer/+UV) for Exp. #1 or Exp. #2; (ii) a 5-fold enrichment in spectral counts for pacCer photoaffinity-labeled samples (pacCer/+UV) in comparison with the controls for Exp. #1 or #2; (iii) a spectral count ratio for pacCer/+UV over pacGlcCer/+UV samples of ≥1 for Exp. #1 or #2. High-confidence ceramide binding proteins were selected based on a spectral count ratio for pacCer/+UV over pacGlcCer/+UV samples of ≥2 for both Exp. #1 and #2.

**Photoaffinity labelling of recombinant protein** – Recombinant proteins were diluted in 50 mM Tris-HCl pH 7.4, 300 mM NaCl to a final concentration of 0.1 µg/µl. 25 µl of this solution was combined with 25 µl of 0.1 µg/µl MBP, which served as internal negative control. The sample was then mixed with 50 µl of a liposome suspension containing 1 mol% pacLipid and incubated for 30 min at 37°C with gentle shaking. For UV cross-linking, samples were transferred into a pre-cooled 96-well plate on ice and irradiated for 90 sec as described above. The samples were transferred to eppendorf tubes and subjected to chloroform-methanol precipitation after addition of 20 µg soybean trypsin inhibitor as carrier to aid protein recovery. The protein pellet was dissolved in 100 µl of 1% SDS in PBS with vigorous shaking for 10 min at 37°C. 20 µl of each sample were used for click reaction for 1 h at
37°C, as described above, except that biotin-N\textsubscript{3} was replaced by 80 µM Alexa Fluor647-N\textsubscript{3}. After addition of 0.25 volume of 5x Sample Buffer, samples were boiled for 5 min at 95°C, subjected to SDS-PAGE and then analysed by in-gel fluorescence and Coomassie staining. To this end, run gels were fixed in 40% ethanol, 10% acetic acid for 30 min and then washed in H\textsubscript{2}O for 1 h at room temperature. Gels were scanned with a Typhoon FLA 9500 (GE Healthcare) with a 635 nm laser and LPR filter. Fluorescence intensities were quantified using ImageQuant TL. Next, gels were stained with Coomassie and the amount of recombinant protein was determined by measuring the staining intensity using Image Lab 5.2 software. Fluorescence intensities of recombinant protein were corrected for background fluorescence in the same lane and then divided by the total amount of protein. The specific fluorescence intensity of UV-irradiated protein was determined after subtraction of fluorescence intensity of non-UV irradiated protein.

**Modelling of StarD7 lipid binding pocket and docking studies** – A database search of SWISS-MODEL (36–39) using the primary sequence of human StarD7 START (40) retrieved human StarD2 START ((41); PDB 1LN1) as the closest related protein sequence (29.19 % identity). The search also yielded CERT START ((42); PDB 2E3R) as a more distant related protein (16.40 % identity). As both StarD2 and StarD7 are known as PC transfer proteins, the crystal structure 1LN1 was chosen as a starting point. Three separate runs using SWISS-MODEL standard parameters were carried out, the resultant homology models were subsequently tested for anomalies by their Ramachandran plots. The best three models, one from each run, were superimposed and energy minimised using MMFF94x force field to yield the final model that was used as a reference. The homology model was tested by docking PC (16:1, 16:1) into the binding pocket using SWISS-DOCK (43, 44). The interaction maps revealed high similarity to the identified residues in the orthologous StarD2 protein. C16:0-ceramide was then docked into the refined model as described above. Note that a significant difference in volume of the ligand binding pockets between StarD7 (~2298 Å\textsuperscript{3} 1LN1) and CERT (~1650 Å\textsuperscript{3}, 2E3P and 2E3R; CHIMERA, http://www.cgl.ucsf.edu/chimera/docs/morerefs.html) resulted in different binding modes of ceramide. Only those modes that showed a mixture of hydrophilic and hydrophobic interaction were considered for this study. Protein structures were displayed using Pymol software.

**Lipid transfer assay** – For lipid transfer assays, 14 pmol of CERT START or 70 pmol of StarD7-isoform II protein in 50 µl Transfer Buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl) were mixed with 40 µl of 10 mM acceptor liposomes on ice. The reaction was started by addition of 10 µl donor liposomes followed by incubation for 20 min at 37°C while shaking mildly. To stop the reaction, 20 µl of agglutinin (2.5 mg/ml in Transfer Buffer) were added and samples were incubated on ice for 15 min. For control samples, the protein was mixed with acceptor liposomes as above, but kept on ice and the donor liposomes were added just after addition of agglutinin. Donor liposomes were removed by
centrifugation at 16,000 g for 5 min at 4°C, and 100 µl of the supernatant were transferred to a fresh eppendorf tube. Either C18-ceramide (d18:1/18:0, Avanti no. 860518) or DOPC were added in appropriate amounts as internal standard. Lipids were extracted by addition of 320 µl CHCl₃:MeOH (1:2) and 340 µl H₂O with subsequent centrifugation at 16,000 x g for 5 min at room temperature. The organic phase was collected in a fresh eppendorf tube and the samples were subjected to a second round of extraction by addition of 100 µl CHCl₃ followed by the same treatment as above. The organic phase was dried down in a centrifugal evaporator and lipids were dissolved in 10 µl CHCl₃. For mass spectrometry analysis, 1 µl of superDHB matrix (50 mg/ml in 30% acetonitrile, 0.1% TFA) was spotted on a stainless steel MALDI plate and dried before spotting 1 µl per lipid sample. The spots were analysed in a Bruker UltrafleXtreme™ MALDI-TOF/TOF instrument in positive ion mode. Peptide calibration standard II (Brucker) was used for calibration.

RESULTS

A search for cytosolic ceramide binding proteins using bifunctional ceramide analogue pacCer yields CERT

As part of our ongoing efforts to identify novel effector proteins involved in ceramide-mediated stress signalling and apoptosis, we synthesized a bifunctional analogue of ceramide containing a photoactivatable diazirine and clickable alkyne group in its N-linked 15-carbon-long acyl chain (pacCer; Fig. 1A). UV irradiation of the diazirine group generates a highly reactive pacCer intermediate that can form a covalent linkage with proteins in its direct vicinity. Click chemistry is then used to react the alkyne group with a fluorophore or biotin as reporter molecule to allow visualization or affinity purification of photoaffinity labelled proteins, respectively (Fig. 1B; (29)). To test whether pacCer can be used to capture ceramide binding proteins, cytosolic fractions prepared from Chinese hamster ovary CHO, human cervix carcinoma HeLa and mouse melanoma GM95 cells were incubated with liposomes containing 5 mol% of pacCer and subjected to UV crosslinking. Photoaffinity labelling experiments with cytosol and liposomes containing 5 mol% of a bifunctional analogue of glucosylceramide (pacGlcCer; Fig. 1A) served as control. Following a click reaction with biotin-N₃, photoaffinity labelled cytosolic proteins were visualized by immunoblotting using an antibiotin antibody. As shown in Fig. 1C, a subset of cytosolic proteins from each cell line could be UV-crosslinked with both lipid probes. However, some cytosolic proteins appeared to have a higher affinity for pacCer than pacGlcCer and vice versa.

To identify soluble proteins that preferentially bind ceramide, the cytosolic fraction derived from GM95 cells was photoaffinity labelled with pacCer or pacGlcCer and subjected to a click reaction with biotin-N₃. Next, biotinylated proteins were affinity-purified using NeutrAvidin beads, separated by SDS-PAGE, subjected to in-gel tryptic digestion and identified by mass spectrometry. Two
inddependent experiments yielded in total 67 proteins that were either exclusively present or enriched in affinity-purified fractions of pacCer-incubated and UV-irradiated cytosol (Supplemental Table S1). Nearly 30% of these proteins (20 out of 67) were marked as high-confidence ceramide binding proteins based on a spectral count ratio for pacCer/+UV over pacGlcCer/+UV samples of ≥2 in both experiments (Table 1). These proteins participate in a wide range of cellular processes, including membrane trafficking (sorting nexins), signal transduction (Unc-119 proteins), DNA damage response pathways (DNA damage binding protein-1, DnaJ homolog DnaJc7) and protein ubiquitination (COP9 signalosome subunit). A possible involvement of these proteins in ceramide-dependent cellular processes is currently under investigation and will be discussed elsewhere. Reassuringly, the list of high-confidence ceramide binding proteins also included ceramide transfer protein CERT, a cytosolic protein responsible for non-vesicular delivery of newly synthesized ceramides from the ER to the site of sphingomyelin production in the trans-Golgi (16). The latter finding qualifies pacCer as a suitable tool to capture ceramide binding proteins from a complex proteome.

**Probing the lipid-binding pocket of CERT using bifunctional lipid analogs**

CERT, also referred to as StarD11, is a member of the Steroidogenic Acute Regulatory Transfer (START) domain containing proteins (45). Members of this family are defined by the presence of a conserved ~210 amino acid sequence that folds into an α/β helix-grip structure forming a hydrophobic cavity for ligand binding. A crystal structure of the START domain of CERT with one ceramide molecule buried in its amphiphilic cavity has been solved at 1.40-Å resolution (42). At the far end of the cavity, the amide and hydroxyl groups of ceramide form a hydrogen bond network with specific amino acid residues that play key roles in ceramide recognition. For instance, the carboxy-group of Glu446 forms two hydrogen bonds with the amide-nitrogen and C1-hydroxyl group of ceramide. In addition, the oxygen atom of Asn504 forms a hydrogen bond with the C3-hydroxyl group of ceramide (Fig. 2A). This led us to examine the impact of alanine substitutions of these residues on pacCer labelling of CERT START produced in *E. coli*. Contrary to recombinant maltose binding protein (MBP), CERT START could be readily UV-crosslinked with pacCer (Fig. 2B,F). Substitution of Glu446 with Ala had no obvious impact on pacCer labelling of the protein. In contrast, substitution of Asn504 for Ala virtually abolished photoaffinity labelling, also in combination with the E446A substitution (Fig. 2B,F). These results are largely consistent with the previous observation that the ceramide extraction activity of recombinant CERT START is severely compromised by a N504A substitution and only partially affected by the E446A substitution (42). Interestingly, Ala substitution of a Trp residue exposed on the protein’s exterior near the entry site of the amphiphilic cavity, i.e. Trp473, resulted in a marked increase in pacCer labelling. However, pacCer labelling of CERT STARTW473A was abolished upon substitution of Glu446 and Asn504 for Ala (Fig. 2B).
Together, the above data indicate that photoaffinity labelling of CERT START with pacCer occurs within the protein’s lipid-binding pocket and that hydrogen bonding between Asn504 and the C3-hydroxy group of pacCer is particularly critical for stabilizing the protein-lipid interaction. Indeed, removal of the C3-hydroxy group strongly reduced the ability of pacCer to label CERT START whereas removal of the C1-hydroxy group actually enhanced photoaffinity labelling (Fig. 2C-E). Furthermore, UV-crosslinking experiments with bifunctional analogs of diacylglycerol (pacDAG), phosphatidylethanolamine (pacPE), phosphatidylycholine (pacPC) and sphingomyelin (pacSM) revealed that photoaffinity labelling of CERT START with pacCer is specific (Fig. 2F). Altogether, our findings demonstrate the suitability of pacCer as chemical probe to trace ceramide binding proteins and map their lipid-binding pocket.

**Lipid specificity profiling of CERT-related proteins indicates that StarD7 binds ceramide**

The human genome encodes 15 START domain-containing proteins that can be categorised into 6 sub-families based on sequence homology within the START domain (45). According to this phylogenetic classification, proteins in the same clade mainly show specificity towards a common lipid ligand (Fig. 3A). As the lipid ligands of StarD9 and members of the Rho-GAP START group (i.e. StarD8, StarD12 and StarD13) are not known, we first investigated whether any of these putative lipid carriers bind ceramides. To this end, we produced their START domains in *E. coli* and carried out pacCer labelling experiments on the purified recombinant proteins, in the presence of MBP as internal control. This revealed that, contrary to CERT START, none of these proteins displayed any notable affinity for ceramide (data not shown). We then asked whether any of the START domains of the CERT-related phospholipid carrier proteins (i.e. StarD2, StarD7 and StarD10) bind ceramide in addition to their phospholipid ligand. As expected, START domains of the PC transfer proteins StarD2 (PCTP; (46)) and StarD10 (47) preferentially labelled with pacPC over all other bifunctional lipid analogs tested (Fig. 3B,C). These domains could be labelled only modestly with pacSM and lacked any pronounced affinity for pacCer, pacDAG or pacPE, even though StarD10 has been shown to possess dual specificity lipid transfer activity for PC and PE *in vitro* (47). Strikingly, the START domain of StarD7, which was so far thought to only bind PC (40), consistently displayed a very strong photoaffinity labelling signal with pacCer (Fig. 3B,C). Photoaffinity labelling of StarD7 START with pacPC was also pronounced while pacDAG, pacSM and pacPE each gave rise to substantially lower labelling intensities. As PC and ceramide are structurally very different lipids, we next asked whether their interaction with StarD7 involves a shared lipid-binding pocket.

**The lipid-binding pocket of StarD7 has dual specificity for PC and ceramide**

As there is no detailed structural information on the START domain of StarD7, we created a 3D homology model based on the crystal structures of the closely-related START domains of CERT ((42); PDB 2E3R) and PC transfer protein StarD2 ((41); PDB 1LN1). StarD7 START shares 17% and
28% sequence identity with the START domains of CERT and StarD2, respectively, whereas the latter two domains share 22% sequence identity. Next, the structure of C16:0-ceramide or di-palmitoleoyl-PC was docked to the StarD7 START lipid-binding pocket (see Material and Methods for further details). In the PC-bound model, a conserved Arg residue (Arg189) makes direct contact to the lipid’s phosphoryl group, probably by forming a salt bridge with the help of Asp193 (Fig. 4A,B), in line with what has been reported for PC when bound to StarD2 (41). Moreover, the quaternary amine of PC interacts with Trp215 and Tyr228, which likely form part of an aromatic cage similar to the one described in the START domain of StarD2 (Fig. 4A,B). In StarD10, the Arg-Asp pair is conserved and embedded in a consensus sequence (YRKKWDD) that is also present in StarD2 and StarD7, but absent in CERT (Fig. 4A). Contrary to this, the residues that form the aromatic cage are not conserved in StarD10, which might explain why pacPC labelling of this protein is overall much less efficient than for StarD2 and StarD7 (Fig. 3B).

To validate our model of the StarD7 PC binding site, we next substituted Arg189, Trp215 and Tyr228 with other residues using site-directed mutagenesis and then analysed the impact of each substitution on PC binding by photoaffinity labelling of the mutant proteins with pacPC. Substitution of Arg189 with Gln (R189Q) caused a major (~80%) reduction in pacPC labelling, indicating that Arg189 is a critical determinant of PC binding by StarD7 (Fig. 4C,F). Substitution of Trp215 or Tyr228 with Arg (W215R, Y228R) in each case essentially abolished pacPC labelling. The marked impact of the latter substitutions is likely due to the introduction of a positive charge close to the quaternary amine within the PC head group, which would strongly interfere with its binding. In our initial model, Tyr267 did not show up directly as being part of the PC binding pocket in StarD7 but was nevertheless assumed to be part of an aromatic cage around the quaternary amine of PC, analogous to how StarD2 binds PC (41). To verify this assumption, we tried a slightly different approach by substituting Tyr267 on the one hand for a neutral residue, i.e. Ala, and on the other hand for another aromatic but charged residue, i.e. His. Substitution of Tyr276 with Ala (Y267A) reduced the intensity of pacPC labelling by 80% as compared to wild type, while the Y267H substitution was slightly less effective (Fig. 4C,F). Together, these data indicate that Trp215, Tyr228 and Tyr276 in StarD7 likely form an aromatic cage that coordinates the quaternary amine of the PC head group whereas Arg189 stabilizes PC binding by forming a salt bridge with the lipid’s phosphoryl group.

In the ceramide-bound model, Arg189 is predicted to also contribute to ceramide binding by forming a hydrogen bond with the lipid’s C3-hydroxyl group (Fig. 4D). However, substitution of Arg189 with Gln (R189Q), if anything, enhanced photoaffinity labelling of StarD7 START with pacCer (Fig. 4E,F) while having either no or a slightly negative impact on labelling with pacDAG, pacPE and pacSM (Fig. 5). We reasoned that the C3-hydroxyl group of ceramide might also form a hydrogen bond with a glutamine at position 189. Instead, we found that the C3-hydroxyl group is fully dispensable for
ceramide binding, as StarD7 START could be equally well labelled with pacCer, C3-deoxy-pacCer or C3-deoxy-N-methyl-pacCer (Fig. 4G,H). This indicates that Arg189, a critical determinant of PC binding, does not significantly contribute to ceramide binding. In contrast, substitution of any of the residues that form the aromatic cage (i.e. Trp215, Tyr228 and Tyr267) clearly reduced pacCer labelling, with the introduction of a positive charge (i.e. W215R, Y228R) having the strongest impact (Fig. 4E,F). Substitution of Tyr228 for Arg also reduced pacDAG labelling (Fig. 5), suggesting that StarD7 START can also accommodate DAG. However, the efficiency of pacCer labelling consistently exceeded that of pacDAG. From this we conclude that StarD7 has a significantly higher affinity for ceramide than for DAG.

Collectively, our data indicate that the START domain of StarD7 provides a common binding pocket for PC and ceramide, which includes residues that form an aromatic cage involved in coordinating the quaternary amine of PC. How exactly these residues contribute to ceramide binding remains to be established, but based on our results it seems unlikely that hydrogen bonding of the ceramide head group makes a major contribution to the interaction. We also uncovered differences regarding the involvement of the conserved Arg residue in the PC binding pocket, which proofed to be dispensable for ceramide binding.

Photolabelling of CERT and StarD7 with pacCer in the presence of excess ceramide

To obtain complementary evidence that photolabelling of the START domains of CERT and StarD7 with pacCer occurs at an authentic ceramide-binding site, we also examined whether pacCer labelling of these protein domains could be competitively inhibited by natural C16:0-ceramide. Using the START domains of wild-type CERT and the ceramide-binding deficient mutant CERTR189Q as references, we determined that liposomes containing 0.5 mol% of pacCer (instead of the usual 1 mol%) in combination with an UV crosslinking time of 90 sec gave the highest specific labelling efficiency. As shown in Fig. S1A and S1B, lowering the pacCer concentration to 0.25 mol% or shortening the UV crosslinking time to 30 sec diminished the specific labelling efficiency so that these conditions were discarded for the competition experiments. Photolabelling of CERT with 0.5 mol% pacCer in the presence of 10 to 40-fold excess of ceramide (i.e. pacCer:Cer, 0.5:5 to 0.5:20 mol%) showed a trend toward inhibition that was not statistically significant (Fig. S1C,D). Addition of excess ceramide did also not lead to any significant reduction in pacCer labelling of either StarD7 or the PC-binding deficient mutant StarD7R189Q, which was included to facilitate ceramide binding (see Fig. 4E,F). While these results are hard to reconcile with our site-directed mutagenesis data and argue against the notion that pacCer labels an authentic ceramide-binding site in CERT and StarD7, it deserves mention that competitive inhibition of ligand photolabelling could also not be demonstrated.
for known ligand-receptor interactions such as the GABA receptor and neurosteroids ([48]; see also Discussion). As the competition experiments did not yield any conclusive insights, we next set out to examine possible functional implications of a dual binding specificity of StarD7 for ceramide and PC.

**Ceramide modulates the PC transfer activity of StarD7**
To elucidate the functional relevance of ceramide binding by StarD7, we first asked whether StarD7 acts as a bona fide ceramide transfer protein. Thus, we analysed the ability of its START domain to catalyse the transfer of ceramide between liposomes using a MALDI-based read out assay. As we wanted to avoid any competing effect of the liposome backbone lipids on PC or ceramide transfer, we chose a mixture of phosphatidylglycerol (PG) and PE for the liposomes in our assay. With this assay at hand, ceramide transfer by the START domain of CERT as well as PC transfer by the START domain of StarD7 could be readily monitored in a time dependent manner (Fig. 6A). For the START domain of CERT, the transfer activity was approximately 8 pmol ceramide/pmol protein transferred per min, which is comparable to previously published data (16). As expected, substitution of Asn504 for Ala in the START domain of CERT abolished ceramide transfer activity (Fig. 6A,B). The START domain of StarD7 showed an activity of approximately 7 pmol PC transferred per pmol protein per min (Fig 6A), a rate that is substantially higher than the one reported by Horibata et al. (40). This discrepancy is likely due to the fact that in their assay, Horibata et al. (40) measured the transfer of radiolabelled PC between liposomes containing bulk amounts of unlabelled PC. In line with our photoaffinity labelling experiments, substitution of Arg189 for Gln in the START domain of StarD7 strongly reduced its PC transfer activity (Fig. 6A,B). In contrast to CERT START, neither wild-type StarD7 START nor the R189Q mutant displayed any measurable ceramide transfer activity (Fig. 6B).

It should be noted that, owing to its hydrophobicity, the detection limit of ceramide by MALDI is considerably higher than that of PC. Thus, a low ceramide transfer activity may go unnoticed in our assay. Still, the foregoing experiments demonstrate that under conditions where CERT actively transports ceramide, StarD7 does not, even though it displays a robust PC transfer activity under the same conditions. As our site-directed mutagenesis and photoaffinity labelling studies indicate that StarD7 START has dual affinity for PC and ceramide, we wondered whether ceramide binding could influence its PC transfer activity. To test this idea, we next performed PC transfer assays with donor liposomes containing ceramide in 2-fold excess over PC. As shown in Fig. 6C, ceramide addition caused a significant (~40%) reduction in StarD7-mediated PC transfer. By contrast, including DAG in 2-fold excess over PC in donor liposomes had no obvious impact on PC transfer by StarD7, in line with our finding that StarD7 preferentially labels with pacCer over pacDAG (Figs. 3-5). Moreover, addition of ceramide did not affect PC transfer by StarD2 (Fig. 6C), a StarD7-related protein lacking affinity for pacCer (Fig. 3). Besides providing complementary evidence that StarD7 has dual specificity for PC and ceramide, these results indicate that ceramide modulates the PC transfer activity.
of StarD7, presumably by acting as a competitive inhibitor of PC binding to the protein’s START domain.

**StarD7 is dispensable for ceramide-induced apoptosis in SMSr-depleted cells**

We previously showed that acute inactivation of sphingomyelin synthase-related protein SMSr/SAMD8 causes a rise in ER ceramides and their mistargeting to mitochondria, triggering a mitochondrial pathway of apoptosis (35). How ER ceramides reach mitochondria in cells lacking functional SMSr is not known. StarD7 has been reported to serve a crucial role in mitochondrial import of PC (31, 40). Given our current finding that StarD7 has dual affinity for PC and ceramide, we wondered whether the protein might participate in the mitochondrial translocation of ER ceramides to commit SMSr-deficient cells to death. To address this, we created two independent HeLa cell-lines in which expression of StarD7 was eliminated using CRISPR/Cas9 technology (StarD7-KO#1 and #2). Wild-type HeLa cells treated with SMSr-targeting siRNAs (siSMSr) readily underwent apoptosis, as evidenced by proteolytic cleavage of caspase substrate poly-ADP ribose polymerase-1 (PARP1; Fig. 7). No PARP1 cleavage was observed when cells were treated with non-silencing RNA (siNS). Importantly, removal of StarD7 in HeLa cells did not lead to reduced PARP1 cleavage upon siSMSr-treatment. From this we conclude that StarD7 is not a critical component of the mechanism by which ER ceramides can reach mitochondria to induce apoptosis in SMSr-depleted cells.

**DISCUSSION**

In here, we report the successful application of a photoactivatable and clickable ceramide analogue, pacCer, to capture ceramide-binding proteins and unravel the structural basis by which these proteins recognize ceramide. This approach yielded CERT-related PC transfer protein StarD7 as a novel ceramide binding protein. Previous work revealed that StarD7 is required for efficient PC import by mitochondria and has a critical role in mitochondrial function and morphogenesis (31, 40, 49). Combining site-directed mutagenesis and photoaffinity labelling experiments, we show that the START domain of StarD7 harbours a common binding site for PC and ceramide. While StarD7 lacks robust ceramide transfer activity *in vitro*, we observed that its ability to shuttle PC between two membranes is strongly affected by ceramide. Thus, StarD7 qualifies as a candidate effector protein of ceramide, a lipid known for its ability to initiate a variety of mitochondria-mediated cytotoxic effects (50–52).

The suitability of pacCer as chemical tool to capture ceramide binding proteins from a complex proteome is reflected by the identification of CERT among the photoaffinity labelled proteins in a cytosolic fraction of mouse melanoma GM95 cells. Interestingly, our primary set of pacCer-labelled proteins also included the 65kDa regulatory subunit A of protein phosphatase PP2A, a ceramide-
inducible serine/threonine phosphatase with potent tumour suppressor activity (53, 54). As this subunit also displayed affinity for pacGlcCer (Supplemental Table S1), it did not pass our stringent selection criteria for high-confidence ceramide binding proteins (Table 1). Nevertheless, whether direct binding of ceramide to PP2A subunit A is part of the mechanism by which the phosphatase is activated merits further investigation. High-confidence ceramide binding proteins included proteins involved in membrane trafficking (sorting nexins), signal transduction (Unc-119 proteins), DNA damage response pathways (DNA damage binding protein-1, DnaJ homolog DnaJc7) and protein ubiquitination (COP9 signalosome subunit). Addressing whether the cellular activities of these proteins are responsive to ceramides is subject to ongoing investigations. StarD7 was not among the ceramide binding proteins originally identified by photoaffinity labelling of a cytosolic fraction prepared from GM95 cells. However, lipid specificity profiling of the recombinant protein with our bifunctional lipid probes clearly indicated that StarD7 has dual specificity for ceramide and PC. In this respect, it deserves mention that StarD7 primarily localizes to mitochondria and distributes only to the cytosol when cells are grown to very high density (40). As this was not the case for the GM95 cells used to prepare a cytosolic fraction, StarD7 likely escaped detection in our screen for cytosolic ceramide binding proteins. In addition, it is unlikely that this screen reached saturation.

Remarkably, our efforts to demonstrate that photolabelling of the START domains of CERT and StarD7 with pacCer occurs at an authentic ceramide-binding site using natural ceramide as competitive inhibitor were unsuccessful. Even addition of ceramide in 40-fold access over pacCer did not result in any statistically significant reduction in photolabelling. We noticed that also other known or predicted ligand-protein interactions could not be validated by competitive inhibition of ligand photolabelling (48, 55). Possible reasons for why competitive inhibition may be hard to demonstrate in certain cases include the irreversible nature of photolabelling, a gradual depletion of the photoactivatable analogue by solvent labelling and potential differences in binding affinities between the analogue and its natural counterpart for the target protein (56). A further complication potentially relevant to our studies is that ceramide forms gel-like domains in synthetic phospholipid bilayers (57), which may reduce its capacity to serve as effective competitive inhibitor in pacCer photolabelling experiments.

As complementary approach to demonstrate that pacCer retains the physico-chemical properties important for recognition by ceramide binding proteins, we took advantage of crystal structures of the START domain of CERT in complex with ceramides of different acyl chain lengths (42). In these structures, Asn504 is predicted to form a hydrogen bond with the C3-hydroxyl of ceramide, which appears critical for efficient CERT-mediated ceramide transport (42). Consistent with this notion, substitution of Asn504 for Ala virtually abolished pacCer labelling of the CERT START domain. Moreover, removal of the C3-hydroxyl group from pacCer strongly reduced its ability to label CERT
START. Two other residues, Glu446 and Gln467, form a hydrogen bond network with the amide and C1-hydroxyl groups of ceramide (42). Surprisingly, removal of the C1-hydroxyl group actually enhanced pacCer labelling of CERT START whereas substitution of Glu446 to Ala did not have any obvious impact. Hence, we anticipate that the multilateral hydrogen bonding, involving the amide and C1-hydroxyl groups, is more adaptable to changes in one of its constituents. Our finding that substitution of Trp473, a residue located in the Ω1 loop near the entry of the amphiphilic cavity, increased pacCer labelling was unexpected, as previous work revealed that the membrane affinity of CERT STARTW473A is reduced and that ceramide extraction by a CERT STARTW473A/W562A mutant is almost completely blocked (42, 58). The Ω1 loop of CERT START is thought to function as a gate that opens the amphiphilic cavity upon interaction of Trp473 and also Trp562 with the target membrane (42, 58). Conceivably, substitution of Trp473 for Ala may lead to an enhanced pacCer labelling by causing a constitutive opening of the amphiphilic cavity. However, addressing the precise role of Trp473 in the transfer cycle of CERT will require a more detailed functional analysis.

Our finding that StarD7 has dual specificity for PC and ceramide is striking given the considerable structural differences between the two lipids. A homology model based on crystal structures of the closely related PC transfer protein StarD2 allowed us to map potential structural determinants of PC binding in the START domain of StarD7, and verify their functional relevance by site-directed mutagenesis and photoaffinity labelling analysis. Our data indicate that Trp215, Tyr228 and Tyr276 in StarD7 START form an aromatic cage that coordinates the quaternary amine of the PC head group and that Arg189 promotes PC binding by forming a salt bridge with the lipid’s phosphoryl group, analogous to the PC binding site described in StarD2 (41). Whereas Arg189 is fully dispensable for ceramide binding, substitution of any of the residues forming the aromatic cage caused a significant reduction in pacCer labelling, indicating that PC and ceramide share a common binding site. However, given that StarD2 lacks affinity for ceramide, it is clear that ceramide recognition by StarD7 involves additional structural determinants.

Other examples of lipid transfer proteins with dual lipid specificity include the mammalian oxysterol binding protein OSBP and its yeast homologue Osh4p, which catalyse non-vesicular transport of both sterols and phosphatidylinositol-4-phosphate (PI4P; (59–61)). Molecular dynamics simulations revealed that the binding energy of the Osh4p-PI4P pair is mostly mediated by electrostatic interactions between specific polar residues and the lipid head group. For the Osh4p-sterol pair, on the other hand, binding is achieved by van der Waals contacts that are dispersed over the entire lipid molecule, involving numerous Osh4p residues (61, 62). Our data suggest that similar principles may govern the dual specificity of StarD7 for PC and ceramide. Indeed, PC binding was overall more sensitive to single residue substitutions than ceramide binding. Moreover, neither hydroxyl group in ceramide proved essential for its interaction with StarD7. As StarD7 displays only a reduced affinity
for DAG, it appears that ceramide recognition relies, at least in part, on the lipid’s sphingoid backbone. Given that its lipid-binding START domain can accommodate both PC and ceramide, it is remarkable that StarD7 does not display strong affinity for SM. However, we found that residues that form the aromatic cage that coordinates the quaternary amine of PC are also critical for ceramide binding. Thus, it is conceivable that SM shows only low affinity for StarD7 because its phosphocholine head group precludes specific binding of the ceramide backbone to the protein’s START domain. Moreover, the dynamic volume of SM likely deviates significantly from that of PC. Indeed, even minor changes in the lipid backbone can have considerable impact on the dynamic volume of phospholipids by altering the P-N head group angle (63). This effect may reduce the affinity of the phosphocholine head group for the lipid-binding pocket of StarD7 in SM as compared to PC. Further insights into the different binding modes of PC and ceramide will require crystal structures or molecular dynamics simulations of StarD7 bound to these ligands.

As opposed to OSBP and Osh4p, which catalyse non-vesicular transport of both PI, P and sterols, we were unable to detect any StarD7-mediated transfer of ceramides with a protein that showed robust PC transfer activity in vitro. Nevertheless, ceramides caused a significant reduction in StarD7-mediated PC transfer in vitro whereas PC transfer by StarD2 was unaffected. DAG had no obvious impact on PC transfer by StarD7, in agreement with our finding that StarD7 START displays only a reduced affinity for this lipid. The most straightforward explanation for these findings is that ceramide, contrary to DAG, effectively competes with PC for binding to the START domain of StarD7. As StarD7 has a crucial role in mitochondrial import of PC from the ER (31, 40, 60), we also addressed a potential involvement of StarD7 in the mitochondrial translocation of ER ceramides and subsequent induction of apoptosis in cells depleted of SMSr (35). This revealed that StarD7 is fully dispensable for apoptosis induction in SMSr-depleted cells. While our current findings do not exclude that StarD7 acts as a ceramide transfer protein in vivo, they raise the possibility that StarD7-mediated PC transport is subject to negative regulation by ceramides.

PC is a vital component of mitochondria. As mitochondria lack the enzymes necessary for PC synthesis, they rely on PC import for proper function. Loss of StarD7 causes a significant reduction in mitochondrial PC levels, impaired respiratory activity, overproduction of reactive oxygen species, and disrupted cristae structures (31, 32). StarD7 resides in the outer mitochondrial membrane (OMM) and is believed to shuttle PC between ER and OMM at ER-mitochondria contact sites (40, 64). As ceramide biosynthesis occurs in mitochondria-associated ER membranes (MAMs; (61, 62)) and is up-regulated in response to various stress stimuli (8, 9), it is conceivable that the local concentration of ceramides at ER-mitochondria contact sites can rise to a level where it affects StarD7-mediated mitochondrial PC import. Moreover, it is well known that a rise in mitochondrial ceramide levels has a detrimental impact on mitochondria function and is associated with overproduction of reactive oxygen
species and stress-induced mitochondrial apoptosis (50–52, 67). In view of our current findings, the notion that ceramides may exert their toxic effects in part by acting through StarD7 merits further investigation.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.
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Table 1. Candidate ceramide binding proteins.

| #    | Accession #     | Name                                      | MW kDa | control |       |       |       |       |       |       |
|------|-----------------|-------------------------------------------|--------|---------|-------|-------|-------|-------|-------|-------|
| 1    | IPI00321734     | Lactoylglutathione lyase Glo1             | 21     | 0       | 0     | 0     | 12    | 0     | 2     | 0     | 0     | 5     | 0     | 1     |
| 2    | IPI00230084     | Aldehyde dehydrogenase -7a1              | 59     | 0       | 0     | 0     | 14    | 0     | 0     | 1     | 9     | 2     | 1     |
| 3    | IPI00622364     | Sorting nexin-1                          | 59     | 0       | 0     | 0     | 18    | 2     | 8     | 1     | 8     | 4     | 3     |
| 4    | IPI00109212     | Sorting nexin-2                          | 58     | 0       | 0     | 0     | 16    | 0     | 4     | 0     | 10    | 2     | 0     |
| 5    | IPI00131315     | Unc-119 homolog A                        | 27     | 0       | 0     | 0     | 10    | 0     | 1     | 0     | 6     | 0     | 1     |
| 6    | IPI00225371     | Unc-119 homolog B                        | 28     | 0       | 0     | 0     | 9     | 0     | 0     | 0     | 5     | 0     | 0     |
| 7    | IPI00111167     | Ceramide transfer protein CERT           | 71     | 0       | 0     | 0     | 5     | 0     | 2     | 0     | 3     | 0     | 1     |
| 8    | IPI00348414     | Ado, 2-aminoethanethiol dioxygenase      | 28     | 0       | 0     | 0     | 10    | 1     | 0     | 0     | 6     | 0     | 0     |
| 9    | IPI00421223     | Tropomyosin alpha-4 chain                | 28     | 0       | 0     | 0     | 5     | 0     | 2     | 0     | 6     | 1     | 1     |
| 10   | IPI00131870     | COP9 signalosome subunit 3               | 48     | 0       | 0     | 0     | 8     | 3     | 2     | 0     | 3     | 1     | 1     |
| 11   | IPI00134334     | Prostaglandin reductase-2                | 38     | 0       | 0     | 0     | 9     | 1     | 2     | 0     | 4     | 1     | 1     |
| 12   | IPI00314510     | Aspartoacylase-2                         | 35     | 0       | 0     | 0     | 5     | 0     | 1     | 0     | 4     | 0     | 0     |
| 13   | IPI00331707     | Hydroxymethylglutaryl-CoA synthase       | 58     | 0       | 0     | 0     | 12    | 2     | 4     | 0     | 2     | 1     | 0     |
| 14   | IPI00310658     | Aldo-ketoreductase-1c13                  | 37     | 0       | 0     | 0     | 7     | 0     | 1     | 0     | 4     | 1     | 0     |
| 15   | IPI00111959     | CTP synthase-1                           | 61     | 0       | 0     | 0     | 5     | 0     | 2     | 0     | 3     | 1     | 0     |
| 16   | IPI00114818     | Sec14-like protein-2                     | 46     | 0       | 0     | 0     | 4     | 0     | 0     | 0     | 3     | 0     | 0     |
| 17   | IPI00316740     | DNA damage binding protein-1             | 127    | 0       | 0     | 0     | 16    | 0     | 8     | 0     | 3     | 1     | 0     |
| 18   | IPI00223861     | Peroxisomal N(1)-acetyl-spermine/spermidine oxidase | 55  | 0       | 0     | 0     | 6     | 0     | 1     | 0     | 3     | 0     | 0     |
| 19   | IPI00128880     | Importin-4                               | 119    | 0       | 0     | 0     | 18    | 1     | 8     | 1     | 6     | 3     | 3     |
| 20   | IPI00331385     | DnaJ homolog DnaJc7                     | 65     | 0       | 0     | 0     | 10    | 0     | 3     | 0     | 2     | 2     | 0     |
Fig. 1. Screen for ceramide binding proteins using bifunctional lipid analogs.
(A) Bifunctional analogs of ceramide (pacCer) and glycosylceramide (pacGlcCer) containing a photoactivatable diazirine group (pink) and clickable alkyne group (green).
(B) General outline of a screen for soluble ceramide binding proteins. Cytosolic fractions from mammalian cells are incubated with liposomes containing 5 mol% of pacCer or pacGlcCer and then subjected to UV irradiation. Click chemistry is then used to label the alkyne group in the pacLipid with biotin-\textit{N}_3 or Alexa Fluor647-\textit{N}_3 (not shown), allowing the respective identification or visualization of cross-linked protein-lipid complexes.
(C) Cytosolic fractions from CHO, HeLa and GM95 cells were incubated with liposomes containing pacCer, pacGlcCer or no pacLipid (−) for 30 min at RT, subjected to UV irradiation and then clickreacted with biotin-\textit{N}_3. Samples were analyzed by immunoblotting using an anti-biotin antibody (IB) or by Coomassie staining (CB).
Fig. 2. Probing the lipid-binding pocket of CERT using bifunctional lipid analogs.

(A) Structure of the START domain of CERT in complex with ceramide (PDB 2E3O). The START domain is displayed in rainbow-coloured ribbon representation and the ceramide molecule as spheres, with green, red and blue colour representing C, O and N atoms, respectively. The small inset highlights residues involved in coordinating the ceramide molecule in the lipid-binding pocket.

(B) Wild-type (WT) and point-mutants of CERT START were produced in E. coli, purified and then incubated with liposomes containing 1 mol% of pacCer for 30 min at 37°C. Samples were either UV-irradiated (+) or kept in the dark (-), clickreacted with Alexa Fluor647-N₃ and then analysed by in-gel-fluorescence (IGF) and Coomassie staining (CB).

(C) Bifunctional analogs of C1-deoxy-ceramide (1-DO-pacCer), C3-deoxy-ceramide (3-DO-pacCer) and C3-deoxy-N-methyl-ceramide (3-DO-NMe-pacCer).

(D) Wild-type (WT) CERT START were mixed with maltose-binding protein (MBP), incubated with liposomes containing 1 mol% of pacCer, 1-DO-pacCer, 3-DO-pacCer or 3-DO-NMe-pacCer, and processed as in (B).
(E) Quantitative analysis of photoaffinity labelling of CERT START by deoxy derivatives of pacCer relative to control (pacCer). The labelling intensity of CERT START by pacCer was set at 100%. Data shown are the means ± S.D. of three independent experiments.

(F) Wild-type (WT) and point-mutants of CERT START were mixed with MBP, incubated with liposomes containing 1 mol% of pacCer, pacDAG, pacPC or pacSM, and processed as in (B).
Fig. 3. Lipid specificity profiling of CERT-related StarD proteins.
(A) Phylogenetic tree of the human START domain-containing family of lipid transfer proteins, grouped by their known lipid ligands and additional functional domains. Note that the closest relatives of CERT (StarD11) are the PC-carrier proteins StarD2, StarD7 and StarD10. The phylogenetic tree
was produced with ClustalW and Phylodendron using protein sequences of START domains predicted by PROSITE in UniProt accession numbers: StarD1 (P49675), StarD2 (Q9UKL6-1), StarD3 (Q14849), StarD4 (Q96DR4), StarD5 (Q9NSY2), StarD6 (P59095), StarD7 (Q9NQZ5), StarD8 (Q92502), StarD9 (Q9P2P6), StarD10 (Q9Y365), CERT (Q9Y5P4), StarD12 (Q96QB1), StarD13 (Q9Y3M8), StarD14 (Q8WXJ4-1), StarD15 (Q8WYK0).

(B) START domains of CERT, StarD2, StarD7 and StarD10 were produced in *E. coli*, purified, mixed with MBP, incubated with liposomes containing 1 mol% of pacCer, pacDAG, pacPC, pacPE or pacSM, and processed as in Fig. 2B.

(C) Quantitative analysis of photoaffinity labelling of STAR domains by pacLipids as in (B). Maximum labelling intensity was set at 100%. Data shown are the means ± error range of two independent experiments.
Fig. 4. The lipid-binding pocket of StarD7 has dual specificity for PC and ceramide.

(A) Sequence alignment of lipid-binding regions in the START domains of StarD2, StarD7, StarD10 and CERT. Residues predicted to form a salt bridge involved in coordinating the phosphoryl-group of PC are marked in green. Residues predicted to form an aromatic cage around the quaternary amine of PC are marked in yellow.

(B) Model of the START domain of StarD7 in complex with PC. The START domain is displayed in rainbow-coloured ribbon representation and the PC molecule as spheres with green, red, orange and blue colour representing C, O, P and N atoms, respectively. The small inset highlights residues involved in coordinating the PC molecule in the lipid-binding pocket. See text for further details.

(C) Wild-type (WT) and point-mutants of StarD7 were produced in E. coli, purified, mixed with MBP, incubated with liposomes containing 1 mol% pacPC and processed as in Fig. 2B.
(D) Model of the START domain of StarD7 in complex with ceramide with colour code as in (B). See text for further details.

(E) Wild-type (WT) and point-mutants of StarD7 were mixed with MBP, incubated with liposomes containing 1 mol% pacCer and processed as in Fig. 2B.

(F) Quantitative analysis of photoaffinity labelling of StarD7 by pacLipids as in (C) and (E). Labelling intensity of WT-StarD7 was set at 100%. Data shown are the means ± error range of two independent experiments.

(G) WT-StarD7 and MBP were mixed, incubated with liposomes containing 1 mol% of pacCer, 1-DO-pacCer, 3-DO-pacCer or 3-DO-NMe-pacCer, and processed as in Fig. 2B.

(H) Quantitative analysis of photoaffinity labelling of StarD7 by pacLipids as in (G). Labelling intensity of WT-StarD7 by pacCer was set at 100%. Data shown are the means ± SD of three independent experiments.
Fig. 5. Lipid specificity profiling of StarD7 mutants deficient in PC and ceramide binding.

(A) Wild-type StarD7, PC-binding deficient mutant StarD7^{R189Q} and PC/ceramide-binding deficient mutant StarD7^{Y228R} were mixed with MBP, incubated with liposomes containing 1 mol% pacCer, pacDAG, pacPC, pacPE or pacSM, and processed as in Fig. 2B.

(B) Quantitative analysis of photoaffinity labelling of StarD7 by pacLipids as in (A). Labelling intensity of WT-StarD7 by pacCer was set at 100%. Data shown are the means ± S.D. of three independent experiments.
Fig. 6. PC transfer activity of StarD7 is negatively influenced by ceramide.

(A) Time course of PC transfer by StarD7 and StarD7^{R189Q} (top) and of ceramide transfer by CERT START and CERT START^{N504A} (bottom). Lipid transfer from donor liposomes containing 10 mol% PC or ceramide was measured as described in Materials and Methods. Data shown are the means ± error ranges of two or three independent experiments, as indicated.

(B) Quantitative analysis of the PC (top) and ceramide (bottom) transfer activity of CERT, StarD7 and StarD7^{R189Q} was performed as described in Materials and Methods. Data shown are the means ± error ranges of two independent experiments.

(C) Quantitative analysis of the PC transfer activity of StarD2, StarD7 and StarD7^{R189Q} using donor liposomes containing 5 mol% PC in the absence (-) or presence of 10 mol% ceramide (Cer) or diacylglycerol (DAG). The analysis was performed as described in Materials and Methods. Data shown are the means ± S.D. of at least three independent experiments. ***p < 0.001; paired t-test.
Fig. 7. StarD7 is dispensable for apoptosis induction in SMSr-depleted cells.
Wild-type (WT) and StarD7-KO#1 and StarD7-KO#2 HeLa cells were mock-treated or treated with siNS or siSMSr for 3 days. The cells were lysed and subjected to immunoblot analysis using antibodies against PARP, StarD7, SMSr and mitochondrial marker p60Mito. FL, full length; CL, cleaved.