Further characterization of mammalian ceramide kinase: substrate delivery and (stereo)specificity, tissue distribution, and subcellular localization studies

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Abstract **Recombinant human ceramide kinase (*Hs*CERK) was analyzed with regard to dependence on divalent cations and to substrate delivery, spectrum, specificity, and stereoselectivity. Depending on the chain length of the ceramide, either albumin for short-chain ceramide or a mixed micellar form (octylglucoside/cardiolipin) for long-chain ceramide was preferred for the substrate delivery, the former resulting in higher activities. Bacterially expressed *Hs*CERK was highly dependent on Mg^{2+} ions, much less on Ca^{2+} ions. A clear preference for the D-*erythro* isomer was seen. Various *N*-acylated amino alcohols were no substrate, but *N*-hexanoyl-1-*O*-hexadecyl-2-desoxy-2-amino-*sn*-glycerol and *N*-tetradecanoyl-2*S*-amino-1-butanol were phosphorylated, suggesting that the secondary hydroxy group is not required for recognition. The properties of *Hs*CERK, expressed in CHO cells, were similar to those of the bacterially expressed protein, including the Mg^{2+} dependence. In mouse, the highest activities were found in testis and cerebellum, and upon subcellular fractionation the activity was recovered mainly in the microsomal fraction. This fits with the plasma membrane localization in CHO cells, which was mediated by the N-terminal putative pleckstrin domain. No evidence for phosphorylation of ceramide by the recently described multiple lipid kinase was found. The latter kinase is localized in the mitochondria, but no firm conclusions with regard to its substrate could be drawn. —**Van Overloop, H., S. Gijsbers, and P. P. Van Veldhoven. **Further characterization of mammalian ceramide kinase: substrate delivery and (stereo)specificity, tissue distribution, and subcellular localization studies. *J. Lipid Res.* 2006.** 47: **268–283.**

Supplementary key words *N*-acyl serinol • sphingosine • lipid kinase • ceramide phosphate • pleckstrin • testis • anandamide

Sphingolipids, ubiquitous constituents of eukaryotic cells, are important signaling molecules with essential roles in cell proliferation, differentiation, and apoptosis (1). Ceramide, the initial product of the sphingomyelin cycle, functions as a second messenger in a variety of biological processes, such as cell differentiation and apoptosis (2). Ceramide can be hydrolyzed by ceramidase(s) to produce sphingenine. Ceramide and sphingenine can be phosphorylated to ceramide-1-phosphate (Cer1P) and sphingenine-1-phosphate (SeP), respectively. The phosphorylated lipids influence various biological processes and are recognized by specific phosphatases (3, 4). The responsible kinases, ceramide kinase (CERK) (5) and sphingosine kinase [type 1 (6–9) and type 2 (10)], belong to a family of kinases related to diacylglycerol kinases.

Cer1P was initially described as the product of a Ca^{2+}-stimulated kinase that copurified with brain synaptic vesicles (11). A regulatory function in the secretion of neurotransmitters, by promoting membrane fusion, was suggested. Phagocytosis of IgG-coated erythrocytes by human neutrophils was found to be associated with an increase of Cer1P (12). A mitogenic, as well as an anti-apoptotic, effect was also attributed to Cer1P (13, 14). By inhibition of acid sphingomyelinase, leading to an increase in ceramide level, Cer1P blocks both DNA fragmentation and caspase activation (15). Cer1P was also shown to mediate arachidonic acid release and prostanoid synthesis and to be a direct activator of cytosolic phospholipase A_2 (16). Recently, the stimulation of Ca^{2+} entry through voltage-operated calcium channels by Cer1P was demonstrated in rat pituitary cells (17).

Compared with the effects of Cer1P, less is known about CERK. Reports about the subcellular distribution of the kinase activity are contradictory (5, 11), and detailed in-

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Abbreviations: CERK, ceramide kinase; Cer1P, ceramide-1-phosphate; CL, cardiolipin; CMC, critical micellar concentration; DMS, *N,N*-dimethylsphingenine; EST, expressed sequence tag; EtOH, ethanol; GFP, green fluorescent protein; *Hs*CERK, human ceramide kinase; LDAO, lauryldimethylammonium *N*-oxide; MuLK, multisubstrate lipid kinase; C_2-ceramide, *N*-acetyl-sphingenine; C_6-ceramide; *N*-hexanoyl-sphingenine; OG, octyl-β-D-glucopyranoside; SeP, sphingenine-1-phosphate.

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formation about CERK substrate specificity and stereoselectivity has not been reported. Given the recent report about another mammalian kinase, multisubstrate lipid kinase (MuLK), acting on ceramide and diacylglycerol as the main substrate (18), additional information on CERK, and MuLK, will be beneficial for understanding ceramide signaling. Here, we report on some properties of these enzymes, whose cDNAs were assembled during the cloning of human sphingosine kinases (19).

MATERIALS AND METHODS

Synthesis of lipids

Sphinganine, sphingenine stereoisomers, and *N*-octyl-sphingenine were obtained from Avanti Polar Lipids, Sigma, or Toronto Research Chemicals. Other lipids, amino alcohols, and amino acids (analytical grade) were from Sigma, Aldrich, or Acros Organics, except for *N*,*N*-dimethylsphingenine (DMS; from Alexis), 1-*O*-hexadecyl-2-desoxy-2-amino-*sn*-glycerol, 1-*O*- and 3-*O*-octadecyl-*sn*-glycerol (from Bachem), anandamide (Larodan) and CAY1044 (from Cayman). FTY720 was a gift from Novartis Pharma (Basel, Switzerland).

N-[1-^{14}C]C_6-sphingenine was prepared as follows. Na-[1-^{14}C] hexanoate (4.5 μmol; specific radioactivity, 55.4 μCi/μmol; ARC), diluted with cold Na-hexanoate (6.5 μmol), was dried overnight with P_2O_5 under vacuum. After adding 240 μl of tetrahydrofuran containing 11 μmol of 2,6-dichlorobenzoic acid, the solution was stirred for 2 h at room temperature followed by the addition of 660 μl of 146 mM triethylamine in dichloromethane containing sphingenine (16.5 μmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (55 μmol). After a N_2 flush, the reaction mixture was stirred overnight at room temperature. Phase separation was induced by adding 4.84 ml of chloroform, 5.17 ml of methanol, and 4.95 ml of water, and the lower phase was washed with 5.5 ml of methanol and 10 mM NaOH (1:1, v/v). The lower phase was dried, and the ceramide produced was further purified by preparative TLC (silica G60; Merck) with chloroform-methanol-acetic acid (95:5:1, v/v) as solvent. The same method was used for the synthesis of *N*-[1-^{14}C]C_2-sphingenine using [1-^{14}C]acetate (sodium salt; specific radioactivity, 56.5 μCi/μmol; ARC).

Other amides were made by acylation of the aminoalkanols/diols with the appropriate acylchlorides, obtained by thionylchloride treatment of the fatty acid, adopted from Weis and Raizman (20). Briefly, the acylchloride (2 mmol) was dissolved in 1 ml of dimethylformamide, to which the aminoalkanol/diol (2 mmol), dissolved in 4 ml of dimethylformamide-pyridine (95:5, v/v), was added. After 2 h at room temperature, the mixture was quenched with 5 ml of 1 N HCl, and the amides were extracted twice into 5 ml of diethylether. To remove any formed *O*-acylation, the dried ether extract was dissolved in 2 ml of 40% methylamine-ethanol (EtOH; 1:1) and heated to 60°C for 1 h. Water (4 ml) and EtOH (4 ml) were added, and the amides were extracted into 2 × 5 ml of diethylether. After drying, the crude amides were dissolved in chloroform-diisopropylether (6:4, v/v), applied to silica-SepPAK (5 g; Waters), and eluted with chloroform-diisopropylether (6:4 or 9:1, v/v). Purity was analyzed by TLC in chloroform-methanol-acetic acid (90:10:1 and 60:40:1, v/v) and mass spectrometry analysis (Finnigan MAT TSQ 70; electrospray ionization; positive and negative mode). Structures were confirmed by daughter scan analysis.

N-Acylated amino acids were obtained by treating the amino acid (1 mmol, dissolved in 5 ml of 0.2 M $NaHCO_3$) with *N*-tetradecylhydroxysuccinimide (1 mmol, dissolved in 5 ml of tetrahydrofuran), adopted from Lapidot, Rappoport, and Wolman (21). After overnight reaction, 5 ml of 1 M HCl was added and amides were extracted into diethylether. The dried organic layer was dissolved in chloroform-methanol (2:1, v/v) and applied on a NH_2-BondElut cartridge (500 mg; Varian). After a wash with chloroform-methanol (2:1 and 1:9, v/v), amides were eluted with methanol-acetic acid (95:5). TLC analysis was done in chloroform-methanol-acetic acid (80:18:2, v/v).

Cloning and expression of lipid kinases

By BLAST homology searches (22), human and murine expressed sequence tags (ESTs) that code for proteins related to yeast sphingosine kinases (23) were identified and clustered into five different groups (19) (unpublished data). Related to the human ESTs, the inserts of IMAGE (24) clones 2392073 and 6185601 (UK-HGMP Resource Center, Hinxton, England), followed later by clone hk01650 (KIAA1646; Kazusa DNA Research Institute, Chiba, Japan), which all belonged to cluster 4 [lipid kinase 4 (LK4)], were sequenced (ALF DNA sequencer) with fluorescent plasmid primers and designed internal primers. Clone hk01650, despite its large insert (4,171 bp) (25), did not contain the putative start codon[3]; clone 2392073 missed both 5′ and 3′ information and is likely produced by erratic splicing, as it contains some intron sequence; clone 6185601 contained both the start and stop codons but also two deletions, 11 and 64 bp, the first resulting in a premature stop (at bases 449–451 of the open reading frame). The insert of IMAGE clone 649534, which belonged to cluster 2 [lipid kinase 2 (LK2)], was sequenced in a similar way and contained the full open reading frame (1,269 bases). The mouse counterpart was derived from IMAGE clone 2631716.

Expression vectors were made as described in **Table 1**, according to standard protocols (26). For PCR, *Pfx* polymerase (GibcoBRL) was used, whereas Top10F′ *Escherichia coli* cells were used as host for recombinant work and for protein expression. For the expression of pETM-30-encoded proteins, *E. coli* BL21(DE3) was used.

Bacterially expressed *Hs*LK4/CERK was obtained from Top10F′ *E. coli* cells transformed with plasmid pPVV072, grown at 37°C in growth medium (0.5% m/v yeast extract, 1.0% m/v tryptone in water, pH 7) medium supplemented with tetracycline (15 μg/ml) and ampicillin (50 μg/ml), and induced at an optical density of 0.6 with 0.2% (w/v) arabinose. The bacteria were harvested 4 h after induction (6,200 *g* for 10 min), resuspended in PBS containing a mix of protease inhibitors (25 ml/100 ml culture), and sonicated on ice (Branson Sonifier B115, microtip). Aliquots of 1 ml were frozen in liquid nitrogen, stored at −80°C, and diluted 50-fold in PBS containing protease inhibitors before measuring. The specific activity in these preparations, using *N*-[^{14}C]C_6-D-*erythro*-sphingenine bound to BSA, ranged from 17.7 to 29.2 nmol/min/mg protein (three different lysate batches). In later experiments, the lysate was centrifuged at 10,000 *g* for 10 min, the supernatant was discarded, and the pellet was resuspended in PBS plus protease inhibitors (25 ml/100 ml culture), resulting in 2- to 3-fold higher specific activities (68 nmol/min/mg protein; membrane batch).

For eukaryotic expression of human ceramide kinase (*Hs*CERK), CHO cells were cultured on 100 mm plates in α-Minimal Essential Medium (GibcoBRL) supplemented with 10%

[3] Because of this fact, and by using another reading frame, this clone was predicted to code for KIAA1646 protein (AB051433).

TABLE 1. Plasmids and their construction used in this work

Code	Description	Generation of Insert	Primers	Acceptor	Company/ Reference
pPVV069	His_6-*Hs*CERK$_{78–537}$	*1*) PCR on clone hk01650	5′-GCCCTCGAGATGGAAAAGCCTTACGCCTTTTAC	*Xho*I-*Hind*III	Invitrogen
		2) *Xho*I-*Hind*III	5′-CTAAAGCTTCAGTTTGTGAGCAGGACGCC	pBAD-HisA	
pPVV070	GST-*Hs*CERK$_{78–537}$	*1*) PCR on hk01650	5′-CGGGAATTCATGGAAAAGCCTTACGCTTTTAC	*Eco*RI-*Xho*I	Pharmacia
		2) *Eco*RI-*Xho*I	5′-CTTCTCGAGCAGTTTGTGAGCAGGACGCC	pGEX4T-1	
pPVV071	His_6-*Hs*CERK$_{1–138}$[a]	*1*) PCR on clone 6185601	5′-CAATACTCGAGATGGGGGCGACGGGGGCG	*Xho*I-*Hind*III pBAD-HisA	
		2) *Xho*I-*Hind*III	5′-CTAAAGCTTCAGTTTGTGAGCAGGACGCC		
pPVV072	His_6-*Hs*CERK$_{1–537}$	*1*) Remove *Xho*I-*Pst*I 120 bp fragment		pPVV069	
		2) Insert 345 bp *Xho*I-*Pst*I fragment from pPVV071			
pHVO001	*N*-FLAG-*Hs*CERK$_{1–537}$	*Bam*HI-*Hind*III fragment of pPVV072		*Bam*HI-*Hind*III pCMV-Tag 2B	Stratagene
pHVO002	GFP-*Hs*CERK$_{1–537}$	*Bam*HI-*Hind*III fragment of pPVV072		*Bgl*II-*Hind*III pEGFP-C1	Clontech
pHVO006	His_6-*Hs*CERK$_{219–537}$	*Sal*I-*Hind*III fragment of pPVV072		*Xho*I-*Hind*III pBadHisA	
pHVO008	His_6-GST-*Hs*CERK$_{269–537}$	*Nco*I-*Hind*III fragment of pPVV072		*Nco*I-*Hind*III pETM-30	EMBL Heidelberg (G. Stier)
pHVO010	*N*-FLAG-*Hs*CERK$_{116–537}$	*Pst*I-*Hind*III fragment of pPVV072		*Pst*I-*Hind*III pCMV-Tag 2B	
pSG004	His_6-*Hs*LK2$_{1–422}$	*1*) PCR on clone 649534	5′-CCGGATCCTCGAGAATCTCTCGAAGATG	*Xho*I-*Hind*III	Invitrogen
		2) *Xho*I-*Hind*III fragment	5′-CCCAAGCTTCCCGGGGTCTTCTGCTGCTCA	PBADHisA	
pSG005	*Hs*LK2$_{1–422}$-Myc-His	*1*) PCR on clone 649534	5′-CCGGATCCGCCGTGCAAATCTCTCGAAGAT	*Bam*HI-*Xho*I	Invitrogen
		2) *Bam*HI-*Xho*I fragment	5′-AATCTCGAGCTGGGTGGGGCTTGTGAG	pcDNA3.1/ Myc-His	

*Hs*CERK, human ceramide kinase.
[a] Because of a deletion, a shift in reading frame starting at amino acid 138.

(v/v) fetal bovine serum (GibcoBRL), L-glutamine (Invitrogen), and antibiotics/antimycotics (Invitrogen). The CHO cells were transfected using the Lipofectamine Plus Reagent (Invitrogen) or the polyethylenimine procedure (27). The cells were harvested 24 h after transfection, dissolved in 0.5 ml of PBS containing a mix of protease inhibitors, sonicated on ice (3 × 10 s), frozen in liquid nitrogen, and stored at −80°C. The kinase activity, corrected for activity in cells transfected with the appropriate empty vector, with *N*-[^{14}C]C_6-D-*erythro*-sphingenine bound to BSA, varied between 5.4 (Lipofectamine transfection) and 0.92 (polyethylenimine transfection) nmol/min/mg protein, being severalfold higher than the endogenous activity (60 ± 20 pmol/min/mg protein).

The other lipid kinase, *Hs*LK2/MuLK, was expressed in bacteria transformed with pSG004 and in CHO cells transfected with pSG005 (or empty vector). For the latter, stable transfectants were selected by growing the cells in the presence of 400 μg/ml G418.

Antisera

His_6-GST-*Hs*CERK$_{269–537}$ fusion protein was expressed in *E. coli* BL21 cells, transformed with pHVO008, and induced with 3 mM isopropylthio-β-galactoside for 4 h at 37°C (1 liter of culture medium). The harvested bacteria (Kontron; 6,200 *g* for 10 min) were resuspended in lysis buffer (6 M guanidinium chloride, 100 mM NaH_2PO_4, 10 mM Tris, and10 mM imidazole, pH 8.0) and gently stirred for 1 h at 4°C. After removal of debris (Kontron; 7,750 *g* for 15 min), nickel-nitrilotriacetic acid agarose resin (Qiagen) was added to the supernatant, and the mixture was gently stirred for 30 min at 4°C. Beads were collected and washed with 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, and 10 mM imidazole, pH 7.0. Bound fusion proteins were eluted with elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, and 250 mM imidazole, pH 7.0). Eluted proteins were mixed with Freund's complete adjuvant (or incomplete for boosters) and injected subcutaneously into rabbits. Ten days after the fifth booster, the rabbit was bled. For affinity purification, the serum was incubated with blot strips containing a His_6-tagged truncated *Hs*CERK, obtained from Top10F′ *E. coli* cells transformed with pHVO006 and induced with arabinose 0.2% (w/v), and purified via nickel-nitrilotriacetic acid agarose Sepharose and SDS-PAGE/blotting. Specific antibodies were eluted from the strips with Gentle Elute Buffer (Pierce).

Anti-*Hs*LK2/MuLK was obtained by immunizing rabbits with the bacterially expressed and purified His_6-tagged protein. The generation of anti-human Pex14p (28), anti-green fluorescent protein (GFP) (29), and anti-myc (30) has been described.

Tissue distribution and subcellular studies

Tissues obtained from mouse (male C57/Bl6 N) or rat (male Wistar) were homogenized in 0.25 M sucrose, 5 mM Mops/NaOH, pH 7.2, and 0.1% (v/v) EtOH (HM) using Dounce (Potter-Elvehjem) homogenizers or a Polytron device (Kinematica). Subcellular fractions enriched in nuclei, mitochondria, lysosomes and peroxisomes, microsomal vesicles, and cytosol were prepared from testis or brain tissue homogenates, made with Potter-Elvehjem homogenizers, as described previously for rat liver (31). For the fractionation of brain tissue (cerebellum and medulla oblongata), myelin was removed (32) by resuspending the lysosome and peroxisome fraction (derived from 5 g starting wet weight) in 2 ml of 0.85 M sucrose, 5 mM Mops/NaOH, pH 7.2, and 0.1% (v/v) EtOH, followed by overlaying with 2 ml of homogenization medium. After centrifugation at 108,000 *g* (Beckman SW55) during 45 min, the supernatant, containing interfering myelin, was discarded. Marker enzyme and protein measurements were done as described previously for glutamate dehydrogenase (33), catalase (31), acid phosphatase (33), glucose-6-phosphatase (33), carboxylesterase (31), lactate dehydrogenase (33), proteins (31), and CERK.

For subcellular localization studies by immunofluorescence microscopy, CHO cells were grown on cover slips on 12-well plates and transfected with selected vectors. Monolayers were fixed at 24 h after transfection with 4% (w/v) paraformaldehyde, permeabilized with 1% Triton X-100 in PBS, blocked with 5% (w/v) BSA in PBS, and processed for immunostaining as described previously (34). Antibodies used were diluted in 1% (w/v) BSA in PBS. Primary antibodies were anti-FLAG M2 (Stratagene), anti-KDEL (Affinity BioReagents), anti-biotin (Rockland), anti-GFP, anti-*Hs*CERK, anti-myc, and anti-Pex14p; secondary antibodies were anti-rabbit IgG Cy3, anti-mouse IgG FITC, and anti-rabbit AP (Sigma). To visualize mitochondria, cells were exposed to 2 μM Red Mitotracker (Red.CM-H_2Xros; Molecular Probes) for 45 min before fixation. Fluorescence was observed with a Leica DMR microscope equipped with FITC/RSGFP/Bodipy/Fluo3/DIO and Texas Red filters.

Kinase assays

To measure CERK, different assays, based on the recovery of labeled Cer1P in the organic phase when the assay mixture was subjected to a lipid extraction under acid conditions, were used. The substrate was presented bound to albumin (EtOH/BSA assay), mixed with CHAPS (detergent assay), or inserted in a mixed detergent/phospholipid micelle [octyl-β-D-glucopyranoside/cardiolipin (OG/CL) assay].

For the standard EtOH/BSA assay, a 50 μl aliquot of bacterial or CHO lysates, or homogenate or subcellular fraction (appropriately diluted in HM), was mixed with 150 μl of reaction mixture. To prepare the reaction mixture, 20 nmol of substrate was dried at the bottom of a screw-capped glass tube, dissolved in EtOH (1% of final assay volume), and mixed with 4 volumes of BSA (resulting in a molar ceramide/BSA ratio of 2.5). Final concentrations were 100 μM substrate, 40 μM BSA, 5 mM ATP, 50 mM Mops/NaOH, pH 7.2, 3 mM $MgCl_2$, 40 mM NaF, 1 mM DTT, and 100 μM orthovanadate. Either labeled substrate {*N*-[1-^{14}C]C_6-sphingenine (~50,000 dpm/nmol), *N*-[1-^{14}C]C_2-sphingenine (~50,000 dpm/nmol), *N*-[1-^{14}C]C_{16}-sphingenine (~40,000 dpm/nmol)} or [γ-^{32}P]ATP (~7,000 dpm/nmol) was used. Purity and specific activity of the ATP were determined by ultraviolet light absorbance and TLC on polyethyleneimine-cellulose plates (35). For the other assays, the dried ceramide was solubilized in 40 μl of 2% CHAPS (detergent assay) or 40 μl of 3.75% OG/2.5 mM CL (OG/CL assay), followed by sonication in a water bath and addition of the other solutions. For inhibition studies, the lipid substrate concentration was decreased to 25 μM and the inhibitor was tested at 100 μM, and both were dissolved together.

After an incubation of 15 min at 37°C, reactions were stopped by the addition of 1.5 ml of chloroform-methanol (1:2, v/v) and phase-separated by adding 1 ml of chloroform and 0.7 ml of a 0.5 N HCl/2 M NaCl solution. When using labeled substrate, a 1 ml aliquot of the lower phase was transferred to a glass tube and dried with nitrogen. The residue was dissolved in 60 μl of chloroform-methanol (4:1, v/v), immediately followed by spotting 20 μl on a Silica 60G glass plate with a concentration area (Merck). When using [γ-^{32}P]ATP, 250 μl of the lower phase was semiautomatically spotted. Plates were developed in chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1, v/v). Spots of the phosphorylated products, visualized by autoradiography (Amersham Biosciences MP film), were scraped from the plates into 0.5 ml of 1% SDS and counted by liquid scintillation chromatography.

All measurements were corrected for the presence of endogenous kinase substrates, possessing after phosphorylation similar relative mobility values as (truncated) Cer1P (assays done plus/minus substrate) and/or endogenous kinase (transfections/transformations with empty vector), unless they proved to be negligible under the particular assay condition.

The phosphorylation of other lipids was determined in a similar manner, using one of the delivery modes described above. When sphingoid bases/amino alcohols were tested, alkaline phase separations were used as described previously for sphingosine kinase measurements (19, 36).

The activity of the putative lipid kinase was analyzed by similar procedures in the presence of $MgCl_2$ or $CaCl_2$. In addition, substrate was also delivered to the enzyme dissolved in DMSO (final concentration, 2%).

Analysis of cellular lipids

CHO cells were grown on six-well plates to 50% confluence in the medium described above and transfected with plasmid pHVO001 or the appropriate empty vector pCMV-Tag 2B using the Lipofectamine Plus Reagent (Invitrogen). The medium was replaced by phosphate-free Dulbecco's modified Eagle's medium (ICN), supplemented with 0.2% (v/v) Ultroser (GibcoBRL), Glutamax (GibcoBRL), and antibiotics/antimycotics (Invitrogen) 20 h after transfection. After 6 h, cells were given 2 ml of fresh phosphate-free medium containing 20 μCi of ^{32}P and C_6- or C_2-ceramides, added as EtOH/4% BSA (1:4, v/v) complexes (final concentration, 5 μM), or N-C_{16}-ceramide, dissolved in dodecane-methanol (37). After 15 h of incubation, medium was removed and monolayers were washed twice with PBS, scraped in 2 × 1 ml of methanol (38), and transferred to extraction tubes. Phase separation was introduced by the addition of 2 ml of chloroform and 1.8 ml of a 0.5 N HCl/2 M NaCl solution to the combined methanol solutions. An aliquot (250 μl) of the chloroform layer was spotted onto a silica plate and developed in chloroform-methanol-25% NH_4OH (60:35:8, v/v) (first dimension), allowed to dry, turned 90° and run in acetone, and subsequently in the same direction developed in chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1, v/v). Radioactive spots were revealed by scanning (P-Imager; Molecular Dynamics) or by autoradiography.

RESULTS AND DISCUSSION

By homology with the yeast sphingosine kinases, up to five different sequences coding for related proteins were

found in the human and mouse EST databases by different groups. Two cDNAs have been shown to encode sphingosine kinases: our cluster 1 (19) corresponded to type 1 sphingosine kinase (7–9), and our cluster 3 corresponded to type 2 (10). The proteins encoded by two other clustered ESTs were tentatively called lipid kinases (cluster 4, accession number AJ457828; cluster 2, accession numbers AJ278150 and AJ401619). The first one, as shown by Sugiura

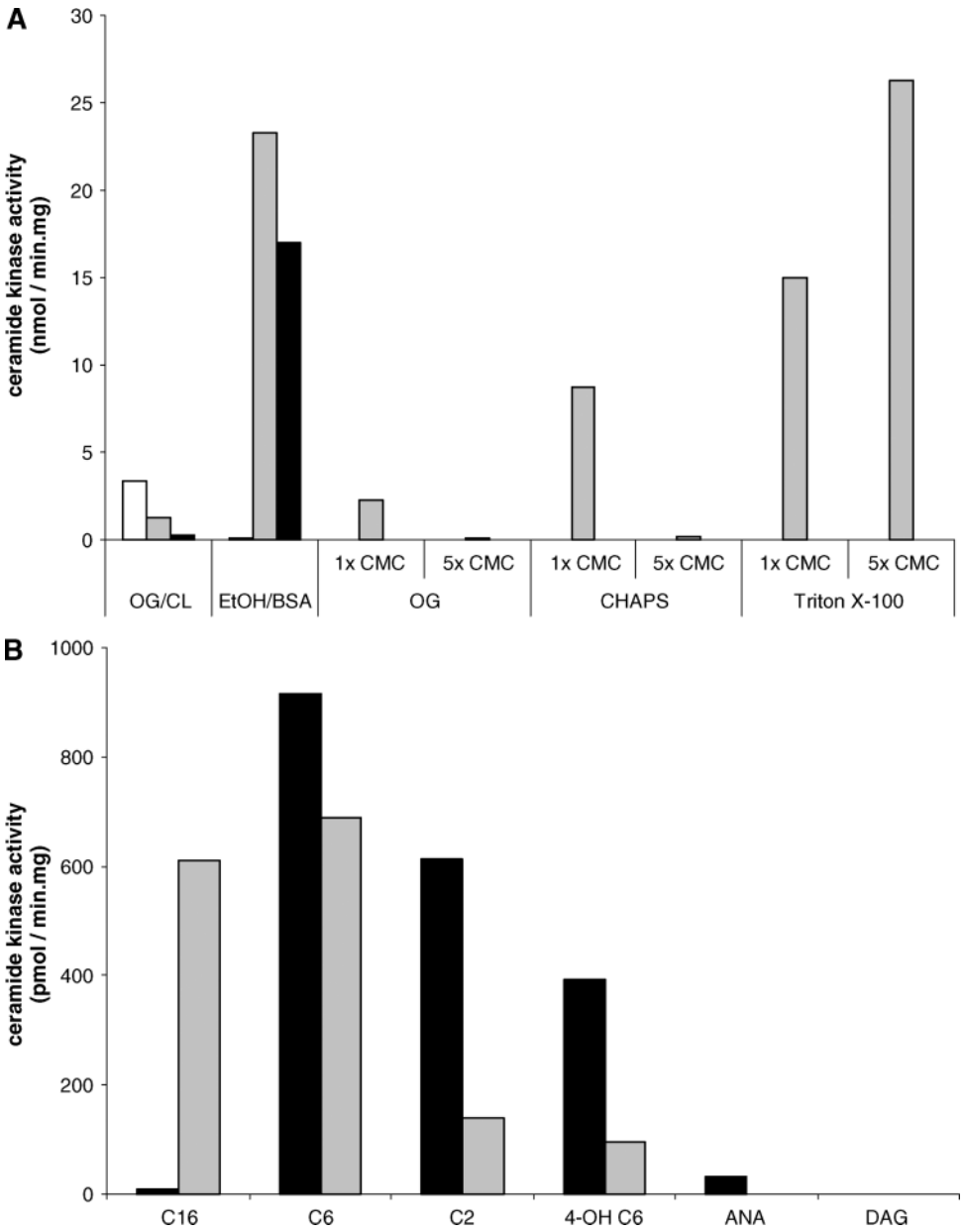

Fig. 1. Effect of substrate delivery on ceramide kinase (CERK) activity. A: Phosphorylation of ceramide (100 μM) was determined in lysates from *E. coli* Top10F′ cells expressing human ceramide kinase (*Hs*CERK; pPVV072) using different solubilization methods and [γ-^{32}P]ATP. The dried C_6-ceramide (gray bars) was dissolved either in ethanol (EtOH) followed by BSA (EtOH/BSA), or with mixed octyl-β-D-glucopyranoside/cardiolipin micelles (OG/CL), or with OG, CHAPS, or Triton X-100, all tested at one or five times their critical micellar concentration (CMC). With lauryldimethylammonium *N*-oxide and *N*-laurylsarcosine, both at 1× and 5× CMC, or with EtOH/BSA combined with detergent, phosphorylation of C_6-ceramide was <1% of the highest activity (data not shown). Activities are expressed as nanomoles of phosphorylated product per milligram of lysate protein per minute. For C_{16}-ceramide (white bar) and C_2-ceramide (black bars), only OG/CL and EtOH/BSA were tested. B: Phosphorylation of ceramide (100 μM) was measured in lysates from CHO cells transfected with *Hs*CERK (pHVO002) in the presence of [γ-^{32}P]ATP, using the EtOH/BSA (black bars) or OG/CL (gray bars) assay as described in Materials and Methods. Activities are expressed as picomoles of phosphorylated product per milligram of lysate protein per minute. ANA, anandamide; Cx, *N*-Cx-sphingenine; DAG, 1,2-*sn*-dioleoylglycerol; 4-OH C6, *N*-hexanoyl-4-OH-sphinganine.

et al. (5), appeared to be CERK, a protein of ~60 kDa (537 amino acids in human; 531 in mouse); the other one was recently claimed to be a MuLK (18), a protein of ~47 kDa (422 amino acids in human; 421 in mouse). Both contain the conserved domains initially described for sphingosine kinases (6), and their relationship to sphingosine kinases has been discussed in the cited references. In the meantime, Tuson, Marfany, and Gonzalez-Duarte (39) found evidence for still another kinase when characterizing the RP26 locus on chromosome 2q31.2-q32.3, one of the loci mutated in autosomal recessive retinitis pigmentosa. Based on the similarity of the predicted gene product, the gene was named ceramide kinase-like gene (CERKL).

To understand some discrepancies between our preliminary data, using bacterially expressed *Hs*CERK, and the data reported by Sugiura et al. (5), based on CERK expressed in HEK293 cells, we further investigated in depth the enzyme characteristics. Unless indicated otherwise, the data reported below were all obtained with fusion proteins containing the full-length protein. In addition, substantial efforts were made to define the substrate of the other kinase (LK2), which we will refer to as MuLK to minimize confusion.

Effect of chain length and substrate delivery on CERK activity

In a first approach, the length of the *N*-acyl chain, using albumin as a carrier for the ceramides, was varied. With His-tagged bacterially expressed CERK and in the presence of Mg^{2+} ions (see below), the highest activity was observed for *N*-hexanoyl-sphingenine (N-C_6-Se), somewhat less for *N*-acetyl-sphingenine (N-C_2-Se) (**Fig. 1A**). The natural substrate, C_{16}-ceramide, was poorly phosphorylated (Fig. 1A). With N-C_6-Se as substrate, almost normal Michaelis-Menten kinetics were observed for recombinant *Hs*CERK (**Fig. 2**). A K_m value of 30 μM and a V_{max} of 35 nmol/min/mg were calculated. For N-C_2-Se, an apparent K_m of 22 μM was obtained, but substrate inhibition was seen at >100 μM. The same chain length dependence was seen when using CHO-expressed kinase, in contrast to Sugiura et al. (5), who reported low activity with N-C_2-Se. Because the data reported by these authors were obtained in a micellar assay, the effect of substrate dissolution and delivery on CERK activity was examined more closely. EtOH/BSA, detergents such as Triton X-100 and CHAPS, and, to a lesser extent, OG and mixed micelles (OG/CL) turned out to be useful for the dissolution/delivery of C_6-ceramide to the bacterially expressed kinase (Fig. 1A). High concentrations of OG and CHAPS [five times the critical micellar concentration (CMC)], however, almost completely inhibited CERK activity. Low concentrations of lauryldimethylammonium *N*-oxide (LDAO) and *N*-laurylsarcosine were also inhibitory. This inhibitory effect was not attributable to an ineffective dissolution of the ceramide (data not shown) (40); moreover, inhibition was also obvious when LDAO (1× CMC), OG (5× CMC), or CHAPS (5× CMC) was added to assays containing BSA-bound ceramide as substrate. With both EtOH/BSA and OG/CL as carriers, C_2-ceramide was less well phosphorylated than C_6-ceramide. Interestingly, C_{16}-ceramide was a better substrate than C_6-ceramide when OG/CL instead of EtOH/BSA was used for its delivery, and

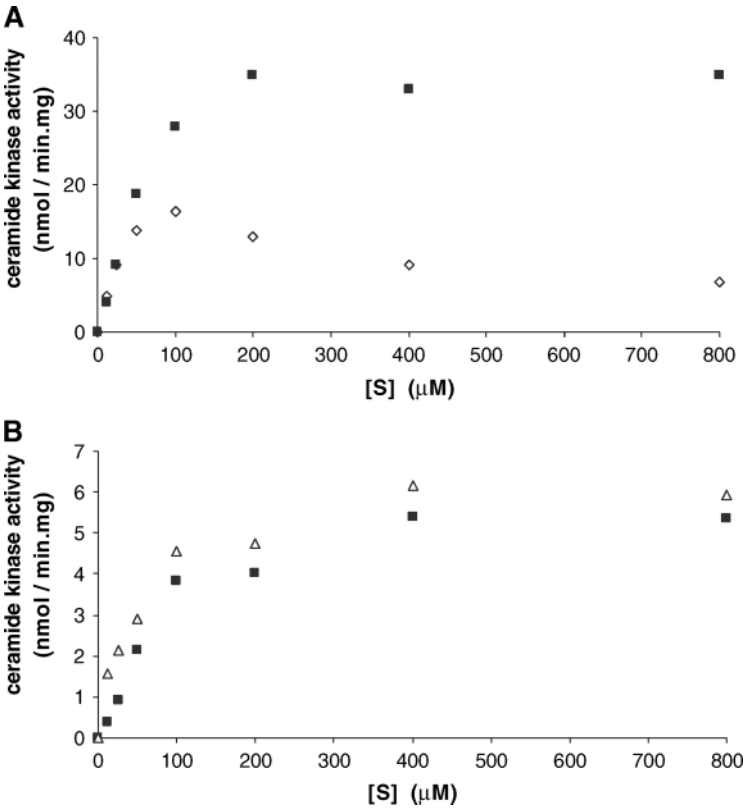

Fig. 2. Enzyme kinetics of recombinant *Hs*CERK. The activity of recombinant $(His)_6$-tagged *Hs*CERK, expressed in Top10F′ *E. coli*, was measured at increasing concentrations of C_2-ceramide (open diamonds), C_6-ceramide (closed squares), and C_{16}-ceramide (open triangles) in the presence of [γ-^{32}P]ATP and solubilized in EtOH/BSA (A) or OG/CL (B). Activities are expressed as nanomoles of phosphorylated product per milligram of protein per minute. Because of borderline activities, data for C_{16}- and C_2-ceramide are not shown in A and B, respectively.

the activities toward C_{16}-ceramide or ceramide containing a 2-hydroxy fatty acid (ceramide IV) were comparable (data not shown).

When these assay conditions were tested with eukaryotically expressed *Hs*CERK, similar results were obtained (Fig. 1B). In the presence of OG/CL, C_{16}- and C_6-ceramide were almost equally well phosphorylated (Fig. 2), and to a much larger extent than N-C_2-Se, in agreement with the findings by Sugiura et al. (5).

Because the activities are substantially higher with truncated ceramides complexed to BSA than in the mixed micellar assay, the former assay was mainly used to study the CERK properties.

Role of divalent ions for CERK activity

In older reports concerning CERK measurements in tissue/cells, a stimulation by Ca^{2+} was reported (11, 41). Also, Sugiura et al. (5) reported a Ca^{2+} dependence for the recombinant CERK, reaching a maximum at ~0.5 mM free Ca^{2+}. In our preliminary trials, performed in the absence of Ca^{2+} but in the presence of Mg^{2+} (see above), high kinase activity was found. Therefore, the influence of Ca^{2+} and Mg^{2+} on *Hs*CERK activity was determined with C_6-ceramide as substrate. In the absence of Ca^{2+}, the activity of CERK, bacterially as well as eukaryotically expressed, was increased by Mg^{2+} in a concentration-dependent manner (**Fig. 3A**). In the absence of Mg^{2+}, CERK was stimulated by Ca^{2+}, although to a lesser extent than by Mg^{2+}, and inhibited at >1 mM Ca^{2+} (0.04 mM free Ca^{2+}) (Fig. 3B). The highest *Hs*CERK activity was measured in the presence of Mg^{2+} and without Ca^{2+} for both forms of expressed kinase (Fig. 3C). Sugiura et al. (5) also described a stimulation by Mg^{2+}, which was less than by Ca^{2+}. The reason for this discrepancy with our data is not clear. Because the Mg^{2+} dependence is seen for both the EtOH/BSA and

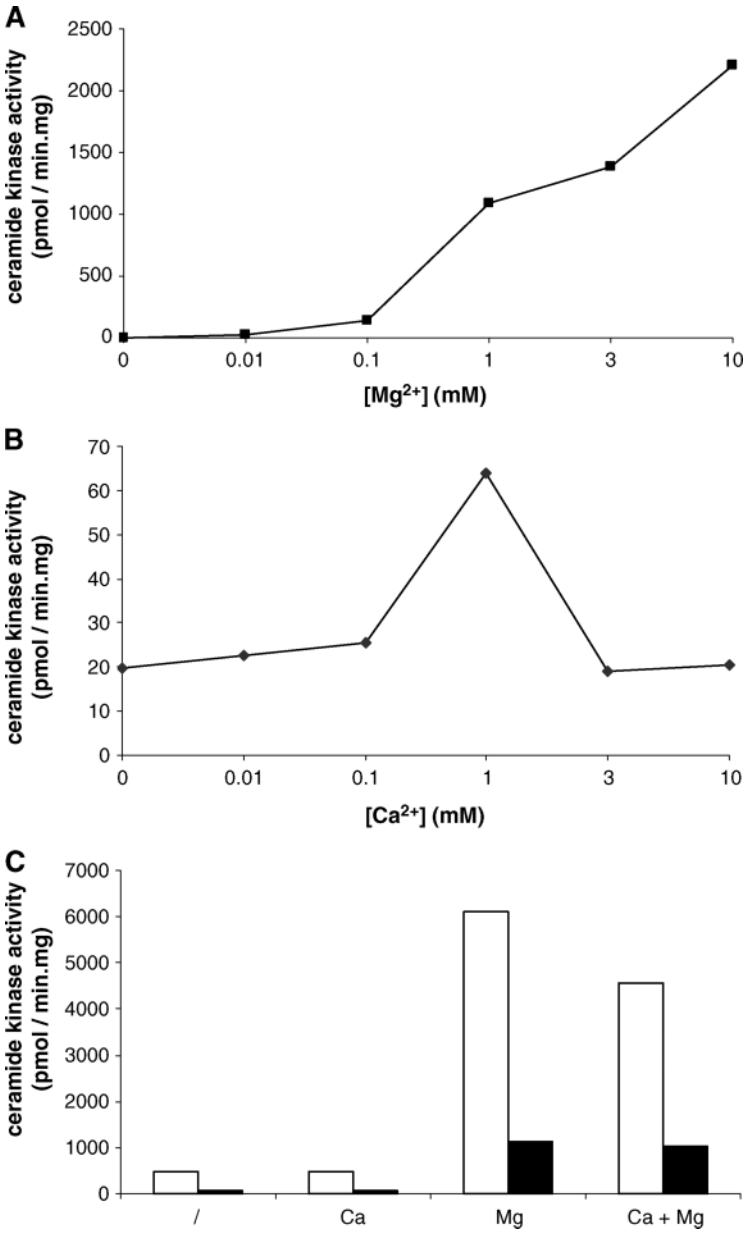

Fig. 3. Effect of Mg^{2+} and Ca^{2+} on CERK activity. CERK activity was measured with 100 μM [^{14}C]C_6-ceramide as substrate and solubilized with OG/CL under standard conditions except for the presence of 0.2 mM EDTA. Activities are expressed as picomoles of phosphorylated C_6-ceramide formed per milligram of lysate protein per minute. A, B: CERK activity in lysate from CHO cells transfected with *Hs*CERK (pHVO002) was measured in the presence of increasing Mg^{2+} concentrations in the absence of Ca^{2+} (A) or in the presence of increasing Ca^{2+} concentrations in the absence of Mg^{2+} (B). Profiles similar to these, but at higher specific activities, were observed when using lysate from *E. coli* Top10F′ cells expressing *Hs*CERK (pPVV072) (data not shown). C: CERK activity in the absence or presence of Mg^{2+} (3 mM) and/or Ca^{2+} (1 mM) in a membrane fraction prepared from *E. coli* Top10F′ cells expressing *Hs*CERK (pPVV072) (open bars) or lysate from CHO cells transfected with *Hs*CERK (pHVO002) (closed bars).

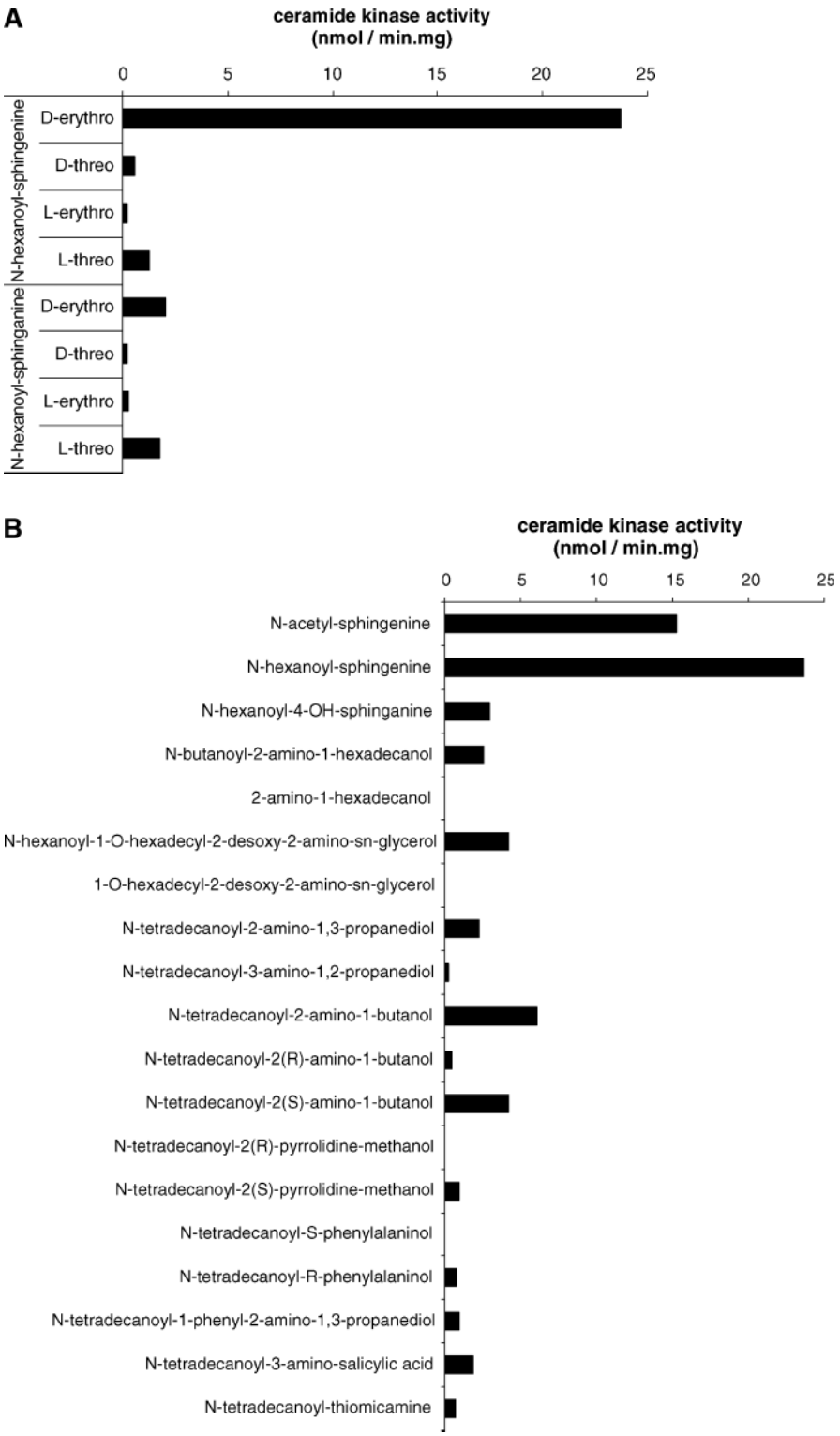

Fig. 4. Substrate specificity of CERK. Phosphorylation of the different ceramide stereoisomers (A) and ceramide analogs (B), each at 100 μM, was determined in lysate from *E. coli* Top10F′ cells expressing *Hs*CERK (pPVV072) in the presence of [γ-^{32}P]ATP using the EtOH/BSA procedure. Activities are expressed as nanomoles of phosphorylated product per milligram of protein per minute. Unless specified otherwise, amino alcohols and amides are racemic, whereas sphingoid bases and derivatives possess the D-*erythro* configuration. *N*-tetradecanoyl-3-amino-1-propanol, *N*-tetradecanoyl-4-amino-1-butanol, *N*-tetradecanoyl-3-hydroxypyrrolidin, *N*-tetradecanoyl-*R*-serine, *N*-tetradecanoyl-*S*-serine, *N*-tetradecanoyl-2-benzylserine, *N*-tetradecanoyl-3-phenylserine, *N*-tetradecanoyl-3-amino-tyrosine, *N*-tetradecanoyl-1-amino-2-naphthol-4-sulfonic acid, *N*-tetradecanoyl-3-hydroxy-anthranilic acid, *N*-tetradecanoyl-4-aminophenol, *N*-tetradecanoyl-diethanolamine, and *N*-hexadecanoyl-ethanolamine were not phosphorylated (data not shown).

the OG/CL assay, it is not attributable to the presence of acidic phospholipids, known to bind Mg^{2+} as well as Ca^{2+} ions. It is also not caused by differences in the ATP concentrations used. At all ATP concentrations tested (0.5–20 mM), Mg^{2+} resulted in 10- to 20-fold higher activities than Ca^{2+} (data not shown). In the presence of 3 mM Mg^{2+}, a K_m of 400 μM was obtained for ATP, substantially higher than the reported value of 32 μM using natural ceramides (5). Also, the pH profiles were analyzed with 3 mM Mg^{2+} with or without Ca^{2+} (1 or 3 mM). A broad pH optimum between 6.2 and 7.8 was seen, and the activity of the (bacterially expressed) kinase was always higher in the presence of Mg^{2+}, using either the EtOH/BSA or the OG/CL assay (data not shown). As a consequence, the regulation of CERK activity by calcium levels might occur to a lesser extent than proposed (42).

Substrate stereoselectivity and specificity of CERK

To test the stereoselectivity of *Hs*CERK, the different stereoisomers of both C_6-ceramide and C_6-dihydroceramide were initially compared. For the unsaturated compounds, the D-*erythro* form was clearly preferred. The absence of the double bond in the D-*erythro* isomers decreased the activity almost 10-fold, but for the saturated substrates, both D-*erythro* and L-*threo* isomers were phosphorylated equally well (**Fig. 4A**). Also with the unsaturated substrates, the L-*threo* isomer was recognized (see below). The presence of a hydroxy group at position 4 of the base (*N*-C_6-4-hydroxy-sphinganine) did not affect the activity (compared with *N*-C_6-sphinganine) (Figs. 1B, 4B). In agreement with others (5), neither diacylglycerol nor sphingoid bases (or related bases) were phosphorylated by *Hs*CERK. The presence of an amide bond seems essential, because *N*-octyl-sphingenine, also known as ceramine (43), was not a substrate (Fig. 4B). As expected, *N*-C_6-psychosine was not a substrate.

In subsequent experiments, the influence of a selected collection of ceramide/sphingenine analogs was tested, first for possible inhibitory action, then as substrate. None of the synthetic ceramide analogs showed a great degree of inhibition. The most potent inhibitors found were sphinganine, sphingenine, and DMS (data not shown), in agreement with the data reported by Sugiura et al. (5). For sphingenine, an IC_{50} value of 40 μM was obtained in the EtOH/BSA assay (data not shown). In this respect, the severe inhibitory effect of LDAO, bearing some structural similarity to DMS, should be recalled. It also shows that one should be careful when interpreting the cellular effects of DMS, which blocks not only sphingosine kinase (44, 45), as assumed, but also CERK. Of particular interest were ceramide analogs containing a carboxy group (*N*-acylated amino acids), which could be inhibitory given their negative charge similar to the physiological reaction product. None of these, however, was inhibitory.

Only a few of the tested analogs were substrates for CERK (Fig. 4B). If the sphingoid base was shortened to the first three C-atoms (*N*-C_{14}-2-amino-1,3-propanediol), the analog was still a substrate. According to Bieberich, Kawagushi, and Yu (46), the *N*-C_{16} analog of this compound (called *N*-acylated serinol) acts as a ceramide mimic. When added to cultured NG108-15 or F-11 cells, it causes ceramide increase and apoptosis. *N*-Acyl serinol is a moderate inhibitor of acidic ceramidase and can compete with ceramide for glucosyl transfer (46), but competition with the kinase, as shown here, could also contribute to the changes in ceramide. When reversing the amino and secondary hydroxy group (*N*-C_{14}-3-amino-1,2-propanediol), the amide is poorly phosphorylated. Interestingly, the hydroxy group at carbon 3 of the base is not necessary for recognition by CERK, because *N*-C_4-2-amino-1-hexadecanol, *N*-C_{14}-2-amino-1-butanol, and *N*-C_6-1-*O*-hexadecyl-2-desoxy-2-amino-*sn*-glycerol are good substrates. Indirectly, these data support the notion that the primary hydroxy group of ceramide is phosphorylated by the kinase. To document the importance of the amino group configuration, both *R*- and *S*-isomers of *N*-C_{14}-2-amino-1-butanol were synthesized and tested: the 2*S*(D) isomer is clearly a better substrate than the 2*R*(L) isomer (Fig. 4B). Mixing experiments suggest that the 2*R* compound, although a poor substrate, can have a stimulatory effect (data not shown). These data agree with the selectivity seen toward the D-*erythro* isomer of C_6-ceramide and explain the recognition of the L-*threo* isomers, both corresponding to the configuration of the *S* isomer at carbon 2. The recognition of *N*-C_6-1-*O*-hexadecyl-2-desoxy-2-amino-*sn*-glycerol is interesting (Fig. 4B). Previously, it was shown that the etherlipid analog, 1-*O*-hexadecyl-2-desoxy-2-amino-*sn*-glycerol, was efficiently used by human sphingosine kinase type 1 (19). Hence, it is striking that both this base and sphingosine fit into the catalytic site of sphingosine kinase, and after *N*-acylation, into that of CERK, despite some structural differences, underlining the evolutionary relationship between both kinases. On the other hand, the *N*-acylated form of the immunosuppressive drug FTY720, another sphingosine analog that can be phosphorylated by sphingosine kinase types 1 and 2 (47–49), was not recognized by CERK.

Finally, we were interested to learn whether anandamide (*N*-arachidonoyl-ethanolamine) was phosphorylated or not. Anandamide, an endocannabinoid, can be considered as structurally related to ceramide, but unlike the other analogs used here, it is naturally occurring (50). It was a substrate, albeit a very poor one, of CERK (Fig. 1B).

The selectivity for the natural D-*erythro* substrates and its substrate spectrum, as analyzed here in detail, accentuates the physiological role of this kinase, classifying it as a true CERK. It should be emphasized that short-chain ceramides such as N-C_2-Se, so often used as a water-soluble ceramide analog in cellular studies, and ceramide mimics such as *N*-acyl serinol can be efficiently phosphorylated by CERK. According to Sugiura et al. (5), *N*-C_2-sphingenine is a poor substrate; however, as shown here, the affinity is very much dependent on the way the substrate is delivered to the enzyme (see below).

Tissue distribution of *Hs*CERK

Having established optimum assay conditions for CERK using recombinant proteins, its presence was measured in

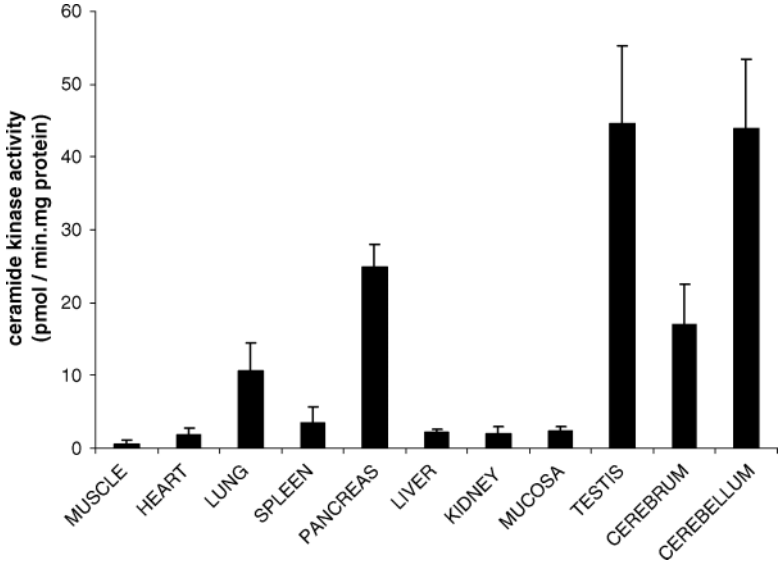

Fig. 5. Tissue distribution of CERK activity in mouse. CERK activity was determined in mouse tissue homogenates using 100 μM C_6-ceramide, solubilized with EtOH/BSA, and using 200 μg of protein per assay. Activities are expressed as picomoles of phosphorylated C_6-ceramide per milligram of protein per minute. The values shown are means ± SEM of separate measurements performed on three male mice, 60–90 days of age. The same activity distribution pattern was seen with C_2-ceramide, complexed to BSA, or C_{16}-ceramide, solubilized with OG/CL, as substrate (data not shown).

different mouse tissues. In all tissues, Cer1P was formed, suggesting a ubiquitous expression of CERK. The level of kinase activity varied substantially, however, being markedly high in testis and cerebellum, followed by pancreas and cerebrum, and negligible in skeletal muscle, using either EtOH/BSA (**Fig. 5**) or mixed micelles (data not shown) to deliver the substrate. This distribution, however, does not fully reflect the expression of CERK, which, based on Northern analysis, is high in mouse testis, heart, and spleen (5). The lack of correlation between mRNA, protein, and activity levels can be attributable to many factors, including the presence of an inhibitor or activities affecting the substrate or its product. In heart and lung homogenates, unless diluted appropriately, residual ATPase activity interfered with the kinase measurements, despite the use of NaF and orthovanadate (H. Van Overloop, L. Van den Bossche, and P. P. Van Veldhoven, unpublished data). When analyzing the kinase activity in testis, we noticed the formation of an additional ^{32}P-labeled spot that, based on relative mobility value, represents SeP, but this was only seen in BSA-based assays (data not shown). Presumably, ceramide bound to BSA is more accessible to hydrolysis (by ceramidases) than when inserted in a mixed micelle. If formed, sphingenine is efficiently phosphorylated in testis homogenates, a tissue in which we previously reported a high sphingosine kinase activity when using BSA-bound substrates (36). Unfortunately, the amounts of CERK in tissues are too low to be convincingly visualized by immunoblotting (data not shown).

Another possibility to explain the differences between activity measurements and Northern analysis could be the presence of (an)other kinase(s) acting on ceramide, given the presence of different lipid kinase genes in mammalian genomes. Recently, Waggoner et al. (18) reported on a new kinase, which they described as a MuLK, acting on ceramide and diacylglycerol as main substrates, but also on monoacylglycerol. This kinase is highly expressed in pancreas, followed by brain and liver. Another putative lipid kinase was cloned by Tuson, Marfany, and Gonzalez-Duarte (39), who analyzed the causative genes for retinitis pigmentosa. A protein, encoded by one of these genes and displaying some similarity to CERK, was named CERK-like protein. However, this might be rather misleading. The similarity to *Hs*CERK is limited (26% amino acid identity), no information on its substrate spectrum or ATP dependence is available (39), and the protein does not seem to act on ceramide (51). This "kinase" has a restricted expression pattern in human: based on RT-PCR, it is moderately expressed in pancreas, kidney, lung, and retina and almost not expressed at all in heart and muscle (39); based on real-time PCR, it is highly expressed in brain, followed by kidney and trachea (51).

The kinase described as MuLK was cloned some years ago in our laboratory and named putative lipid kinase 2 (accession numbers AJ278150 and AJ401619). Despite many attempts (52), both with bacterially and eukaryotically expressed proteins, no substrate, when corrected for background or endogenous kinase, could be demonstrated.[4] The reason for the lack of activity is not clear; no PCR-

[4] Several lipophilic phosphorylatable compounds, including all four sphinganine and sphingenine stereoisomers, 4-hydroxysphinganine, (truncated) (dihydro)ceramides, (truncated) 1,2-*sn*-diacylglycerol, 1- or 2-monoacylglycerols, 1-monoalkylglycerols, and phosphatidylinositol, as well as less well-studied kinase substrates (tocopherol, cholesterol, farnesol) or putative substrates (galactosylceramide, anandamide), have been tested as substrates under different delivery modes (EtOH/BSA, octyl-β-D-glucopyranoside/cardiolipin, CHAPS, DMSO) in the presence of Ca or Mg ions, with bacterially expressed kinase, lysates of CHO cells stably transfected with *Hs*LK2-Myc-His, mitochondria isolated from such cells, or in vitro-transcribed/translated kinase. Compared with the appropriate controls, no remarkable differences were noticed. In addition, in lipid extracts from CHO cells cultured in the presence of labeled glucose (precursor of glycerolipids) or serine (precursor of sphingolipids) or uploaded with ^{32}P, no additional or enhanced radiolabeled products were revealed when analyzed by two-dimensional TLC/autoradiography and compared with cells stably transfected with the empty vector. On the other hand, these experiments showed that *E. coli* diacylglycerol kinase can phosphorylate anandamide and that coupled transcription/translation systems phoshorylate sphingoid bases, diacylglycerol, and ceramide (P. P. Van Veldhoven and K. De Greef, unpublished data).

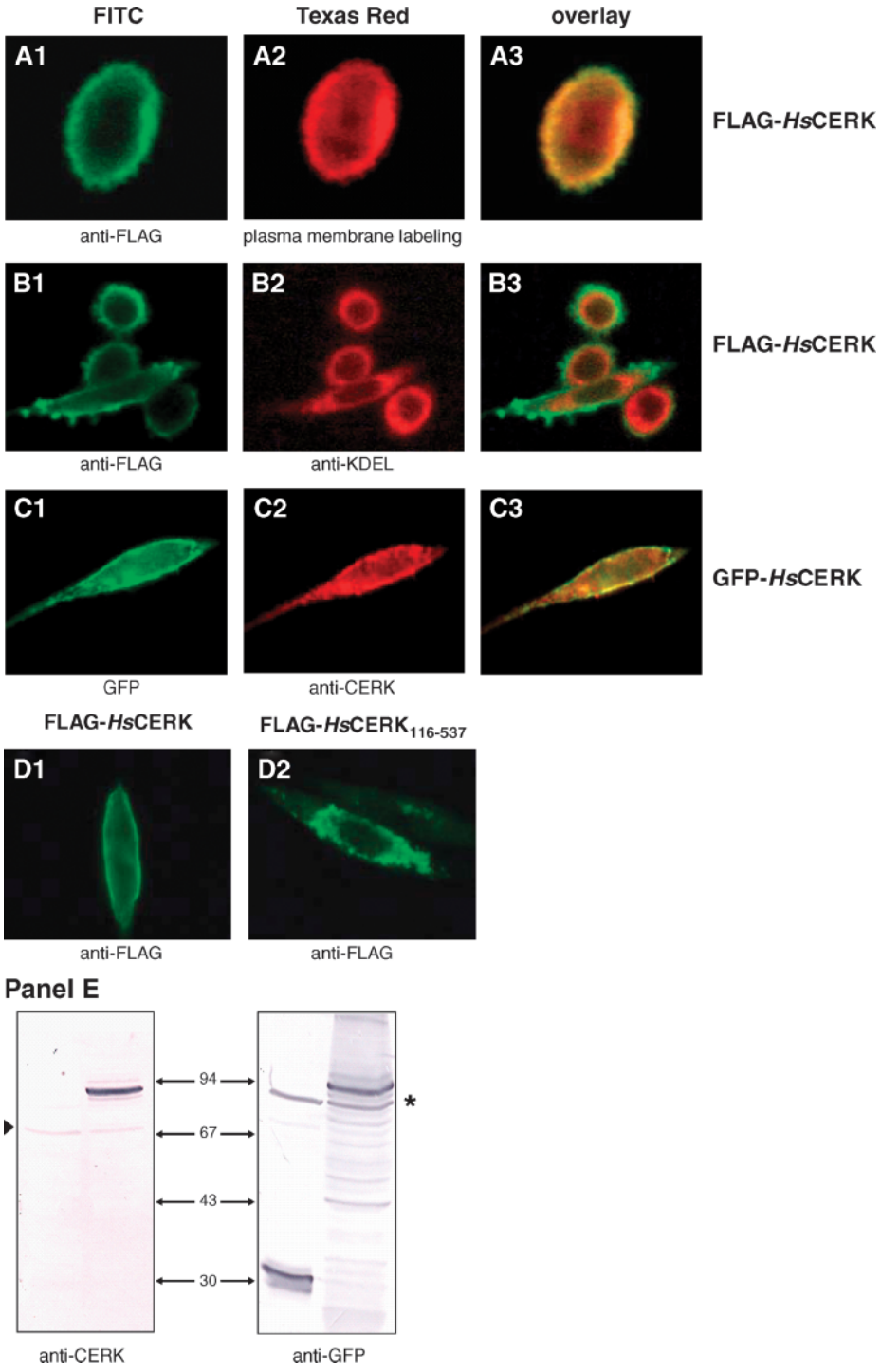

Fig. 6. Subcellular localization of *Hs*CERK. CHO cells were analyzed 24 h after Lipofectamine-mediated transfection with plasmids encoding a full-length FLAG-*Hs*CERK fusion (pHVO001) (A, B, D1), green fluorescent protein (GFP)-*Hs*CERK (pHVO002) (C), *N*-truncated FLAG-*Hs*CERK$_{116\text{-}537}$ (pHVO010) (D2), or appropriate control vectors (data not shown) by direct (C1) or indirect (A1, B1, B2, C2, D1, D2) immunofluorescence microscopy. FLAG fusions were stained with monoclonal mouse anti-FLAG M2 (Stratagene)/anti-mouse FITC (A1, B1, D1, D2); plasma membrane was visualized with the Image-iT^{TM} LIVE plasma membrane labeling kit (Molecular Probes) (A2); endoplasmic reticulum was visualized with rabbit anti-KDEL (Affinity BioReagents)/anti-rabbit Cy3 (B2), and CERK was visualized with affinity-purified rabbit anti-CERK/anti-rabbit Cy3 (C2). Overlays are shown in A3, B3, and C3. In E, CHO cells transfected with pEGFP-C1 (lane 1) or pHVO002, coding for a GFP-*Hs*CERK fusion (lane 2), were analyzed 24 h after transfection by immunoblotting using anti-CERK antibody or anti-GFP. Both antibodies immunodecorated a band of the expected size for the fusion protein (87.7 kDa). Presumably, anti-CERK also recognizes the endogenous CERK in CHO cells (left panel, arrowhead); the asterisk in the right panel indicates a protein cross-reacting with anti-GFP. Migration of protein standards, expressed in kDa, is indicated.

introduced errors appear to be present in the plasmids used, and the cDNAs cloned by Waggoner et al. (18) were based on our database entries. It is possible that the tag, although at different ends in the bacterial and eukaryotic constructs, interferes with the kinase activity. Because of the lack of experimental detail on the way MuLK was constructed (18), we cannot explore this possibility. Another consideration is that the majority of our data were obtained with the human kinase, whereas those reported by Waggoner et al. (18) relate to the murine kinase, characterized by one additional amino acid (Q_{271}).

The expression of MuLK in human tissues, estimated by RT-PCR techniques by Waggoner et al. (18), is highest in pancreas, followed by brain, liver, and kidney. Although MuLK, expressed in CHO cells, was recognized by our antiserum, we failed to see signals of the correct size (~50 kDa) upon immunoblotting of various rat or mouse tissues, indicating low expression. Given the tissue distribution of MuLK, if active, it is rather unlikely that it contributes to ceramide phosphorylation under our assay conditions. In addition, the fact that the same tissue distribution was seen when using EtOH/BSA or mixed micelles to deliver the substrate, either short- or long-chain ceramides (Fig. 5; data not shown), also suggests that the measured activity resides in a single enzyme. Moreover, the subcellular localization of MuLK is different from that of CERK (see below).

Subcellular localization of *Hs*CERK and *Hs*MuLK

The enzyme(s) catalyzing the phosphorylation of ceramide was (were) previously reported to be membrane-associated (11, 41). Upon overexpression of CERK in HEK293 cells, again a membrane-bound activity was found (5). Mitsutake et al. (42) recently showed that *Hs*CERK was predominantly localized in the cytosol of RBL-2H3 cells but was present at the plasma membrane in a few cells, and Carre et al. (53) reported on a Golgi localization in Cos-1 and HUVEC cells and translocation of *Hs*CERK to the plasma membrane upon osmotic swelling. In our hands, tagged forms of *Hs*CERK (FLAG-*Hs*CERK and EGFP-*Hs*CERK fusions), upon expression in CHO cells, were mainly localized to the plasma membrane, based on colocalization with appropriate markers (**Fig. 6A**), whereas no evidence for an ER association was found (Fig. 6B).

Immunostaining of such cells with purified anti-CERK antibody confirmed this localization pattern (Fig. 6C). An N-truncated form of CERK, lacking the first 115 amino acids, failed to associate with the plasma membrane (Fig. 6D) and was not active. These findings are in agreement with data published by Carre et al. (53) showing that removal of the first 123 amino acids, which mediate binding to liposomes (pleckstrin domain), abolished activity and that the truncated protein became cytosolic. In fact, bacterially expressed CERK lacking the first 77 amino acids, either as a His_6 or GST fusion, was also inactive (H. Van Overloop and P. P. Van Veldhoven, unpublished data), although it contained the conserved domains situated more C terminally. Therefore, the N-terminal region seems to be required for enzyme activity/folding. In CHO cells overexpressing CERK, formation of Cer1P from endogenous ceramides could be seen upon ^{32}P uploading, and exogenously added C_6-ceramide, as well as C_2-ceramide, were efficiently converted to their phosphate esters (**Fig. 7**). Exogenously added long-chain ceramide, however, was not phosphorylated, likely because of poor uptake.

The localization of the other cloned kinase was analyzed by expressing it with a C-terminal Myc-His_6 tag. Waggoner et al. (18) reported murine MuLK-EGFP to be associated with undefined endomembranes. Our colocalization stud-

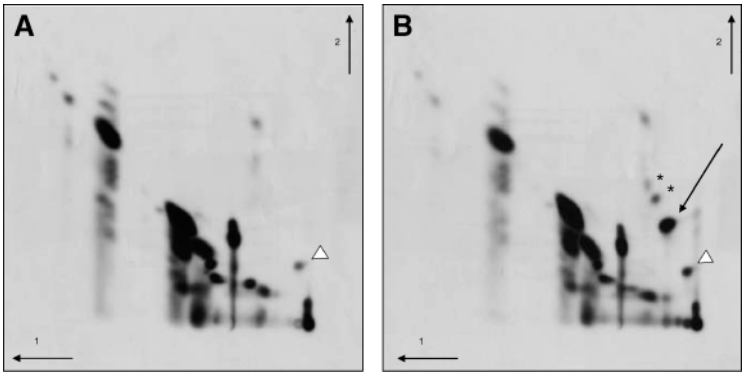

Fig. 7. C_6-ceramide is phosphorylated by CHO cells overexpressing *Hs*CERK. C_6-ceramide was added to CHO cells without (A) or with (B) overexpression of *Hs*CERK, uploaded with ^{32}P, followed by lipid extraction after overnight incubation. Lipids were analyzed by two-dimensional TLC [first dimension, chloroform-methanol-NH_4OH, 60:35:8 (v/v); second dimension, acetone wash followed by chloroform-acetone-methanol-acetic acid-water, 10:4:3:2:1 (v/v)], followed by detection by autoradiography. Phosphorylated C_6-ceramide is indicated by a solid arrow. Phosphorylation of endogenous ceramides (asterisks) was also seen in CHO cells overexpressing *Hs*CERK without the addition of ceramide (data not shown). The arrowheads indicate the position of sphingenine-1-phosphate, resulting from C_6-ceramide breakdown by ceramidase(s) and subsequent action of endogenous sphingosine kinase. Similar findings were obtained when fortifying the cells with C_2-ceramide (data not shown).

ies clearly reveal an exclusive mitochondrial pattern for the *Hs*MuLK-Myc-His_6 fusion (**Fig. 8**). This is consistent with the presence of an N-terminal targeting sequence and transit peptide, as predicted by different algorithms [SignalP 3.0 (54), http://www.cbs.dtu.dk/services/SignalP/#submission; ProtComp 6.0, http://sun1.softberry.com/berry.phtml?topic=protcompan&group=programs&subgroup=proloc), likely cleaved between positions 31 and 32 (...LYG_{31}-K_{32}HC..) for both human and murine kinase. The submitochondrial localization of *Hs*MuLK was further investigated by immunocytochemistry and selective permeabilization of the plasma membrane. When the fixed cells were exposed to high detergent concentrations [0.03–1% (w/v) Triton X-100], *Hs*MuLK was recognized by anti-myc antibodies (Fig. 8A, C). Likewise, under these conditions, anti-biotin decorated the mitochondrial matrix (Fig. 8D), known to contain various biotinylated proteins (55). However, at 0.015% Triton X-100, no staining of *Hs*MuLK or biotinylated proteins was observed (Fig. 8G). Under these conditions, Pex14p, a peroxisomal membrane protein facing the cytosol (56), was still immunodecorated (Fig. 8H), indicating that the plasma membrane was permeabilized but that MuLK was not accessible. After sonication of transfected CHO cells in hypotonic alkaline medium [10 mM Na-pyrophosphate buffer, pH 9 (57)], the fusion protein was recovered in the high-speed pellet (data not shown), suggesting a tight membrane association, in agreement with Waggoner et al. (18).

To document further the cellular localization of CERK under physiological conditions, ceramide phosphoryla-

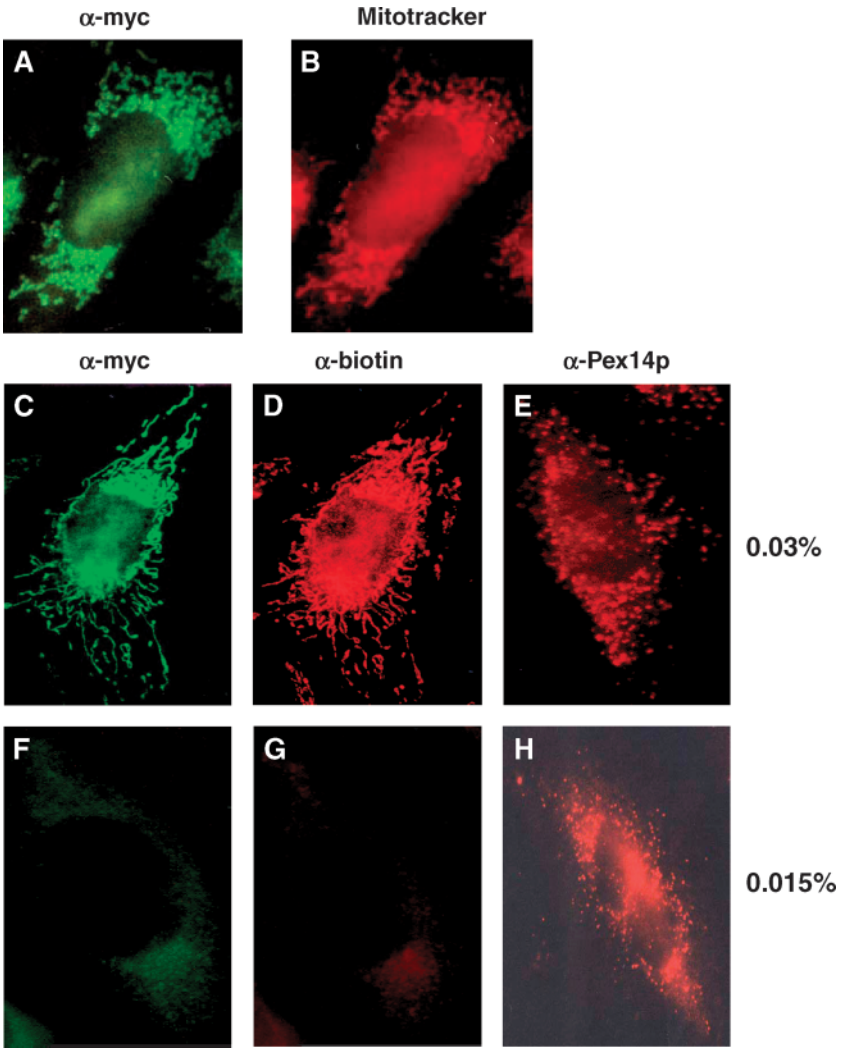

Fig. 8. Subcellular localization of human multisubstrate lipid kinase. CHO cells, stably transfected with pSG005 coding for *Hs*LK2-Myc-His, were analyzed by indirect immunofluorescence microscopy, after permeabilization with 1% (A, B), 0.03% (w/v) (C–E), or 0.015% (w/v) (F–H) Triton X-100, using monoclonal mouse anti-myc as primary antibody and anti-mouse FITC as secondary antibody (A, C, F). Colocalization studies were performed using Mitotracker Red CM-H2×Ros (Molecular Probes) (B) or polyclonal rabbit anti-biotin and anti-rabbit-Cy3 (D, G). In E and H, cells were stained with polyclonal rabbit anti-Pex14p and anti-rabbit Cy3. Control incubations with cells stably transfected with pcDNA/Myc-His showed that the staining with anti-myc was attributable to the expressed fusion protein.

tion was analyzed in subcellular fractions prepared from testis, a tissue with a high CERK activity, and in brain, in which the expression of MuLK is high. The majority of CERK activity was recovered in the so-called microsomal fraction (**Fig. 9**). This fraction is known to contain vesicles derived from plasma membrane and ER, as can be deduced from the enrichment in specific markers (alkaline phosphodiesterase, 2.76-fold for testis and 2.15-fold for brain; carboxylesterase, 3.75-fold for testis and 3.4-fold for brain). Hence, these data are in agreement with the data obtained in cells overexpressing CERK. In the mitochondrial fraction enriched in mitochondria, CERK activity is very low, an additional argument that MuLK, enriched in this fraction, does not contribute to the ceramide phosphorylation described in this study.

To summarize, CERK is a Mg^{2+}-dependent lipid kinase highly selective for D-*erythro* ceramides and can be efficiently measured using BSA-complexed C_6-ceramide. Compounds containing an unsaturated sphingoid base are preferred, but the presence of a secondary hydroxy group is not required. Considerable variation is allowed with regard to the chain length of the base (up to ethanolamine) and of the fatty acid (up to acetyl) of the amide, but the configuration at carbon 2, carrying the amide bond, should be 2*S*. In this respect, one should be aware that N-C_2-Se, a very popular ceramide analog in cellular studies,

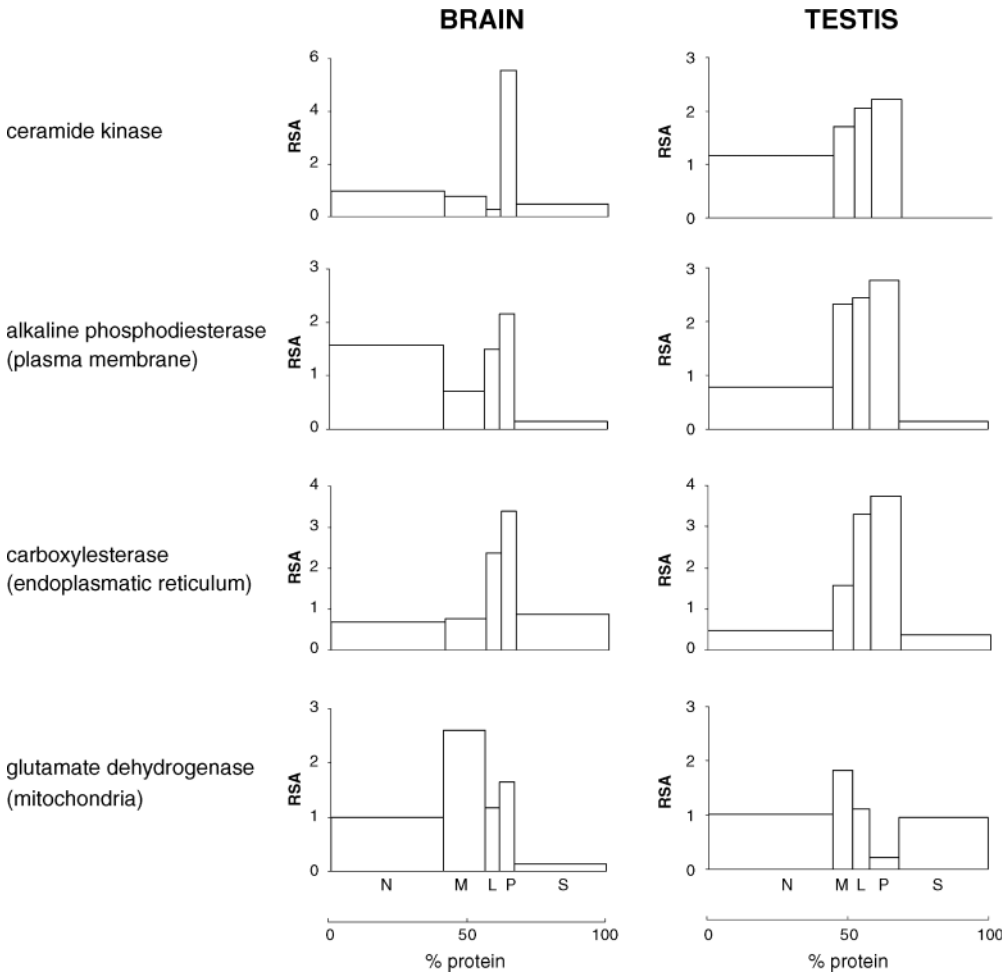

Fig. 9. Subcellular distribution of ceramide phosphorylating activities in rat brain and testis. Fresh rat brain tissue (cerebellum + medulla oblongata) (left panels) and testis (right panels) homogenates were fractionated into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P), and a cytosolic (S) fraction. In each fraction, protein, marker enzymes, and ceramide phosphorylation (100 μM C_6-ceramide, solubilized with BSA/EtOH) were measured. Results are expressed as relative specific activities (RSA) versus cumulative percentage of total protein according to de Duve et al. (64). RSA is defined as the percentage of total activity present in a particular fraction divided by the corresponding percentage of total protein. Recoveries varied between 82% and 102%. CERK activity in rat brain and testis amounted to 27 and 26 pmol/min/mg protein, respectively. Other markers (not shown) included catalase, acid phosphatase, glucose-6-phosphatase, 5′-nucleotidase, and lactate dehydrogenase, with the highest relative specific activity in the L, L, P, P, and S fractions, respectively. Although glutamate dehydrogenase is generally considered a marker for mitochondria, a soluble activity has been reported in nonhepatic tissues (65), likely explaining the rather high amount in the S fraction of testis.

is phosphorylated by CERK and that the amount of C_2-Cer1P formed will depend on the expression level of CERK in the cells studied. The enzyme is mostly active in testis and cerebellum, but its function in these organs is not clear. In brain, it was postulated to be linked to synapse formation. With regard to testis, it is remarkable that many other sphingosine/ceramide-metabolizing enzymes are preferentially expressed in this tissue [sphingosine kinase (36), dihydroceramide desaturase (40)] and that levels of SeP are also high (58). Perhaps this is linked to the continuous process of cell maturation and apoptosis to form gametes in the gonadal tissues (59). The roles of MuLK (18) and certainly of CERKL (39) are unclear. The resemblance of MuLK to *E. coli* 1,2-*sn*-dioleoylglycerol kinase with regard to substrate spectrum, both being unspecific kinases acting on ceramide, diacylglycerol, and monoacylglycerol, and its association with mitochondria, could suggest some ancestral function. The mitochondrial localization of MuLK certainly adds another dimension to sphingolipid signaling. Changes in the mitochondrial integrity, leading to cytochrome*c* release and finally to type 2 apoptosis, have been linked to increases in ceramide (60), and the presence of a mitochondrion-specific neutral ceramidase (61) suggests a specific pool of ceramide in these organelles. Through the action of MuLK, a mitochondrial pool of Cer1P is feasible as well, which could be a mediator in the known ceramide-dependent processes, but this remains to be proven.

Note. Very recently, Bektas et al. (62) published their work on MuLK and claimed that this kinase is not at all active on ceramide, in agreement with our negative data, but is able to phosphorylate monoacylglycerols and diacylglycerols. During the reviewing process, a paper by Wijesinghe et al. (63) on the substrate specificity of CERK appeared in this journal. Using Triton X-100 micelles to deliver the substrate, similar conclusions about D-*erythro* specificity were reached. However, the reported requirements for a sphingosine backbone, at least 12 carbon atoms long, and the secondary hydroxy group for substrate recognition are not supported by our study.

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