Substrate specificity of human ceramide kinase

Dayanjan S. Wijesinghe,* Autumn Massiello,* Preeti Subramanian,* Zdzislaw Szulc,† Alicja Bielawska,† and Charles E. Chalfant[1,*,§]

Department of Biochemistry,* Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298; Department of Biochemistry and Molecular Biology,† Medical University of South Carolina, Charleston, SC 29425; and Research and Development,§ Hunter Holmes McGuire Veterans Administration Medical Center, Richmond, VA 23249

Abstract Previous studies in our laboratory have established ceramide kinase (CERK) as a critical mediator of eicosanoid synthesis. To date, CERK has not been well characterized in vitro. In this study, we investigated the substrate specificity of CERK using baculovirus-expressed human CERK (6×His) and a newly designed assay based on mixed micelles of Triton X-100. The results indicate that the ability of CERK to recognize ceramide as a substrate is stereospecific. A minimum of a 12 carbon acyl chain was required for normal CERK activity, and the 4-5 *trans* double bond was important for substrate recognition. A significant discrimination by CERK was not observed between ceramides with long saturated and long unsaturated fatty acyl chains. Methylation of the primary hydroxyl group resulted in a loss of activity, confirming that CERK produces ceramide-1-phosphate versus ceramide-3-phosphate. In addition, methylation of the secondary hydroxyl group drastically decreased the phosphorylation by CERK. These results also indicated that the free hydrogen of the secondary amide group is critical for substrate recognition. Lastly, the sphingoid chain was also required for substrate recognition by CERK. Together, these results indicate a very high specificity for substrate recognition by CERK, explaining the use of ceramide and not sphingosine or diacylglycerol as substrates.—Wijesinghe, D. S., A. Massiello, P. Subramanian, Z. Szulc, A. Bielawska, and C. E. Chalfant. **Substrate specificity of human ceramide kinase. *J. Lipid Res.* 2005.** 46: **2706–2716.**

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Understanding of the important role played by sphingolipids in the regulation of intracellular processes began with the discovery by Hannun et al. (1) of the inhibition of protein kinase C by sphingosine. Since then, ceramides and a number of their metabolites have been identified to regulate important cellular processes by regulating the activity of key regulatory enzymes (2–9). These include specific serine-threonine kinases (7, 10), protein phosphatases (11), protein kinase Cξ (12), Jun nuclear kinases (13, 14), mitogen-activated protein kinases (15, 16), phospholipase A_2 (17), phospholipase D (18, 19), and protein kinase B (20, 21).

The interconvertable ceramide-derived metabolite ceramide-1-phosphate (C1P) is emerging as an important biological mediator. In mammalian cells, C1P is produced by the phosphorylation of ceramides. To date, ceramide kinase (CERK) is the only enzyme known to catalyze this reaction in mammals, and CERK was first described as a lipid kinase found in brain synaptic vesicles by Bajjalieh and coworkers (22). CERK is a calcium-dependent lipid kinase specific for the phosphorylation of ceramides but not the structurally related diacylglycerol (10, 22, 23). Dressler and Kolesnick (24) also reported the existence of C1P derived from sphingomyelin in HL-60 cells. Later, Kolesnick and Hemer (10) verified the findings of Bajjalieh et al. (22) by reporting CERK activity distinguishable from diacylglycerol kinase activity in HL-60 cells. More recently, Hinkovska-Galcheva et al. (25) and also Rile et al. (26) reported CERK activity in human neutrophils. CERK activity has also been shown in cerebellar granule cells (27) and in epithelium-derived A549 lung carcinoma cells (28).

Increasingly, C1P is reported in the scientific literature as a regulator of intracellular processes. Gomez-Munoz et al. (29) showed that C1P was a potent stimulator of DNA synthesis; it also promoted cell division in rat fibroblasts and blocked apoptosis in bone marrow-derived macrophages (30). Carpio et al. (31) have shown C1P to cause Erk-2 phosphorylation in osteoblastic cells. CERK was also implicated in the process of phagolyzosome formation during phagocytosis (25). Finally, published findings from our laboratory demonstrated, for the first time, a role for C1P in signal transduction in the inflammatory response (28, 32). Incubation of A549 lung carcinoma cells with in-

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Abbreviations: C1P, ceramide-1-phosphate; CERK, ceramide kinase; $cPLA_2$, cytosolic phospholipase A_2; K_m^b, intrafacial Michaelis-Menten constant; Ni-NTA, nickel-nitrilotriacetic acid agarose.

[1] To whom correspondence should be addressed.
e-mail: cechalfant@vcu.edu

terleukin-1β and A23187 (a Ca^{2+} ionophore) caused an induction of C1P levels that coincided with the activation of CERK within the time frame of arachidonic acid release (28). Downregulation of CERK by RNA interference technology caused a concomitant downregulation of arachidonic acid release in response to interleukin-1β, ATP, and A23187. In the presence of exogenous C1P, however, CERK downregulation had no effect on the agonist-induced arachidonic acid release. Further studies also disclosed that C1P interacts with the CaLB/C2 domain of cytosolic phospholipase A_2 ($cPLA_2$), acting as a positive allosteric activator of $cPLA_2$ and enhancing the interaction of the enzyme with phosphatidylcholine (33). These findings established a role for CERK and C1P in the inflammatory pathway. Therefore, with the loss of Vioxx and Bextra as therapeutics for inflammation, the characterization of other mediators of inflammation such as CERK is warranted.

In this study, we examined the structural requirements within the ceramide molecule necessary for recognition and substrate utilization by human CERK. The results disclose that even slight modifications to the sphingoid backbone greatly decrease the ability of the lipid to identify its substrate and to catalyze the reaction. Thus, this study shows a high degree of specificity for ceramide by CERK and identifies key features for the development of sphingoid-based inhibitors of the enzyme.

EXPERIMENTAL PROCEDURES

Materials

Serinol and all general chemicals were purchased from Aldrich. Phytosphingosine (2*S*,3*S*,4*R*), D-*erythro*-$C_{24:0}$, and $C_{24:1}$ were purchased from Avanti. D-*erythro*-C_{16} ceramide and various structural analogs were obtained from the Lipidomics Core at the Medical University of South Carolina and synthesized as described previously (34–37). All lipids were quality controlled by ^{1}H-NMR spectra, recorded using a Bruker AVANCE 500 MHz spectrometer equipped with an Oxford Narrow Bore Magnet. Chemical shifts are given in parts per million downfield from tetramethylsilane as an internal standard, and the listed J values are in hertz. Mass spectral data were obtained in positive ion electrospray ionization mode on a Finnigan LCQ ion trap mass spectrometer. Samples were infused in methanol solution with an electrospray ionization voltage of 4.5 kV and capillary temperature of 200°C. The purity of all synthesized lipids was >95% as estimated by TLC and ^{1}H-NMR analysis.

Recombinant expression of CERK using baculovirus

Recombinant human CERK (hCERK) was expressed in Sf9 cells with a 6×His tag using a baculovirus expression system and purified using a modified protocol as described previously (38, 39). Briefly, Sf9 cells were grown in suspension culture and infected with high-titer recombinant baculovirus at a multiplicity of infection of 1.0 at 72 h after infection. The cells were then harvested, and the cell paste was resuspended in a 10× cell paste volume lysis buffer (10 mM MOPS, pH 7.2, 150 mM KCl, 10% glycerol, 0.5% Triton, 0.01 mg/ml leupeptin, 0.01 mg/ml aprotinin, 0.005 mg/ml pepstatin, and 1 mM PMSF). The cells were broken by 10 strokes with a Dounce homogenizer. The cell lysate was clarified by centrifugation at 16,000 *g* for 45 min at 4°C. The soluble supernatant was batch-bound to 100 ml of nickel-nitrilotriacetic acid agarose (Ni-NTA) superflow resin for 1.5 h at 4°C with nutation. The column was washed first with Ni-NTA wash buffer (10 mM MOPS, pH 7.2, 150 mM KCl, and 10% glycerol) and then with 19.5 mM imidazole at a rate of 4 ml/min until A280 baseline absorbance was achieved. The column was eluted with a step elution to 250 mM imidazole in Ni-NTA wash buffer (10 mM MOPS, pH 7.2, 150 mM KCl, 10% glycerol, and 250 mM imidazole) at 5 ml/min. The fractions were analyzed by SDS-PAGE, and fractions containing significant amounts of $cPLA_{2\alpha}$ were pooled and dialyzed against storage buffer (10 mM Tris, pH 7.2, 0.1 M NaCl, 2 mM EGTA, 50% glycerol, and 1 mM DTT). The dialyzed material was then centrifuged at 39,000 *g* for 30 min to remove precipitation and divided into 10 ml fractions. Recombinant CERK was assayed at 70% purity by Coomassie stain and retained all of the characteristics (e.g., Ca^{2+} sensitivity, pH optimum, etc.) as hCERK overexpressed in mammalian cells (23).

Recombinant expression of CERK in mammalian cells

Recombinant CERK was expressed in HeLa cells by transfecting the plasmid construct (hCERK in pcDNA3.1 His TOPO®) using Effectene reagent (Qiagen Corp.) according to the standard procedure. Briefly, HeLa cells were plated at 40–50% confluence in 150 × 15 mm tissue culture dishes 24 h before transfection. Cells were transfected with 6 μg of plasmid under conditions specified by the manufacturer. After 24 h, the cells were harvested and the crude HeLa extracts were obtained by sonicating in lysis buffer (20 mM MOPS, 2 mM EGTA, 0.5 mM DTT, with added Sigma® P-8340 protease inhibitor cocktail at 1:250 dilution). Ten micrograms of protein of the crude extract was used per assay.

Cell culture

HeLa cells were cultured in DMEM with L-glutamine supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. Incubation conditions were 5% CO_2, 37°C, and 95% humidity.

CERK assays

β-Octylglucoside mixed micellar assay. Ceramide (160 nmol) was micelle-solubilized by sonication in 180 μl of reaction buffer (20 mM MOPS, pH 7.2, 50 mM NaCl, 1 mM DTT, 3 mM $CaCl_2$, 51.24 mM β-octylglucoside, 1 mM cardiolipin, and 0.2 mM diethylenetriaminepentaacetic acid). Ten micrograms of crude human CERK (crude lysate of hCERK overexpressed in HeLa cells) or 0.9 μg of baculovirus expressed CERK was added per reaction and incubated at 37°C for 5 min. The reaction was started by adding 11 μl of ATP {1 μl of [γ-^{32}P]ATP (10 μCi/μl) + 10 μl of 20 mM ATP in 100 mM $MgCl_2$}. All reactions were carried out in triplicate with D-e-C_{16} ceramide as a positive control. The reaction was allowed to continue at 37°C for 40 min and stopped by the addition of 1.2 ml of 1:1 $CHCl_3$/methanol followed by 500 μl of 1 M KCl in 20 mM MOPS. The mixture was then vortexed, and phases were separated by centrifugation at 650 *g* for 5 min. As described previously (40), the organic phase was washed three times with 500 μl aliquots of 1 M KCl, and 100 μl of the organic phase was counted directly. All results (with regard to substrate utilization) obtained with the Triton X-100-based assay (see below) were verified with this previously described CERK assay (40).

Surface dilution assay with Triton X-100 mixed micelles. Briefly, 0.003, 0.006, 0.012, 0.024, 0.048, 0.072, 0.109, and 0.170 mM ceramide solutions were sonicated with 11.5 mM Triton X-100 and 0.5 mM cardiolipin to obtain 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.9, and 1.4 mol% ceramide solutions, respectively. Forty microliters each of these solutions was used per 200 μl reaction containing 20 mM MOPS, pH 7.2, 50 mM NaCl, 1 mM DTT, 3 mM $CaCl_2$, and 0.2 mM diethlenetriaminepentaacetic acid. A total of 0.9 μg of bacu-

lovirus-expressed hCERK or 10 μg of crude hCERK (crude lysate of hCERK overexpressed in HeLa cells) was used per reaction. The reaction was started by adding 11 μl of ATP {1 μl of [γ-^{32}P] ATP (10 μCi/μl) + 10 μl of 20 mM ATP in 100 mM $MgCl_2$}. All reactions were carried out in triplicate. The reaction was incubated at 37°C for 15 min and stopped by the addition of 1.2 ml of 1:1 $CHCl_3$/methanol. The organic phase was washed four times with 500 μl of 1 M KCl in 20 mM MOPS. The mixture was then vortexed, and phases were separated by centrifugation at 650 *g* for 5 min as described previously (40). The amount of [^{32}P]C1P produced was quantified as described previously (40) and confirmed by TLC as described previously (41).

Data analysis

Data analysis was done using Enzyme Kinetics Module™ 1.1 together with SigmaPlot version 8.02A. The mole fraction of ceramide was plotted against reaction velocity (μmol C1P produced/min/mg protein) to obtain intrafacial Michaelis-Menten constant (K_m^b) and V_{max}. The data obtained are means of triplicate data sets ± SEM. Two-way ANOVA was performed using Microsoft Excel.

RESULTS

Previously, CERK was shown to phosphorylate only ceramides and not the closely related lipids, diacylglycerol and sphingosine (10). To determine the molecular basis of the interaction between CERK and its substrate, ceramide, our laboratory focused on key features in the ceramide molecule required for substrate recognition and specificity. These include the stereochemistry at the C_2, C_3, C_4, and C_5 positions, the requirement of the acyl chain length, the effect of the functional groups in the sphingoid backbone, the requirement of the secondary amide bond, and the requirement of the primary and secondary hydroxyl groups (**Fig. 1**). Additionally, the saturation, desaturation, configuration, and hydroxylation of the natural *trans* C_4-C_5 double bond and the role of the alkyl chain of the sphingoid backbone were also investigated (Fig. 1). The data presented here are from the CERK assay using mixed micelles of Triton X-100. The previously described assay for CERK was used to verify these results (data not shown) (40).

Also of note, V_{max}/K_m is used in these studies as a measure of the relative rates of enzymatic reactions for different substrates under identical conditions. Therefore, V_{max}/K_m is a measure of the specificity of an enzyme to different substrates. A statistically significant difference in V_{max}/K_m is generally considered to denote an actual difference in substrate specificity. In this study, we used this parameter to investigate the differences in substrate specificities of CERK, and all differences in substrate specificity mentioned hereafter are statistically relevant.

Stereochemistry

There are two asymmetrical carbon atoms within the ceramide molecule; thus, four stereoisomers are possible. D-*erythro*-C_{16} ceramide (2*S*,3*R*) is the naturally occurring configuration, with L-*erythro*-C_{16} ceramide (2*R*,3*S*) being its enantiomer. Additionally, enantiomeric pairs of the *threo*-diastereoisomers can be formed, L-*threo* (2*S*,3*S*) and D-*threo* (2*R*,3*R*). In this study, only the D-*erythro* configuration of ceramide was converted to C1P. CERK demonstrated no activity toward the other stereoisomers of ceramide. Thus, CERK phosphorylates only the naturally occurring D-*erythro* ceramides (**Table 1**).

The effect of the chain length of the acyl chain

Because sphingosine has been shown to not be a substrate for CERK, we examined various acyl chain lengths of ceramide to determine the minimum size for substrate recognition. We found no statistically significant effects on substrate specificity for chain lengths of $\geq C_{12:0}$ (**Fig. 2A**, **B**, Table 1). With decreasing acyl chain lengths, however ($<C_{12:0}$), CERK showed a proportionate, statistically significant decrease in enzymatic activity, indicated by increasing K_m^b and decreasing specificity (V_{max}/K_m^b). Both C_2 and C_4 ceramide were found not to be substrates for CERK. Thus, a minimum of 14 carbons are required for the full recognition of ceramide as a substrate by CERK, and ceramides with an acyl chain of fewer than six car-

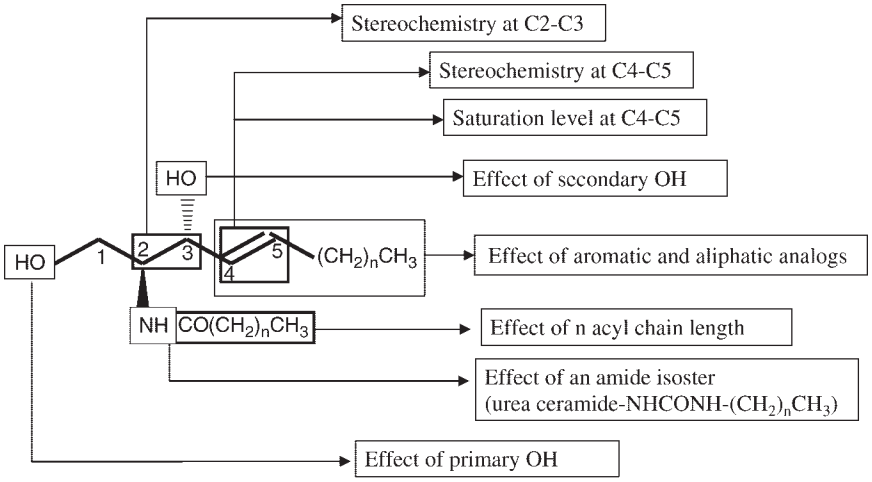

Fig. 1. The investigated structural features of ceramide.

TABLE 1. K_m and V_{max} of all the ceramides and ceramide analogs examined for ceramide kinase (baculovirus-expressed)

Ceramide Tested	K_m[b]	V_{max}	V_{max}/K_m
	mol fraction (μM)	*μmol ceramide-1-phosphate/ min/mg protein*	
D-e-$C_{2:0}$ ceramide	NS	NS	NS
D-e-$C_{4:0}$ ceramide	NS	NS	NS
D-e-$C_{6:0}$ ceramide	0.1109 (299.36)	0.4456	4.0180
D-e-$C_{8:0}$ ceramide	0.0657 (168.77)	0.6843	10.42
D-e-$C_{10:0}$ ceramide	0.0186 (45.49)	0.4408	23.75
D-e-$C_{12:0}$ ceramide	0.0086 (20.82)	0.3357	39.11
D-e-$C_{14:0}$ ceramide	0.0026 (6.26)	0.1590	60.46
D-e-$C_{16:0}$ ceramide	0.0036 (8.67)	0.1882	52.53
D-e-$C_{16:0}$ dehydroceramide	0.0018 (4.33)	0.0338	18.70
D-e-$C_{16:0}$ dihydroceramide	0.0034 (8.19)	0.0723	21.33
D-e-$C_{16:0}$-*cis* ceramide	0.0506 (127.91)	0.5485	10.85
D-e-$C_{16:0}$ (*R*)-α-hydroxyceramide	0.0026 (6.26)	0.0294	11.14
D-e-$C_{16:0}$ (*S*)-α-hydroxyceramide	0.0030 (7.22)	0.0070	2.34
D-e-$C_{18:0}$ ceramide	0.0028 (6.74)	0.1557	56.56
D-e-$C_{18:0}$ phytoceramide	0.0023 (5.53)	0.0451	19.82
D-e-$C_{24:0}$ ceramide	0.0033 (7.95)	0.1626	49.29
D-e-$C_{24:1}$ ceramide	0.0068 (16.43)	0.3059	44.93
1-0-methyl $C_{16:0}$ ceramide	NS	NS	NS
3-0-methyl $C_{16:0}$ ceramide	0.0049 (11.82)	0.0247	5.04
D-e-C_{16} urea ceramide	0.0008 (1.92)	0.0097	11.54
L-e-C_{16} urea ceramide	NS	NS	NS
N-Methyl $C_{16:0}$ ceramide	NS	NS	NS
D-t-$C_{16:0}$ ceramide	NS	NS	NS
L-e-$C_{16:0}$ ceramide	NS	NS	NS
L-t-$C_{16:0}$ ceramide	NS	NS	NS
D-t-$C_{6:0}$ ceramide	NS	NS	NS
L-e-$C_{6:0}$ ceramide	NS	NS	NS
L-t-$C_{6:0}$ ceramide	NS	NS	NS
(*R*)α-hydroxy-C16-1,3-acetoinide of ceramide	NS	NS	NS
(1*S*,2*S*-B13)	NS	NS	NS
(1*R*,2*R*-B13)	NS	NS	NS
N-Palmitoyl-serinol (C16 serinol)	NS	NS	NS

K_m[b], intrafacial Michaelis-Menten constant. Values represent means from three separate determinations.

bons are not recognized as substrates. These data indicate that ceramides with chain lengths observed to occur in cells are preferred substrates of CERK (42–44).

Saturation state of the acyl chain

The main forms of ceramide found in mammalian cells are D-e-$C_{16:0}$ ceramide and D-e-$C_{24:1}$ ceramide (42–44). In this study, we examined whether CERK possessed any selective characteristics for saturated or unsaturated acyl chains of ceramide, using D-e-$C_{24:0}$ ceramide and D-e-$C_{24:1}$ ceramide (**Fig. 3**, Table 1). Although the saturated ceramide showed a lower K_m compared with the unsaturated ceramide, this was accompanied by a proportionate change in the V_{max}. As a result, no statistically significant difference was observed in the specificity constant (V_{max}/K_m[b]). Thus, differences in the K_m and V_{max} may be attributed to the higher solubility of the monounsaturated form. A similar pattern was observed between D-e-$C_{18:0}$ ceramide and D-e-$C_{18:1}$ ceramide (data not shown). Thus, CERK seems to equally recognize ceramides containing both saturated and unsaturated fatty acyl chains.

Effect of α-hydroxylated fatty acids

Ceramidase, another ceramide-utilizing enzyme, is capable of using ceramides with α-hydroxy fatty acids as a substrate (45). Therefore, the ability of CERK to use α-hydroxylated ceramides was also investigated (Table 1). CERK was able to phosphorylate both *R* and *S* D-e-$C_{16:0}$ α-hydroxyl ceramides but demonstrated a lower specificity constant compared with the naturally occurring long-chain ceramides (Table 1). Thus, CERK possesses the ability to phosphorylate α-hydroxylated ceramides, and an α-hydroxylated C1P may exist in cells.

Sphingoid moiety

The sphingoid backbone modifications shown in Fig. 1 were introduced into the ceramide molecule. First, the requirement for the 4-5 double bond within the sphingoid moiety was examined. Saturation of the 4-5 double bond produces D-*erythro*-dihydroceramide, an anabolic precursor of ceramide, which has been shown to be biologically inactive in cells (36). CERK showed significantly reduced specificity ($P < 0.005$) toward the dihydroceramides compared with ceramide (**Fig. 4A**, Table 1).

To further examine the necessity of the 4-5 double bond of ceramide for use as a substrate for CERK, this moiety was further unsaturated to D-*erythro*-dehydro-C_{16} ceramide, producing a triple bond between the 4 and 5 carbons of the sphingoid backbone. This compound also showed a decreased specificity ($P < 0.005$) compared with the natural ceramides (Fig. 4A, Table 1). Because natural ceramide has the 4-5 double bond in the *trans* configura-

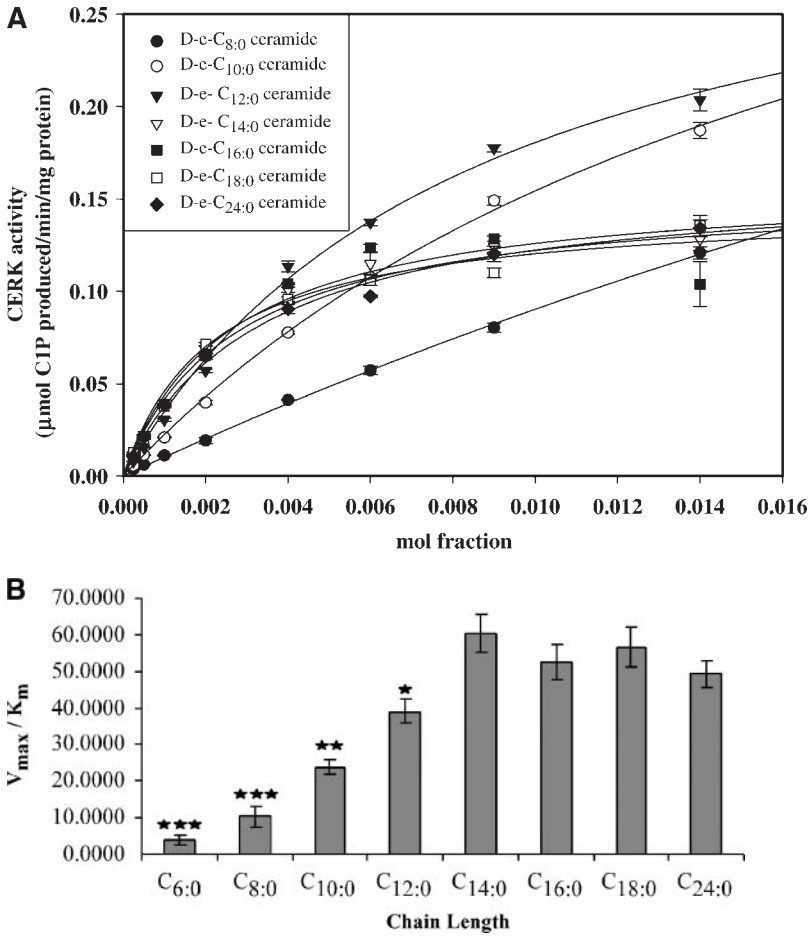

Fig. 2. The length of the acyl chain affects the substrate specificity of ceramide kinase (CERK). A: Michaelis-Menten curves for ceramides of different acyl chain lengths. The specificities of ceramides of different acyl chain lengths were assayed at 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.9, and 1.4 mol% as described in Experimental Procedures. The graph depicts micromoles of ceramide-1-phosphate (C1P) produced per minute per milligram of CERK (baculovirus-expressed) versus the mole fraction of ceramide to obtain V_{max} and the intrafacial Michaelis-Menten constant (K_m^b). Data are presented as means ± SEM and are representative of three separate determinations. Note: for both $C_{6:0}$ (not depicted) and $C_{8:0}$, a higher range (concentration of ceramide) was used to establish K_m^b and V_{max} because of their poor substrate specificity constants (higher range concentrations are not depicted). B: Specificity constants for the different acyl chain lengths of ceramide. Data presented are specificity constants (V_{max}/K_m^b) for D-e-$C_{8:0}$, D-e-$C_{10:0}$, D-e-$C_{12:0}$, D-e-$C_{14:0}$, D-e-$C_{16:0}$, D-e-$C_{18:0}$, and D-e-$C_{24:0}$ ceramide ± SEM. Data are representative of three separate determinations. The significance of the differences in specificity constants compared with D-e-$C_{16:0}$ ceramide was examined by two-way ANOVA. * $P < 0.1$, ** $P < 0.01$, *** $P < 0.005$.

tion, the nature of the 4-5 double bond was further examined by modifying the configuration from *trans* to *cis*. CERK also showed a significant decrease ($P < 0.005$) in specificity for this substrate as well (Fig. 4A, Table 1).

Recently, phytoceramides were described in mammalian cells (46–48); thus, the use of phytoceramides (4-hydroxydihydroceramides) as substrates for CERK was also investigated. $C_{18:0}$-phytoceramide showed a decreased specificity constant ($P < 0.005$) compared with $C_{18:0}$ ceramide (Fig. 4B, Table 1). Thus, these data demonstrate that CERK has a marked preference for the saturation level and the *trans* orientation of the double bond at the 4-5 position.

The necessity of other sphingoid structural features was also examined. Clearly, from other published findings, the free sphingoid bases, sphingosine and dihydrosphingosine, are not substrates of CERK (23). The question remains, however, whether the sphingoid base chain is necessary for the recognition of ceramide as a substrate for CERK. To this end, serinol ceramide (a ceramide analog with a truncated sphingosine backbone containing only the C_1–C_3 part from serine) was synthesized and tested in vitro (35). The results show that *N*-hexadecanoyl-serinol (serinol ceramide) was not a substrate (Table 1). The structural features of the sphingoid moiety were further investigated by replacing the sphingoid alkenyl chain with an aromatic phenyl group producing two enantiomers, (1*R*,2*R*)-2-(*N*-myristoylamino)-1-(4′-nitrophenyl)-1,3-propandiol (1*R*,2*R*-B13) and (1*S*,2*S*)-2-(*N*-myristoylamino)-1-(4′-nitrophenyl)-1,3-propandiol (1*S*,2*S*-B13). Neither of these compounds was a substrate for CERK (Table 1). Therefore, the sphingosine

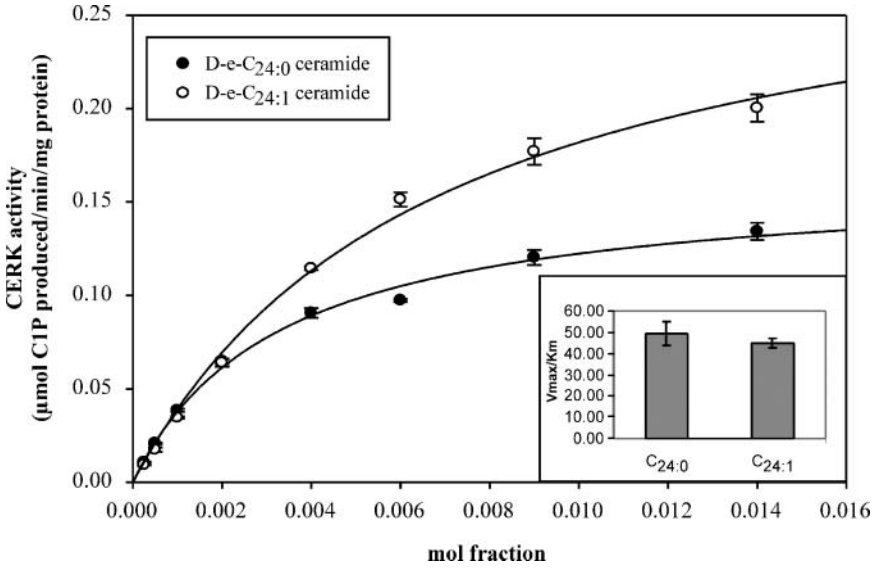

Fig. 3. The saturation state of the acyl chain does not affect the substrate specificity of CERK. Ceramides with saturated ($C_{24:0}$) and unsaturated ($C_{24:1}$) acyl chains were assayed at 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.9, and 1.4 mol% as described in Experimental Procedures. The graph depicts micromoles of C1P produced per minute per milligram of CERK (baculovirus-expressed) versus the mole fraction of ceramide to obtain V_{max} and $K_m{}^b$. Data are presented as means ± SEM and are representative of three separate determinations. Inset: Specificity constants for D-e-$C_{24:0}$ and D-e-$C_{24:1}$ ceramide. Data presented are specificity constants ($V_{max}/K_m{}^b$) for D-e-$C_{24:0}$ and D-e-$C_{24:1}$ ceramide ± SEM. Data are representative of three separate determinations.

chain is necessary for CERK to recognize ceramide as a substrate.

Amide group

To determine the importance of the secondary amide group -NHCO- in the ceramide structure, we synthesized urea isosters of ceramide bearing -NHCONH- instead of the -NHCO- group, producing two enantiomers, 2*S*,3*R* (i.e., D-*erythro*) and 2*R*,3*S* (i.e., L-*erythro*)-urea-C_{16} ceramide. D-*erythro*-urea-C_{16} ceramide was a substrate for CERK but demonstrated significantly lower specificity ($P < 0.005$) compared with the naturally occurring ceramides (**Fig. 5**, Table 1). Also, similar to L-*erythro*-C_{16} ceramide, L-*erythro*-urea-C_{16} ceramide was not a substrate (Table 1). These results indicate that the *N*-acyl-amide bond is required for the complete recognition of the substrate by CERK. Next, the role of the hydrogen atom from the amide bond -NHCO- was examined using *N*-methyl-C_{16} ceramide as a model. This compound was also not a substrate of CERK (Table 1), demonstrating the necessity of the free hydrogen in the secondary amide group for recognition by CERK.

Primary and secondary hydroxyl groups

The necessity of the free primary and secondary hydroxyl groups of the ceramide molecule was examined by using 1-*O*-methyl-C_{16} ceramide and 3-*O*-methyl-C_{16} ceramide (Fig. 1). Methylation of the primary hydroxyl group completely eradicated the ability of CERK to phosphorylate ceramide. Methylation of the secondary hydroxyl group showed dramatically decreased specificity ($P < 0.005$) for the substrate by CERK (Fig. 5, Table 1). To further investigate the importance of the primary and secondary hydroxyl groups, the ability of CERK to phosphorylate $C_{16:0}$-1,3-acetoinide was tested. This compound was also not a substrate of CERK (Table 1). These data confirm that the primary hydroxyl group is the site of phosphorylation by CERK and that the secondary hydroxyl group, although not involved in direct phosphorylation, is still important for substrate recognition.

DISCUSSION

In this study, we demonstrate the strict structural requirements of ceramide for recognition as a substrate by CERK. The understanding of this action of recognition is important for several reasons. First, these studies begin to identify features for ceramide-based inhibitors of CERK, allowing for the possible design of new anti-inflammatory compounds. Second, these studies add to our knowledge of the biochemical mechanisms of CERK and sphingolipid enzymes in general. Lastly, these studies further disclose that this interaction between ceramide and CERK has implications for the physiological regulation of the enzyme.

Previously published kinetic studies on CERK were accomplished using a bulk dilution technique and mixed micelles of β-octylglucoside (49). In this regard, molecular modeling studies show β-octylglucoside to have an irregular surface (50); thus, all of the substrate in the micelle is not presented equally to the enzyme. Furthermore, as ceramides are found in membranes, the true kinetic behavior of the enzyme can only be studied using a model that takes into account both bulk and surface diffu-

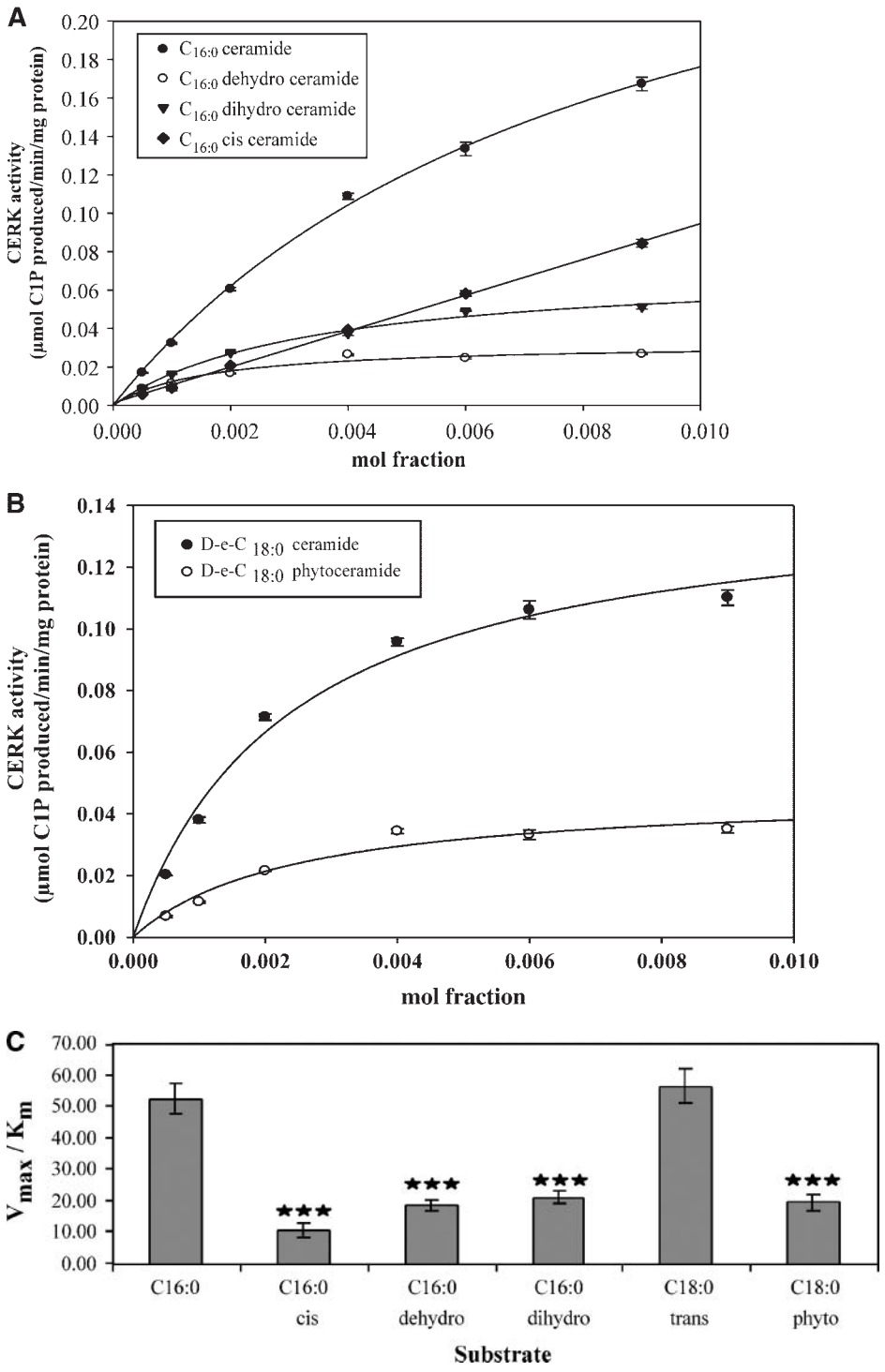

Fig. 4. Alteration of the 4-5 double bond of ceramide significantly affects the recognition of ceramide by CERK. A: Michaelis-Menten curves for C_{16} ceramides with different 4-5 bonds. D-e-$C_{16:0}$ *trans*, $C_{16:0}$ *cis*, $C_{16:0}$ dehydro-, and $C_{16:0}$ dihydro-ceramides were assayed at 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.9, and 1.4 mol% as described in Experimental Procedures. The graph depicts micromoles of C1P produced per minute per milligram of CERK (baculovirus-expressed) versus the mole fraction of ceramide to obtain V_{max} and $K_m{}^b$. Data are presented as means ± SEM and are representative of three separate determinations. B: Michaelis-Menten curves for $C_{18:0}$ ceramide and $C_{18:0}$ phytoceramide. D-e-$_{18:0}$ ceramide and D-e-$C_{18:0}$ phytoceramide were assayed at 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.9, and 1.4 mol% as described in Experimental Procedures. The graph depicts micromoles of C1P produced per minute per milligram of CERK (baculovirus-expressed) versus the mole fraction of ceramide to obtain V_{max} and $K_m{}^b$. Data are presented as means ± SEM and are representative of three separate determinations. C: Specificity constants for the ceramides with different 4-5 bonds. Data presented are specificity constants ($V_{max}/K_m{}^b$) for D-e-$C_{16:0}$ *trans*, $C_{16:0}$ *cis*, $C_{16:0}$ dehydro, $C_{16:0}$ dihydro, and $C_{18:0}$ *trans* ceramides and $C_{18:0}$ phtyo-ceramides ± SEM. Data are representative of three separate determinations. The significance of the differences in specificity constants compared with D-e-$C_{16:0}$ ceramide (for D-e-$C_{16:0}$ dehydro, dihydro, and *cis* ceramide) and D-e-$C_{18:0}$ (for D-e-$C_{18:0}$ phytoceramide) were examined by two-way ANOVA. *** $P < 0.005$.

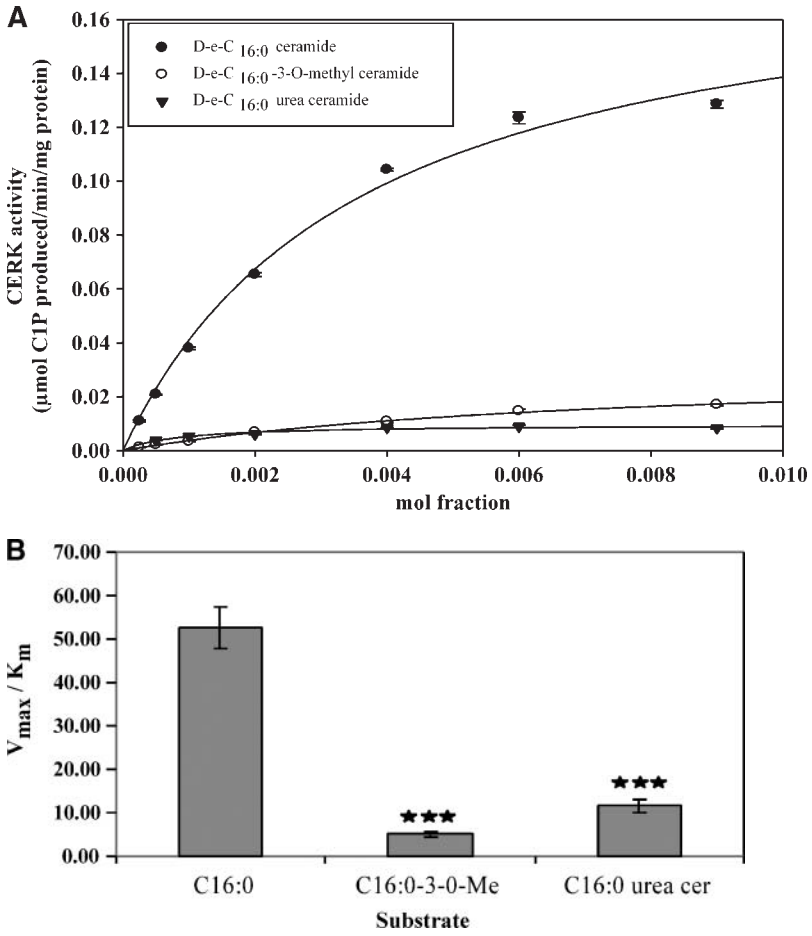

Fig. 5. The secondary amide and the secondary hydroxyl groups of ceramide are important for substrate recognition by CERK. A: Michaelis-Menten curves for $C_{16:0}$, $C_{16:0}$-3-*O*-methyl, and $C_{16:0}$ urea ceramide. D-e-$C_{16:0}$, $C_{16:0}$-3-*O*-methyl, and $C_{16:0}$ urea ceramides were assayed at 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.9, and 1.4 mol% as described in Experimental Procedures. The graph depicts micromoles of C1P produced per minute per milligram of CERK (baculovirus-expressed) versus the mole fraction of ceramide to obtain V_{max} and $K_m{}^b$. Data are presented as means ± SEM and are representative of three separate determinations. B: Comparative specificities of CERK toward modifications to the secondary amide and hydroxyl groups. Data presented are specificity constants ($V_{max}/K_m{}^b$) for $C_{16:0}$, $C_{16:0}$-3-*O*-methyl, and $C_{16:0}$ urea ceramides ± SEM. Data are representative of three separate determinations. The significance of the differences in specificity constants compared with D-e-$C_{16:0}$ ceramide was examined by two-way ANOVA. *** $P < 0.005$.

sion of the enzyme. Therefore, in this study, surface dilution kinetics (33, 51) using Triton X-100 mixed micelles were used to characterize the substrate specificity of CERK. Triton X-100 micelles provide several advantages. First, they provide an inert surface for CERK containing an average of 140 molecules per micelle. Second, the size of the micelles is relatively independent of ionic strength and temperature within the physiological range (52–55). Lastly, mixed micelles up to 20 mol% phospholipids are similar in structure to pure Triton X-100 micelles but proportionally larger (52, 53, 55), and pure Triton X-100 micelles do not coexist with mixed micelles. Therefore, the new Triton X-100-based assay presented here provided an excellent means of studying the kinetics of CERK.

Using this assay, the presented study corroborates the findings of Sugiura et al. (23) in that C_2 ceramide is not a substrate of CERK. In addition, C_4 ceramide was also found not to be phosphorylated by CERK; thus, greater than a C_4 acyl chain is required for recognition by CERK. These previous studies by Sugiura and coworkers (23) also showed C_8 and C_6 ceramides to be good substrates of CERK based on their activity at fixed concentrations, which would be a reflection of the differences in their V_{max}. In contrast, $K_m{}^b$ was taken into account in this study as well as V_{max} to study the substrate specificity ($V_{max}/K_m{}^b$), because this is a more accurate indication in comparing the affinity of an enzyme with different substrates. Therefore, although the V_{max} values of C_6 and C_8 ceramides are in general greater than those of long-chain ceramides, the ratio V_{max}/K_m is dramatically lower by comparison. Sugiura et al. (23) also reported that C_8 dihydroceramide was a good substrate for CERK, with a comparable V_{max} to long-chain ceramides. Our study using naturally occurring D-e-C_{16} ceramides shows that saturation of the 4-5 double bond induces a significant decrease in substrate preference by CERK. Therefore, these studies demonstrate the importance of examin-

ing the K_m and specificity constant for determining substrate preference for lipid kinases.

This study also examined the substrate preference of CERK in depth. As alluded to above, the presence of the 4-5 double bond of the sphingoid moiety is an important feature for recognition by CERK, as dehydroceramide, dihydroceramide, and phytoceramide all showed decreased substrate specificity. In addition, the natural *trans* configuration of the double bond is also important, as the *cis* configuration showed greatly reduced specificity. Thus, the 4-5 double bond of the sphingoid moiety is likely an important contact point for the substrate with CERK. Furthermore, the results disclosed the importance of the sphingoid alkyl chain, because replacement of the alkyl chain with an aromatic phenyl group caused a dramatic loss of phosphorylation. Therefore, the sphingoid moieties of ceramide are essential for substrate recognition and phosphorylation by CERK, explaining why CERK phosphorylates ceramide and not the closely related glycerol lipids like diacylglycerol. For the purpose of designing pharmacologic inhibitors of CERK based on sphingoid moieties, these required features of ceramide that affect the substrate specificities of CERK should be taken into account. For example, at least a six carbon acyl chain should be used, because a shorter acyl chain length is not recognized by CERK. To this end, unpublished findings from our laboratory demonstrate that C_2 ceramide is not an inhibitor of CERK.

The structural requirements for substrate recognition by CERK also appear different from those of other ceramide-utilizing enzymes. CERK showed very high stereospecificity using only the D-*erythro* ceramides as substrates. In contrast, glucosyl ceramide synthase can use both D-*erythro* and L-*erythro* isoforms (56), and Stoffel and Melzner (57) reported that sphingomyelin synthase prefers the L-*threo* isoform over the D-*erythro* isoform. In terms of chain length, neutral ceramidase (45) shows a marked preference for long-chain and unsaturated ceramides. In contrast, CERK does not discriminate between a saturated and unsaturated ceramide. In addition, the phytoceramides 4,5-*cis* ceramide and 3-*O*-methy ceramide were not substrates for ceramidase (45). However, these are substrates for CERK, although the substrate specificity is greatly reduced. CERK does show some similarities to neutral ceramidase in using only the D-*erythro* isoform as a substrate and demonstrating decreased specificity for dihydroceramides. Furthermore, as in the case of neutral ceramidase, CERK does not recognize *N*-methylceramides as substrates. Thus, the recognition of ceramide as a substrate by CERK is more similar to the substrate preferences of ceramidase than to other ceramide-metabolizing enzymes, like glucosylceramide synthase and sphingomyelin synthase. However, there are still clear differences between the substrate preferences of CERK and ceramidase, making CERK unique within the ceramide-metabolizing enzymes.

These studies also suggest a mechanism for the intracellular use of ceramide by CERK. Unpublished findings from our laboratory demonstrate that the major form of C1P found in A549, HeLa, and J774.1 macrophage cells is D-e-$C_{16:0}$ ceramide. As CERK does not discriminate among the long-chain ceramides, the prevalence of particular forms of C1P is likely attributable to substrate availability. Furthermore, a recent report by Baumruker, Bornancin, and Billich (58) localizes CERK to the Golgi apparatus, and ceramide transport protein, the enzyme responsible for actively transporting ceramide to the Golgi apparatus, demonstrates a preference for the D-e-C_{16} ceramide (59). These findings suggest that CERK produces predominantly D-e-$C_{16:0}$ C1P as a result of substrate availability regulated by the transport of ceramides to the Golgi apparatus by ceramide transport protein.

In conclusion, CERK shows very high specificity toward the naturally occurring ceramides. This high specificity allows the enzyme to distinguish between ceramides and other structurally related compounds like sphingosine and diacylglycerol. Furthermore, substrate preference cannot explain the predominance of D-e-$C_{16:0}$ C1P in cells, and CERK demonstrates different requirements for sphingoid moieties than other sphingolipid enzymes. These structural features identified as essential for substrate recognition can now be used as a basis for the rational design of inhibitors to CERK. Because CERK has an established role in the inflammatory pathway as an upstream activator of cPLA$_2\alpha$, inhibitors of CERK may become a new generation of anti-inflammatory drugs. With the cyclooxygenase-2 inhibitors Vioxx [4-(4-methylsulfonylphenyl)-3-phenyl-5H-furan-2-one)] and Bextra [4-(5-methyl-3-phenyl-isoxazol-4-yl)benzenesulfonamide] now withdrawn from the pharmaceutical market, the design of novel anti-inflammatory compounds is a high priority as therapeutics for disorders like arthritis, asthma, multiple sclerosis, and Alzheimer's disease.

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