The structural requirements for ceramide activation of serine-threonine protein phosphatases

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Abstract **The protein phosphatases1 (PP1) and 2A (PP2A) serve as ceramide-activated protein phosphatases (CAPP). In this study, the structural requirements for interaction between ceramide and CAPP were determined. D-*erythro*-C_6 ceramide activated the catalytic subunit of PP2A (PP2Ac) approximately 3-fold in a stereospecific manner. In contrast, saturation of the 4-5 double bond, producing D-*erythro*-dihydro C_6 ceramide, inhibited PP2Ac (IC_{50} = 8.5 μM). Furthermore, phyto C_6 ceramide, D-*erythro*-dehydro C_6 ceramide, and D-*erythro*-*cis*-C_6 ceramide had no effect on PP2Ac activity. Modification of the sphingoid chain also abolished the ability of ceramide to activate PP2Ac. Further studies demonstrated the requirement for the amide group, the primary hydroxyl group, and the secondary hydroxyl group of the sphingoid backbone for activation of PP2Ac through the synthesis and evaluation of D-*erythro*-urea C_6 ceramide, L-*erythro*-urea C_6 ceramide, D-*erythro*-N-methyl C_6 ceramide, D-*erythro*-1-O-methyl C_6 ceramide, D-*erythro*-3-O-methyl C_6 ceramide, and (2S) 3-keto C_6 ceramide. None of these compounds induced significant activation of PP2Ac. Liposome binding studies were also conducted using analogs of D-*erythro*-C C_6 ceramide, and the results showed that the ability of ceramide analogs to influence CAPP (activation or inhibition) was associated with the ability of the analogs to bind to CAPP. This study demonstrates strict structural requirements for interaction of ceramide with CAPP, and disclose ceramide as a very specific regulator of CAPP. The studies also begin to define features that transform ceramide analogs into inhibitors of CAPP.**—Chalfant, C. E., Z. Szulc, P. Roddy, A. Bielawska, and Y. A. Hannun. **The structural requirements for ceramide activation of serine-threonine protein phosphatases. *J. Lipid Res.* 2004.** 45: **496–506.**

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Many lines of evidence have suggested ceramide as an important intracellular regulator of various stress responses and growth mechanisms (1–9). These emerging roles of ceramide necessitate a mechanistic understanding of ceramide action. This has led to the identification of several candidate ceramide-regulated enzymes, including ceramide-activated protein kinase, protein kinase Cζ, cathepsin D, and ceramide-activated protein phosphatase (CAPP) (10–18).

Initially, CAPP was identified as a member of the 2A class of serine-threonine phosphatases (17–19). Subsequently, two serine-threonine phosphatases, protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A), have been shown to be ceramide responsive in vitro and in vivo (16–19). PP2A is found primarily as a heterotrimeric enzyme in cells containing a catalytic subunit (PP2Ac), an A subunit, and a B subunit (possible role in intracellular targeting). PP1 is primarily purified as a heterodimeric enzyme in cells bound to a number of described subunits (e.g., nuclear inhibitor of protein phosphatase-1, pyrimidine tract-binding protein-associated splicing factor). Whether all forms of PP1 and PP2A are activated by ceramide remains to be determined, but the catalytic subunits of these serine-threonine protein phosphatases are now collectively termed ceramide-activated protein phosphatases.

Several in vitro substrates have now been described for CAPPs, including Bcl-2, protein kinase Cα, c-*jun*, SR proteins, and AKT/PKB (20–25). Additional studies also suggest that these substrates are dephosphorylated in response to exogenous ceramides or ceramide-inducing agonists (21, 23). Nevertheless, it is not fully established that these substrates are directly dephosphorylated by CAPP in vivo. A key parameter in determining the specificity and significance of CAPP regulation by ceramide is the study of the specificity of the interaction of these phosphatases with ceramide.

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Abbreviations: PP1, protein phosphatase-1; PP2A, protein phosphatase-2A.

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In this study, we examined the structural requirements within the ceramide molecule necessary for both the activation and association with CAPPs. The results disclose that even slight modifications to the sphingoid backbone greatly decrease the ability of the lipid to interact with and activate CAPP. Thus, the present study shows a high degree of specificity for ceramide by these serine-threonine protein phosphatases, and lends further evidence for the significance of the interaction of ceramide with PP1 and PP2A.

MATERIALS AND METHODS

Serinol and all general chemicals were purchased from Aldrich. (2S, 3S, 4R)-phytosphingosine was 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. ^{1}H-NMR spectra were recorded using a Bruker AVANCE 500 MHz spectrometer equipped with an Oxford Narrow Bore Magnet. Chemical shifts are given in ppm downfield from tetramethylsilane as internal standard, and the listed J values are in Hz. Mass spectral data were obtained in positive ion electrospray ionization (ESI) mode on a Finnigan LCQ ion trap mass spectrometer. Samples were infused in methanol solution with an ESI voltage of 4.5 kV and a capillary temperature of 200°C. The purity of all synthesized lipids was >95% as estimated by TLC and ^{1}H-NMR analysis.

All stereoisomers of C_6-ceramide were prepared from their corresponding sphingosines as described previously (26–28). Sphingosines of specific stereochemical foundations [IUB nomenclature: D-*erythro*-(2S,3R); L-*threo*-(2S,3S)] and the key intermediates *N*- and 1,3-*O*-protected sphingosines were utilized as basic substrates in the synthesis of the target compounds. Starting from a known configurationally stable chiral auxiliary (Garner's aldehyde prepared from L-serine), we synthesized (2S)-sphingosines, 4,5-dihydro-sphingosines and 4,5-dehydro-sphingosines (29–31). Starting from D-serine, we had access to all remaining 2R-isomers (29–31). (2S, 3R, 4Z)-sphingosine, (2S, 3R, 4E)-1-*O*-methyl–sphingosine, (2S, 3R, 4E)-3-*O*-methyl–sphingosine, and (2S, 3R, 4E)-*N*-methyl-sphingosine, as well as all C_{16}-ceramide analogs (see Table 2), were synthesized following a previously described procedure (32, 33). D-myristoylaminophenylpropanol (D-MAPP), L-MAPP and their C_6-analogs were synthesized as previously described (34). Analogs of C_6-ceramide and C_6- and C_{16}-serinol listed in **Tables 1** and **2** were prepared in our laboratory and characterized as shown below.

Synthesis of (2S, 3R)-*N*-hexanoyl-4,5-dehydro-sphingosine (D-*erythro*-dehydro-C_6-ceramide)

This compound was prepared from (2S, 3R)-4,5-dehydro-sphingosine and hexanoyl chloride following a general acylation procedure described previously (26, 28, 32–34). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 94:6, v/v) and crystallized from ethyl acetate-hexane (3:1, v/v) to give a pure D-*erythro*-dehydro C_6 ceramide as a white microcrystalline powder [melting point (mp) 73–75°C, 72% yield]. TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.61. ^{1}H-NMR ($CDCl_3$), δ 6.25 (d, J = 7.4, NH), 4.58 (m, 1H, 3-H), 4.11(dd, J = 11.4, 3.7, 1-H), 4.03 (m, 1H, 2-H), 3.73 (dd, J = 11.3, 3.5, 1-H), 2.23 [m, 4H, C(6)H_2 and $COCH_2$], 1.63 (m, 2H, $COCH_2CH_2$), 1.49 [m, 2H, C(7)H_2], 1.25 (m, 24H, CH_2), 0.86 (t, 6H, J = 7.0, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 813.2 ($[2M + Na]^+$, 100), 613.2 ($[2M + Na + H - COC_5H_{11}]^+$, 65), 396.2 ($[MH]^+$, 38), 378.3 ($[MH - H_2O]^+$, 20). Calculated for $C_{24}H_{45}NO_3$ *m/z* 395.3.

TABLE 1. The structural requirements of ceramide for the activation of trimeric protein phosphatase-2A and protein phosphatase-1cα

Compound	PP2Ac	PP2A	PP1cα
Vehicle control	193	211	180
D-e-C_6 ceramide	537 (2.8)	491 (2.3)	446 (2.5)
D-e-dihydro C_6 ceramide	45 (0.2)	24 (0.1)	32 (0.2)
L-e-dihydro C_6 ceramide	43 (0.2)	21 (0.1)	37 (0.2)
D-e-dehydro C_6 ceramide	192 (1.0)	52 (0.3)	183 (1.0)
D-e-*cis* C_6 ceramide	190 (1.0)	183 (0.9)	171 (1.0)
Phyto C_6 ceramide	213 (1.1)	82 (0.4)	160 (0.9)
C_6-serinol	201 (1.0)	137 (0.7)	181 (1.0)
C_6-D-MAPP	190 (1.0)	53 (0.3)	175 (1.0)
C_6-L-MAPP	202 (1.0)	61 (0.3)	171 (1.0)
D-e-C_6 urea ceramide	198 (1.0)	229 (1.1)	127 (0.7)
L-e-C_6 urea ceramide	210 (1.1)	210 (1.0)	141 (0.8)
N-methyl-D-e-C_6 ceramide	182 (0.9)	200 (1.0)	157 (0.9)
1-*O*-methyl D-e-C_6 ceramide	193 (1.0)	86 (0.4)	173 (1.0)
3-*O*-methyl D-e-C_6 ceramide	196 (1.0)	92 (0.4)	169 (0.9)
3-keto C_6 ceramide	181 (0.9)	99 (0.5)	179 (1.0)

Catalytic subunit of protein phosphatase-2A (PP2Ac), trimeric protein phosphatase-2A (PP2A) (10 mU), and protein phosphatase-1cα (PP1cα) (10 mU) were assayed as described (35) in the presence of 15 μM of the indicated ceramide analogs. Data are presented as mean phosphatase activity in fmoles of ^{32}P release from ^{32}P-MBP per minute (n = 4). Data within parentheses depicts fold stimulation. Data are representative of four separate determinations on two occasions. MAPP, myristoylaminophenylpropanol.

Synthesis of (2S, 3S, 4R)-*N*-hexanoyl-phytosphingosine (phyto-C_6-ceramide)

This compound was prepared from (2S, 3S, 4R)-phytosphingosine and hexanoyl chloride following a general acylation procedure described previously (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 10:1, v/v) following crystallization from ethyl acetate-acetone (1:1, v/v) to give a pure phyto-C_6-ceramide as a white microcrystalline powder (mp 88–90°C, 68% yield). TLC, $CHCl_3$-

TABLE 2. The relative binding affinities of ceramide analogs to PP2Ac

Compound	PP2Ac Binding (Arbitrary Densitometry Units)
D-e-C_{16} ceramide	9160 ± 576
D-e-dihydro C_{16} ceramide	8794 ± 321
D-e-dehydro C_{16} ceramide	1131 ± 267
D-e-*cis* C_{16} ceramide	ND
Phyto C_{16} ceramide	1002 ± 652
C_{16} serinol	ND
C_{16} D-MAPP	ND
C_{16} L-MAPP	ND
D-e-C_{16} urea ceramide	1342 ± 111
L-e-C_{16} urea ceramide	ND
N-methyl-D-e-C_{16} ceramide	ND
1-*O*-methyl-D-e-C_{16} ceramide	1002 ± 213
3-*O*-methyl-D-e-C_{16} ceramide	ND
3-keto C_{16} ceramide	ND

PP2Ac was complexed with D-*erythro*-C_{16} ceramide analogs as described in Materials and Methods. PP2Ac/ceramide complexes were subjected to PAGE-SDS analysis and Western immunoblotting. Data are presented as the mean of arbitrary densitometry units ± SE of the binding of PP2Ac visualized by chemiluminesence after background subtraction. ND = not detected. Data are representative of three separate determinations on two occasions.

MeOH, 10:1, v/v; R_f 0.41; CH_2Cl_2-MeOH, 9:1, v/v; R_f 0.40 (35, 45). EI-MS (CH_3OH; relative intensity, %) *m/z* 853.8 ($[2M + Na]^+$, 35), 416.3 ($[MH]^+$, 100), 398.4 ($[MH - H_2O]^+$, 22). Calculated for $C_{24}H_{49}NO_4$ *m/z* 415.4.

Synthesis of (2S, 3R, 4Z)-*N*-hexanoyl-sphingosine (D-*erythro-cis*-C_6-ceramide)

This compound was prepared from (2S, 3R, 4Z)-sphingosine and hexanoyl chloride following a general acylation procedure described previously (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 94:6, v/v) and isolated as a waxy semisolid (65% yield). TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.57. ^{1}H-NMR ($CDCl_3$), δ 6.21 (d, J = 7.1, NH), 5.60 (dtd, J = 11.3, 9.0, 1.0, 5-H), 5.49 (ddt, J = 11.2, 9.2, 1.5, 4-H), 4.62 (dd, J = 8.5, 4.2, 3-H), 4.1 (dd, J = 11.1, 3.6, 1-H), 3.84 (m, 1H, 2-H), 3.70 (dd, J = 11.2, 3.5, 1-H), 2.21 (t, J = 7.5, $COC\underline{H}_2$), 2.2 (m, 2H, C(6)H_2), 1.65 (m, 2H, C(7)H_2), 1.26 (m, 26H, CH_2), 0.90 (t, 6H, J = 7. 1, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 817.6 ($[2M + Na]^+$, 100), 398.3 ($[MH]^+$, 35), 380.3 ($[MH - H_2O]^+$, 10). Calculated for $C_{24}H_{47}NO_3$ *m/z* 397.4.

Synthesis of (2S, 3R, 4E)-1-*O*-methyl–*N*-hexanoyl-sphingosine (1-*O*-methyl-D-*erythro*-C_6-ceramide)

This compound was prepared from (2S, 3R, 4E)-1-*O*-methyl-sphingosine and hexanoyl chloride following the general acylation procedure described previously (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) and was isolated as a waxy semisolid (62% yield). TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.65. ^{1}H-NMR ($CDCl_3$): δ 6.12 (d, J = 6.7, NH), 5.85 (dtd, J = 11.8, 7.9, 1.6, 5-H), 5.45 (ddt, J = 11.2, 9.6, 1.3, 4-H), 4.13 (m, 1H, 3-H), 4.02 (m, 1H, 2-H), 3.69 (m, 1H, 1-H), 3.55–3.46 (m, 1H, 1-H), 3.32 (s, 3H, OCH_3), 2.20 (m, 2H, $COCH_2$), 2.08 [m, 2H, C(6)H_2], 1.65 (m, 2H, $COCH_2C\underline{H}_2$), 1.24 (m, 26H, CH_2), 0.87 (t, 6H, J = 7.0, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 845.0 ($[2M + Na]^+$, 100), 637.1(75), 411.9 (MH^+, 25), 394.1 ($[MH - H_2O]^+$, 73), 264.2 ($[MH - CH_3OH - H_2O - COC_5H_{11}]^+$, 8). Calculated for $C_{25}H_{49}NO_3$ *m/z* 411.4.

Synthesis of (2S, 3R, 4E)-3-*O*-methyl-*N*-hexanoyl-sphingosine (3-*O*-methyl-C_6-ceramide)

This compound was prepared from (2S, 3R, 4E)-3-*O*-methyl-sphingosine and hexanoyl chloride following the same general acylation procedure (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) and isolated as a waxy white semisolid (60% yield). TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.61. ^{1}H-NMR ($CDCl_3$), δ 6.18 (d, J = 7.7, NH), 5.74 (dtd, J = 11.1,8.9, 1.0, 5-H), 5.35 (ddt, J = 11.2, 9.2, 1.5, 4-H), 3.95 (m, 1H, 1-H), 3.90 (m, 1H, 2-H), 3.83 (dd, J = 7.8, 3.7, 3-H), 3.55 (m, 1H, 1-H), 3.25 (s, 3H, OCH_3), 2.21 (t, 2H, J = 7.6, $COCH_2$), 2.06 [q, J = 7.1, C(6)H_2], 1.62 (m, 2H, $COCH_2C\underline{H}_2$), 1.35 [m, 2H, C(7)H_2], 1.21 (m, 24H, CH_2), 0.87 (t, 6H, J = 7.1, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 877.4 ($[2M + 2Na]^+$, 12), 845.1 ($[2M + Na]^+$, 100), 637.1(15), 434.2($[M + Na]^+$, 15), 411.7 ($[MH]^+$, 8), 380.1(12), 264.2($[MH - CH_3OH - H_2O - COC_5H_{11}]^+$, 5). Calculated for $C_{25}H_{49}NO_3$ *m/z* 411.4.

Synthesis of (2S, 4E)-3-keto-C_6-ceramide (3-keto-C_6-ceramide)

This compound was prepared from D-*erythro*-C_6-ceramide by the selective oxidation of its secondary hydroxyl group following the procedure described for the *N*-acetyl derivative (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) as a waxy semisolid (55% yield). TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.63. ^{1}H-NMR ($CDCl_3$) δ = 7.10 (dt, J = 15.7, 7.1, 5-H), 6.70 (d, J = 6.2, NH), 6.24 (d, J = 15.8, 4-H), 4.87(m, 1H, 2-H), 3.95 (m, 1H, 1-H), 3.80 (m, 1H, 1-H), 2.24 [m, 4H, C(6)CH_2, $COCH_2$], 1.62 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.24 (m, 24H, CH_2), 0.88 (t, 6H, J = 7.0, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 813.4 ($[2M + Na]^+$, 100), 396.2($[MH]^+$, 25). Calculated for $C_{24}H_{45}NO_3$ *m/z* 395.3.

Synthesis of (2S, 3R, 4E)-*N*-methyl-*N*-hexanoyl-sphingosine (N-Me-D-*erythro*-C_6-ceramide)

This compound was prepared from (2S, 3R, 4E)-*N*-methyl-sphingosine and hexanoyl chloride following a general acylation procedure described previously (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 96:4, v/v) as a waxy semisolid (58% yield). TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.58. ^{1}H-NMR ($CDCl_3$); mixture of two conformers, δ = 5.60 (m, 1H, 5-H), 5.42 (m, 1H, 4-H), 4.41 (m, 0.45H, 2-H), 4.11 (t, 0.55H, J = 10.1,3-H), 4.77 (t, 0.45H, J = 10.4, 3-H), 4.0 (dd, 0.45H, J = 3.4, 10.77, 1-H), 3.93 (dd, 0.55H, J = 3.0, 8.1, 1-H), 3.84 (m, 1.45H, 1-H, 2-H), 3.76 (dd, 0.55H, J = 9.0, 10.4, 1-H), 2.95 (s, 1.60H, NCH_3), 2.79 (s, 1.40H, NCH_3), 2.45 (m, 0.9H, $COCH_2$), 2.32 (m, 1.1H, $COCH_2$), 2.0 [m, 2H, C(6)H_2], 1.6 (m, 2H, $COCH_2C\underline{H}_2$), 1.28 (m, 26H, CH_2), 0.88 (t, 6H, J = 7.0, CH_3); EI-MS (CH_3OH; relative intensity, %): *m/z* 1,256.5 ($[3M + Na]^+$, 7), 845.3 ($[2M + Na]^+$, 100), 823.3 ($[2M + H]^+$, 8), 412.3 (MH^+, 10), 394.4 ($[MH - H_2O]^+$, 8). Calculated for $C_{25}H_{49}NO_3$ *m/z* 411.4.

Synthesis of (2S, 3R, 4E)-*N*-[2-(1,3-dihydroxy-4E-octadecene)]N'-hexane-urea (D-*erythro*-C_6-urea-ceramide)

This compound was prepared following the procedure described previously (28, 32, 33). To a solution of (2S, 3R, 4E)-sphingosine (84.0 mg, 0.28 mmol) in anhydrous acetonitril (4 ml) and anhydrous chloroform (3 ml), hexyl isocyanate (0.082 ml, 0.56 mmol) was added, and the mixture was stirred at room temperature under nitrogen for 2 h. After evaporation of the solvents under a reduced pressure, the residue was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 50:4, v/v) following crystallization from acetone-ethyl acetate (1:1, v/v) to give a pure urea isoster of C_6-ceramide as a white microcrystalline powder (mp 95–97°C, 89.1 mg, 75% yield); TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.58. ^{1}H-NMR (MeOD-$CDCl_3$), δ 5.38 (dtd, J = 15.3, 6.5, 1.3, 5-H), 5.35 (ddt, J = 15.3, 6.7, 1.2, 4-H), 3.77 (m, 1H, 3-H), 3.40 (m, 2H, 1-H, 2-H), 3.35 (dd, J = 10.1, 3.5, 1-H), 2.80 (m, 2H, $NHC\underline{H}_2$), 1.72 [m, 2H, C(6)H_2], 1.18 (m, 2H, $NHCH_2C\underline{H}_2$), 1.0 (m, 28H, CH_2), 0.60 (t, 6H, J = 7.1, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 875.4 ($[2M + Na]^+$, 40), 853.4 ($[2M+H]^+$, 55), 427.1 (MH^+, 92), 409.3 ($[MH - H_2O]^+$, 100). Calculated for $C_{25}H_{50}N_2O_3$ *m/z* 426.3.

Synthesis of *N*-hexanoyl-serinol (C_6-serinol)

This compound was prepared from serinol and hexanoyl chloride following a general acylation procedure described previously (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 10:1.5, v/v) following crystallization from acetone-ethyl acetate (1:1, v/v) to give a pure C_6-serinol as white needles (65% yield, mp 100–101°C). TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.22. ^{1}H-NMR (MeOD), δ 3.95 (q, 1H, J = 5.6, 2-H), 3.64 (d, 4H, J = 5.2, 1-H), 2.21 (t, 2H, J = 7.5, $COCH_2$), 1.60 (m, 2H, $COCH_2C\underline{H}_2$), 1.31 (m, 24H, CH_2), 0.90 (t, 3H, J = 7.0, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 400.8 ($[2M + Na]^+$, 100), 303.6($[2M + Na^+H$-

$COC_5H_{11}]^+$, 80), 212.0 ($[M + Na]^+$, 12) 190.0($[MH]^+$, 9). Calculated for $C_9H_{19}NO_3$ *m/z* 189.1.

Synthesis of *N*-hexadecanoyl-serinol (C_{16}-serinol)

This compound was prepared from serinol and palmitoyl chloride following a general acylation procedure described previously (28, 32, 33). The crude product was purified by flash column chromatography (elution with $CHCl_3$-MeOH, 10:1.5, v/v) following crystallization from acetone-ethanol (2:1, v/v) to give a pure C_{16}-serinol as white needles (mp 126–126.5°C, 65% yield); [mp 125.5–126°C (36)]. TLC, $CHCl_3$-MeOH, 10:1, v/v; R_f 0.28. ^{1}H-NMR (MeOD), δ 3.90 (q, 1H, J = 5.2, 2-H), 3.60 (d, 4H, J = 5.4, 1-H), 2.20 (t, 2H, J = 7.6, $COCH_2$), 1.59 (m, 2H, $COCH_2CH_2$), 1.28 (m, 24H, CH_2), 0.89 (t, J = 7.2, CH_3). EI-MS (CH_3OH; relative intensity, %) *m/z* 681.0 ($[2M + Na]^+$, 100), 658.7 ($[2M + H]^+$, 50), 330.1($[MH]^+$, 42). Calculated for $C_{19}H_{39}NO_3$ *m/z* 329.3.

Serine-threonine protein phosphatase assays

Myelin basic protein (MBP) was labeled in a 0.5 ml reaction containing 1 mg of MBP, 50 mM Tris-HCl (pH 7.4), 90 mM $MgCl_2$, 4 mM ATP, 5 mM DTT, 10 mM β-mercaptoethanol, 2 μCi [^{32}P-γ]ATP, and 125 U of protein kinase A. After the components were mixed, the reaction was incubated at 37°C for 2 h. MBP was purified by TCA precipitation and acetone wash as described (35). Phosphatase assay reactions were carried out in 1.5 ml polypropylene tubes in a reaction volume of 100 μl. Ceramides were added to tubes containing PP2Ac purified from rabbit skeletal muscle (Promega Corporation) (10 mU), 50 mM Tris-HCl (pH 7.4), 100 μM $MnCl_2$, and 150 mM NaCl with the ethanol concentration not exceeding 1%. Components were preincubated for 10 min at 30°C. Reactions were initiated with 0.005 μl of [^{32}P]MBP (1 mg/ml) in buffer A. After 10 min at 30°C, the assay was terminated by the addition of 375 μl 60% TCA (ice-cold) followed by the addition of 275 μl fatty acid-free BSA (0.9 mg/ml). After 10 min incubation on ice, reactions were centrifuged at 10,000 *g* for 15 min at 4°C, 250 μl of the supernatant was subjected to β counting, and data were then collected. Human recombinant PP1cα (from *Escherichia coli* expression) (Calbiochem) and rabbit PP2A trimer from skeletal muscle (Calbiochem) were assayed as described for PP2Ac. For PP1cα, 1 unit of activity was defined as the amount of enzyme that will hydrolyze 1.0 nmol of *p*-nitrophenyl phosphate per min at 30°C, pH 7.0. For PP2A trimer and catalytic subunit, 1 unit of activity was defined as the amount of enzyme that will hydrolyze 1.0 nmol of phosphorylase per min at 30°C, pH 7.0.

Ceramide binding assays

In initial studies, we evaluated several methods for ceramide binding, such as lipid-protein overlay assays (35), mixed-vesicle sucrose-loaded vesicle assay (36, 37), and [^{14}C]lipid precipitation assays (38, 39). The lipid-protein overlay assay failed to demonstrate ceramide binding, likely due to low-affinity binding. Protein kinase C also failed to bind diacylglycerol (a neutral lipid with properties similar to ceramide) using this approach. We find that the lipid-protein overlay assay is most useful for high-affinity lipid-protein interactions. With the other binding assays evaluated (mixed vesicles and ceramide precipitation), the nonspecific binding was too high for evaluation of ceramide interaction with PP2A. A large multilamellar vesicle assay (LMV assay) has been used to evaluate the binding of phosphatidylcholine to cytosolic phospholipase A2 and the binding of phosphatidylserine to phosphotyrosine phosphatase-MEG2 (40, 41). Following the published procedure for the LMV assay, vesicles for ceramide and ceramide analogs were produced as described for other lipids (40) by drying 68.3 μl of a 1 mg/ml D-*erythro*-C_{16} ceramide or ceramide analogs in solution under nitrogen per reaction. A solution (100 μl per reaction) of 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl was added, and the lipid was vortexed vigorously for 2 min. Ceramide or ceramide analogs (100 μl) were mixed with buffer A [200 μl of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 100 μM $MnCl_2$]. The binding reaction was initiated by the addition of 100 μl of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 100 μM $MnCl_2$ containing 0.2 μg of PP2Ac. After a 5 min incubation at room temperature, the reaction was centrifuged at 10,000 *g* for 10 min and the supernatant discarded. Laemmli buffer (200 μl) was added to the lipid pellet and resolved on 10% polyacrylamide gels (SDS-PAGE) under denaturing conditions and then transferred to 0.20 μm polyvinylidene difluoride membranes. After blocking overnight at 4°C with 5% nonfat milk in phosphate-buffered saline/0.05% Tween 20 and washing, the membranes were incubated with anti-human PP2Ac antibody (C20) (Santa Cruz Biotechnology) for 2 h at room temperature. The membranes were washed extensively in phosphate-buffered saline/0.05% Tween 20 (washing buffer). Bands were visualized using the appropriate horseradish peroxidase-conjugated anti-mouse IgG antibody and the ECL Western blotting detection system (Amersham Pharmacia Biotech).

RESULTS

To determine the molecular basis of the interaction between PP2Ac and ceramide, we focused on key features in the ceramide molecule required for the activation of the phosphatase. These include the stereochemistry at the C2, C3, C4, and C5 positions, 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, and hydroxylation of the natural *trans* C4-C5 double bond and the role of the alkyl chain of the sphingoid backbone were also investigated (Fig. 1). Short-chain ceramides were chosen for two reasons. First, the solubilities between different structural variants would be more equivalent than with more insoluble long-chain ceramides (all of the D-*erythro*-C_6 ceramide analogs were completely soluble in ethanol and after delivery to aqueous buffer). Second, long-chain ceramides have to be delivered as a mixed vesicle/particle with dodecane (16), and under these conditions, structural variants of ceramide may interact differently with dodecane, i.e., they may fail to produce vesicles or may be insoluble (C. E. Chalfant et al., unpublished findings), thereby making it difficult to distinguish direct effects from indirect ones related to vesicle composition.

Stereochemistry

There are two asymmetrical carbon atoms within the ceramide molecule, and thus, four stereoisomers are possible. D-*erythro*-C_6 ceramide (2S, 3R) is the naturally occurring configuration, with L-*erythro*-C_6 ceramide (2R, 3S) being its enantiomer. Additionally, enantiomeric pairs of the *threo*-diastereoisomers can be formed, i.e., L-*threo* (2S, 3S) and D-*threo* (2R, 3R). As with long-chain ceramides (16), only the D-*erythro* configuration of C_6 analogs activated PP2Ac in a significant manner in the presence of 150 mM

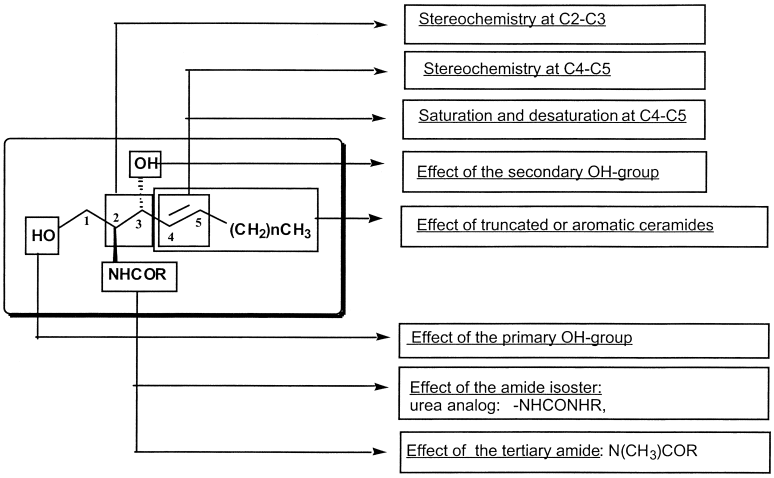

Fig. 1. Scheme of structural modifications of ceramide.

NaCl (**Fig. 2**). Thus, short-chain ceramides are valid for use in specificity studies for CAPPs, inasmuch as they mimic the results of long-chain ceramides (16).

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 that has been shown to be biologically inactive in cells (26). D-*erythro*-dihydro-C_6 ceramide was ineffective in activating PP2Ac in vitro. Furthermore, D-*erythro*-dihydro-C_6 ceramide inhibited PP2Ac activity with an IC_{50} of ~8.5 μM (**Fig. 3**). This inhibition of PP2Ac was not stereospecific, inasmuch as L-*erythro*-dihydro C_6 ceramide inhibited the enzyme to a similar extent with an indistinguishable dose response (Fig. 3). Thus, the inhibition of PP2Ac by dihydroceramide demonstrates less specificity in protein-lipid interaction than does the activation by ceramide.

To further examine the necessity of the 4-5 *trans* double bond of ceramide for activation of PP2Ac, this bond was further unsaturated to D-*erythro*-dehydro-C_6 ceramide, bearing a triple bond between the 4 and 5 carbons of the sphingoid backbone. This compound had no effect on PP2Ac activity in vitro (**Fig. 4**). Natural ceramide has the 4-5 double bond in the *trans*-configuration. To examine whether this configuration was required for activation of PP2Ac by ceramide, 4,5-*cis*-D-*erythro*-C_6 ceramide was synthesized. This compound was also ineffective in activating PP2Ac (Fig. 4). Recently, phytoceramides were described in mammalian cells (42);. therefore the effects of phytoceramides (4-hydroxy-dihydroceramides) were investigated. C_6-phytoceramide also did not induce significant activation of PP2Ac (Fig. 4). Thus, these data demonstrate that the activation of PP2Ac by ceramide has a strict requirement for a *trans*-orientation of 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 demonstrate a lack of activating ability for CAPPs (17, 18, 43). The question remains, however, whether the sphingoid-base chain is necessary for activation of CAPPs by ceramides. To this end, serinol ceramide (a ceramide analog with a truncated sphingosine backbone containing only the C1-C3 part from serine) was synthesized and tested in vitro (44). The results show that *N*-hexanoyl-serinol (serinol ceramide) had no effect on PP2Ac activity (**Fig. 5**). The structural features of the sphingoid moiety were further investigated by replacing the sphingoid-alkenyl chain with an aromatic phenyl group producing two enantiomers, D-*erythro*-2-(*N*-hexanoylamino)-1-phenyl-1-propanol (C_6-D-MAPP) and L-*erythro*-2-(*N*-hexanoylamino)-1-phenyl-1-propanol (C_6-L-MAPP). Neither of these compounds had a significant effect on PP2Ac activity (Fig. 5). There-

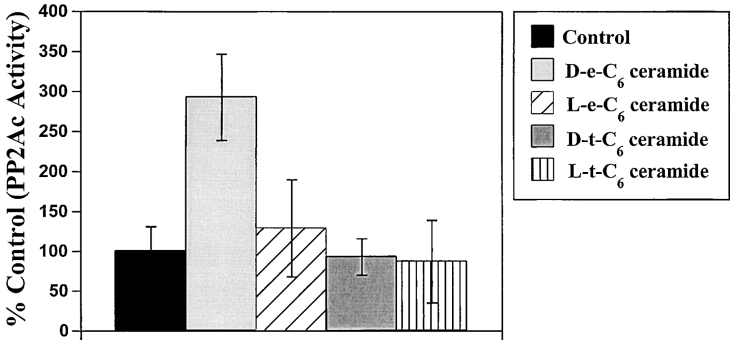

Fig. 2. The effects of stereoisomers of D-*erythro*-C_6 ceramide on the enzymatic activity of protein phosphatase-2Ac (PP2Ac). PP2Ac (10 mU) was assayed as described (35) in the presence of 15 μM of C_6 ceramide stereoisomers. Data are presented as mean percent control ± SE (n = 4) of basal PP2A activity measured by corrected disintegrations per minute of liberated ^{32}P from ^{32}P-myelin basic protein (^{32}P-MBP).

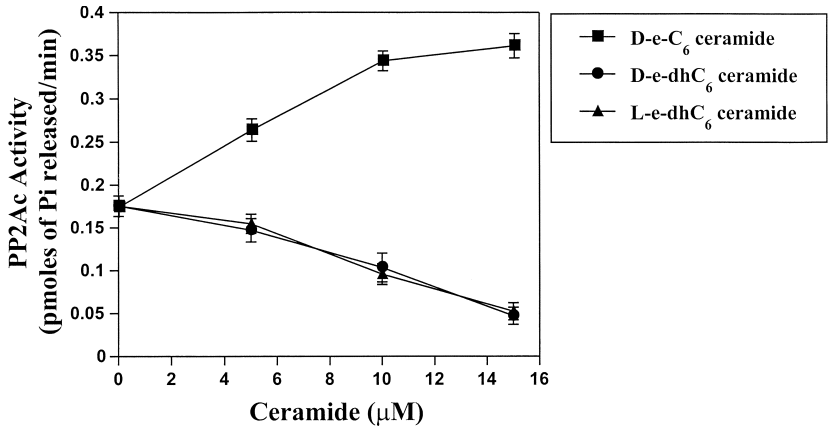

Fig. 3. The effect of dihydroceramide on the enzymatic activity of PP2Ac. PP2Ac (10 mU) was assayed as described (35) in the presence of the indicated concentrations of D-*erythro*-C_6 ceramide, D-*erythro*-dihydro C_6 ceramide (D-*erythro*-dh C_6 ceramide), or L-*erythro*-dihydro C_6 ceramide (L-*erythro*-dh C_6 ceramide). Data are presented as mean PP2Ac activity in pmoles of ^{32}P release from ^{32}P-MBP per min ± SE (n = 4).

fore, the sphingosine backbone chain is necessary for PP2Ac activation.

Amide group

To determine the importance of the secondary amide group, NHCO, in the ceramide structure, we synthesized the ceramide analog D-*erythro*-urea-ceramide bearing the NHCONH group. Also, we evaluated the activity of its enantiomer, L-*erythro*-urea-C_6 ceramide (Fig 1). These compounds had no effect on PP2Ac activity (**Fig. 6**). These results indicate the requirement for the *N*-acyl-amide bond in the activation of CAPPs. Next, the role of the hydrogen atom from the amide bond NHCO was examined using *N*-methyl-C_6 ceramide as a model. This compound also had no effect on PP2Ac activity, thus, demonstrating the necessity of the free hydrogen in the secondary amide group for PP2Ac activation (Fig. 6).

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_6 ceramide and 3-*O*-methyl-C_6 ceramide. Neither of these compounds affected PP2Ac activity (**Fig. 7**). To further examine the role of the secondary hydroxyl group for CAPP activation, (2S) 3-keto-C_6 ceramide was synthesized. This compound again had no effect on PP2Ac activity (Fig. 7). Thus, the primary and secondary hydroxyl groups of the ceramide molecule appear to be required for activation of PP2Ac.

The holoenzyme, trimeric PP2A, and PP1cα demonstrate the same structural requirements of the ceramide molecule for activation

The above results demonstrate that the activation of monomeric PP2Ac by ceramide is very specific, requiring many of the structural features in the sphingoid backbone. PP2A

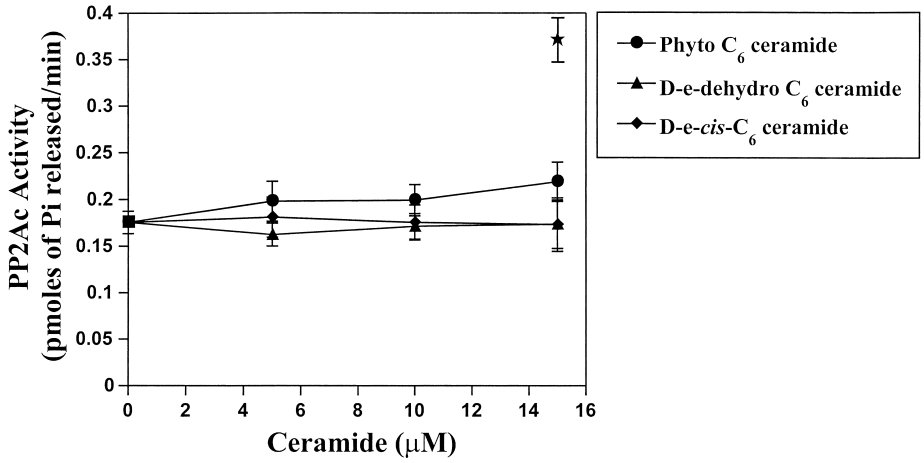

Fig. 4. The structural requirements of the 4-5 double bond. PP2Ac (10 mU) was assayed as described (35) in the presence of the indicated concentrations of the depicted analogs of C_6 ceramide. The asterisk indicates the positive control [D-*erythro*-C_6 ceramide (15 μM)] for these experiments. Data are presented as mean PP2Ac activity in pmoles of ^{32}P release from ^{32}P-MBP per min ± SE (n = 4).

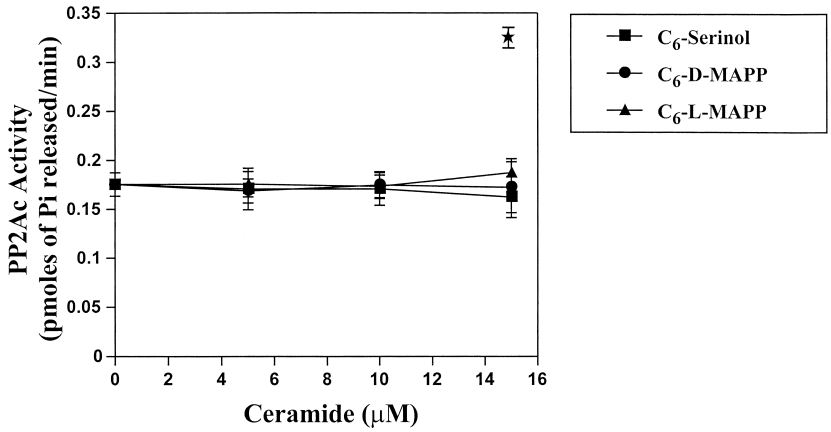

Fig. 5. The structural requirement of the sphingosine chain. PP2Ac (10 mU) was assayed as described (35) in the presence of the indicated concentrations of C_6-serinol, D-*erythro*-2-(*N*-hexanoylamino)-1-phenyl-1-propanol [C_6-D-myristoylaminophenylpropanol (C_6-D-MAPP)], or L-*erythro*-2-(*N*-hexanoylamino)-1-phenyl-1-propanol (C_6-L-MAPP). The asterisk indicates the positive control [D-*erythro*-C_6 ceramide (15 μM)] for these experiments. Data are presented as mean PP2Ac activity in pmoles of ^{32}P release from ^{32}P-MBP per min ± SE (n = 4).

is usually a trimeric complex in cells, and to determine whether this level of specificity extended to the holoenzyme, trimeric PP2A was examined for activation by structural analogs of ceramide. Using the maximal activating dose of D-*erythro*-C_6 ceramide (15 μM), trimeric PP2A demonstrated the same structural specificity for ceramide as the catalytic subunit (Table 1). Thus, the A and B subunits of PP2A do not play a role in the recognition of ceramide.

The structural specificity of ceramide was also determined for the closely related enzyme, PP1cα, also a CAPP (16). Indeed, PP1cα demonstrated the same structural specificity for ceramide as PP2Ac (Table 1). Thus, to date, both CAPPs demonstrate the same specificity for ceramide, which is not unexpected, given the very high homology between the two proteins.

Structural requirements of the ceramide molecule for binding to PP2Ac

The question remained as to why small changes in the ceramide molecule completely abrogated the ability of ceramide to activate CAPPs. Are the structural analogs unable to bind CAPP, or do the structural variants interact with CAPP, but additional contact between each functional group of the ceramide molecule with CAPP is necessary to induce a configurational change leading to an increase in phosphatase activity? To begin to answer these questions, we adopted a vesicle binding assay for naturally occurring long-chain ceramide (D-*erythro*-C_6 ceramide did not form large multilamellar structures) used previously for evaluating binding of phospholipids to proteins (40). In these studies, PP2Ac was incubated with various ceramides, and the enzyme associated with the ceramide particles was quantitated by Western blot analysis. **Figure 8** and Table 2 show the relative binding efficiencies to PP2Ac of selected ceramide analogs. The LMV assay is mainly a qualitative measure of binding, but notably, structural analogs that had no effect on PP2Ac activity (D-*erythro*-dehydro C_{16} ceramide, phyto C_{16} ceramide, D-*erythro*-urea C_{16} ceramide, and 1-*O*-methyl C_{16} ceramide) demonstrated greatly reduced binding compared with D-*erythro*-C_{16}

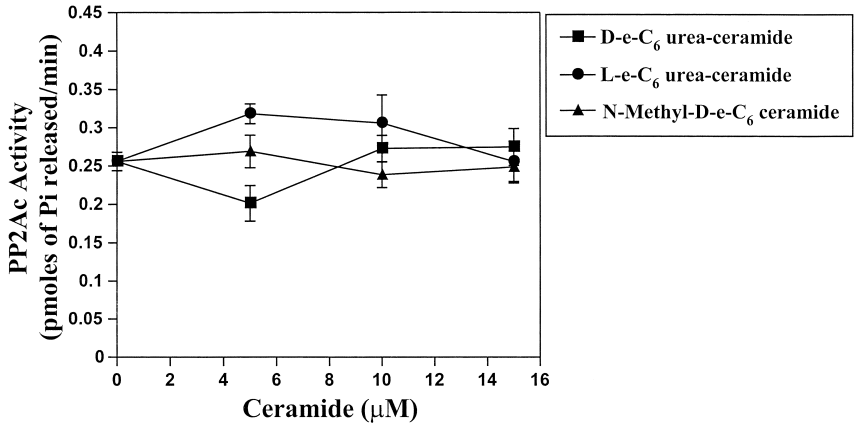

Fig. 6. The structural requirement of the secondary amide group. Protein phosphatase-2A (PP2A) (10 mU) was assayed as described (35) in the presence of the indicated concentrations of C_6 ceramide analogs. Data are presented as mean PP2Ac activity in pmoles of ^{32}P release from ^{32}P-MBP per min ± SE (n = 4).

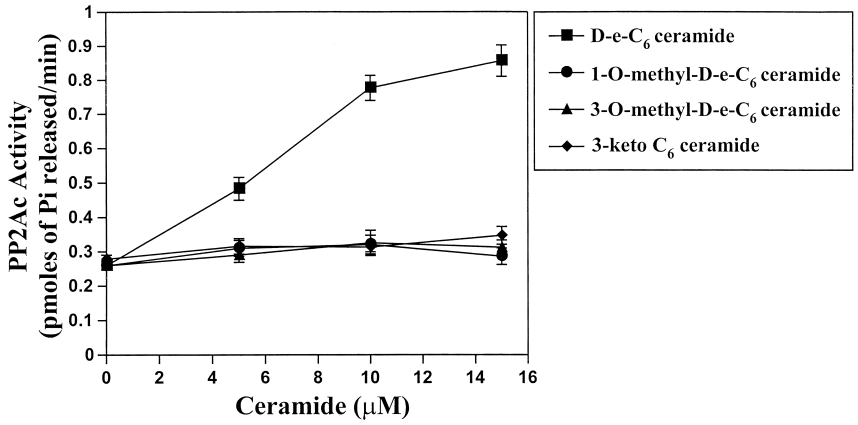

Fig. 7. The structural requirements of the primary and secondary hydroxyl groups. PP2Ac (10 mU) was assayed as described (35) in the presence of the indicated concentrations of C_6 ceramide analogs. Data are presented as mean PP2Ac activity in pmoles of ^{32}P release from ^{32}P-MBP per min ± SE (n = 4).

ceramide. These results demonstrate that the inability of these analogs to affect PP2Ac activity is due to an inability to interact with the enzyme.

Interestingly, D-*erythro*-dihydroceramide, which inhibited PP2Ac, demonstrated binding similar to that of the natural D-*erythro*-ceramide. Therefore, for ceramides to affect (activate/inhibit) the enzymatic activity of PP2Ac, ceramide must bind/interact with the enzyme.

Effects of ceramide analogs on activation of PP2Ac by D-*erythro*-C_6 ceramide

To determine whether ceramide analogs that bind PP2Ac affect the responsiveness of the enzyme for natural ceramide, we examined the effects on CAPP activity of a ceramide analog (D-*erythro*-C_6 urea ceramide) that had no effect on PP2Ac activity and demonstrated low binding efficiency, and a ceramide analog (D-*erythro*-dihydro C_6 ceramide) that on its own inhibited PP2Ac and demonstrated binding efficiency similar to that of natural ceramide. These two analogs were co-added with various doses of D-*erythro*-C_6 ceramide. D-*erythro*-C_6 urea ceramide had no effect on ceramide-induced activation of PP2Ac (**Fig. 9**). On the other hand, D-*erythro*-dihydro C_6 ceramide reduced the basal as well as the ceramide-induced activity of PP2Ac, but did not affect the total fold change in the increase in PP2Ac activity by ceramide (Fig. 9).

DISCUSSION

This study demonstrates strict structural requirements of ceramide for activation of serine-threonine protein phosphatases. The understanding of this allosteric action is important for several reasons. First, it adds to our knowledge of the biochemical mechanisms of CAPP activation. Second, the high specificity for ceramide that these phosphatases possess explains why closely related lipids have no effect on phosphatase activity. Third, these studies disclose that this allosteric interaction between ceramide and serine-threonine protein phosphatases may have implications in regard to other ceramide-interacting proteins (e.g., ceramidase). Finally, these studies begin to identify features of ceramide-based inhibitors of CAPP.

The results presented in this study demonstrate that many of the unique structural features of ceramide are necessary for activation of serine-threonine protein phosphatases. A strict requirement for the structure of the natural (2S, 3R, 4E) ceramide for activation of the phosphatases was demonstrated. This explains the lack of activation by diacylglycerol that has been previously demonstrated (17, 18, 43). Specifically, the 4-5-*trans* double bond within the sphingoid backbone was found to be necessary. In fact, saturation of the 4-5 double bond of ceramide to dihydroceramide produced an inhibitory compound. This suggests a role for the dihydroceramide

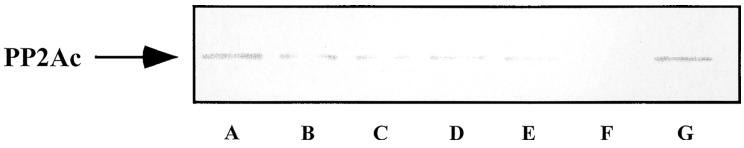

Fig. 8. The binding of ceramide analogs to PP2Ac. PP2Ac was complexed with D-*erythro*-C_{16} ceramide analogs as described in the Materials and Methods section. Depicted are the data obtained from PP2Ac/ceramide complexes subjected to PAGE-SDS analysis and Western immunoblotting for PP2Ac. A: D-*erythro*-C_{16} ceramide; B: D-*erythro*-C_{16} urea-ceramide; C: D-*erythro*-dehydro C_{16} ceramide; D: phyto-C_{16} ceramide; E: D-*erythro*-1-*O*-methyl-C_{16} ceramide; F: (2S) 3-keto-C_{16} ceramide; G: D-*erythro*-dihydro C_{16} ceramide. Data are representative of three separate determinations on two occasions.

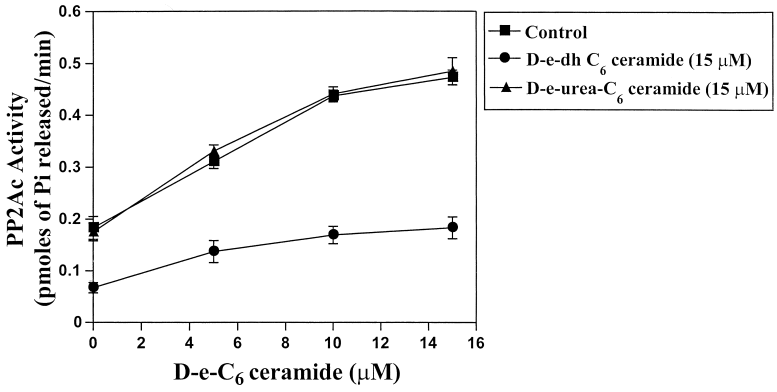

Fig. 9. The effects of D-*erythro*-C_6 urea ceramide and D-*erythro*-dihydro C_6 ceramide on the ability of ceramide to activate PP2Ac. PP2cA (10 mU) was assayed as described (35) in the presence of the indicated concentrations of C_6 ceramide and in the presence of either vehicle control (ethanol), D-*erythro*-dihydro C_6 ceramide (15 μM), or D-*erythro*-C_6 urea ceramide (15 μM). Data are presented as mean PP2Ac activity in pmoles of ^{32}P release from ^{32}P-MBP per min ± SE (n = 4).

desaturase of the de novo ceramide pathway in regulating the activity of serine-threonine phosphatases, such that the action of the desaturase converts dihydroceramide, an inhibitor of CAPP, to ceramide, an activator of CAPP. Hydroxylation of the C4 carbon (production of phytoceramide) also greatly reduced the activation of serine-threonine protein phosphatases, as did further desaturation of the C4-C5 double bond to dehydroceramide. Furthermore, the 4-5 double bond of ceramide must be in the *trans* configuration, inasmuch as 4,5-*cis*-D-*erythro*-C_6 ceramide had no effect on CAPP activity. Thus, this 4-5-*trans* double bond is absolutely required for orientating the ceramide molecule for interaction/activation of CAPPs. The necessity of the amide group, as well as the primary and secondary hydroxyl groups, was also examined, and *O*-methyl analogs of ceramide had no effect on serine-threonine protein phosphatase activity. Thus, for activation to occur, serine-threonine protein phosphatases must interact with ceramide at several points on the molecule, suggesting a complex interaction via a hydrogen bonding network system between the sphingoid backbone, possibly through a "binding pocket" within the enzyme, reflecting the appropriate requirement for the natural ceramide structure (**Fig. 10**). The binding data support this hypothesis by demonstrating the necessity of an intact sphingoid backbone to interact with PP2Ac for activation of the enzyme to occur, because the lack of activation (or inhibition) correlated well with reduced binding to PP2Ac.

The use of the LMV assay to examine the interaction of ceramide with protein targets is a novel approach for studying protein-ceramide interactions. The assay has been used primarily to qualitatively examine the interaction of phospholipids with various proteins (e.g., $cPLA_2$). Other standard lipid binding assays, such as solid support binding assays (lipid-protein overlay assays) and sucrose-loaded vesicle binding assays, were not useful in our preliminary studies. The fat blot requires high affinity of interaction between lipid and protein, and we noticed that neutral lipids (such as diacylglycerol or ceramide) do not layer well on nitrocellulose, but tend to form rings. The sucrose-loaded vesicle assay and immunoprecipitation assays gave a very high background. Therefore, the LMV assay emerged as a suitable qualitative assay that proved to be useful in discriminating between lipids that bind PP2A and those that do not. Importantly, the demonstration that reduced binding of ceramide analogs to PP2Ac correlates with a lack of effect on enzyme activity serves to validate the use of the LMV assay for examining ceramide interactions with CAPP.

Additional data demonstrated that dihydroceramide had no effect on the ceramide dose response, only on the total basal and stimulated activity. This finding suggests that dihydroceramide may interact with PP2Ac through a site different from the activating ceramide binding site. Alternatively, the lack of stereospecificity for the inhibition of the PP2Ac by dihydroceramide may suggest two separate domains for interaction of ceramide and dihydroceramide with PP2Ac, i.e., a domain for nonspecific hydrophobic interactions with the sphingoid base (e.g., sphingosine or dihydrosphingosine) and fatty acid chains, and a domain for specific interaction with the sphingosine backbone. On the other hand, this latter hypothesis is somewhat argued against by two lines of evidence: first, a lack of inhibition was observed in other ceramide analogs, such as 1-*O*-methyl ceramide and 3-keto ceramide; and second, 3-keto dihydroceramide inhibited PP2Ac to the same extent as did dihydroceramide (C. E. Chalfant et al.

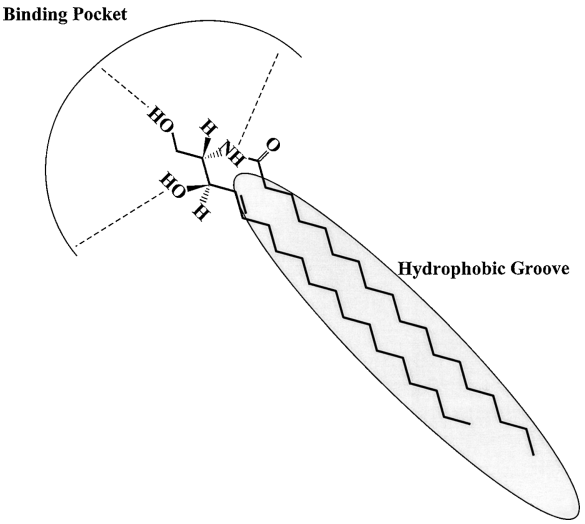

Fig. 10. Hypothetical scheme of the interaction between ceramide and PP2Ac.

unpublished observations). Thus, the inhibition is specific for the dihydroceramide form. This interaction with CAPP does lack the strict specificity for the sphingoid backbone that activation requires, because methylation of the secondary hydroxyl group and changes in stereochemistry have no effect on the ability of dihydroceramide to inhibit CAPP. Interestingly, differences in inhibition profiles between PP2Ac and the trimeric form of PP2A were observed. Unlike PP2Ac, the trimeric form of PP2A was significantly inhibited by other ceramide analogs with modifications to the 4-5 double bond (e.g., D-*erythro*-dehydro C_6 ceramide and D-*erythro*-C_6 phytoceramide). Furthermore, trimeric PP2A was also inhibited by D-*erythro*-MAPP and L-*erythro*-MAPP as well as all of the primary and secondary hydroxyl analogs. Inasmuch as trimeric PP2A is the natural form of the enzyme, these data likely represent the "true" nature of the enzyme and this inhibition is via interaction with either the A subunit or the B subunit. It is also of interest that D-MAPP and L-MAPP inhibit PP2A, because D-MAPP is an inducer of apoptosis, in which PP2A has been reported to play a role. On the basis of the data presented in this study, the increase in cellular endogenous ceramide produced in response to the inhibition of ceramidases by D-MAPP would overcome this inhibition.

A very curious set of results emerged when comparing the structural requirements of ceramide for the activation of serine-threonine protein phosphatases and the requirements for interaction with neutral ceramidase, one of few ceramide-interacting proteins that has been examined extensively for its interaction with ceramide. We find that the structural requirements are very similar (32, 33). For example, neutral ceramidase requires the 4-5-*trans*-double bond, the primary and secondary hydroxyl, and the NHCO amide group for substrate recognition (32). This raises the interesting possibility that both proteins, which do not show significant sequence homology, may share similar ceramide-interacting domains. Obviously, additional ceramide-interacting enzymes need to be examined for structural specificity (both allosteric and catalytic interactions), and specific domains in the proteins need to be identified.

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