Protein Kinase C-induced Activation of a Ceramide/Protein Phosphatase 1 Pathway Leading to Dephosphorylation of p38 MAPK*

Recently we showed that, in human breast cancer cells, activation of protein kinase C by 4β-phorbol 12-myristate 13-acetate (PMA) produced ceramide formed from the salvage pathway (Becker, K. P., Kitatani, K., Idkowiak-Baldys, J., Bielawski, J., and Hannun, Y. A. (2005) J. Biol. Chem. 280, 2606–2612). In this study, we investigated intracellular signaling events mediated by this novel activated pathway of ceramide generation. PMA treatment resulted in transient activation of mitogen-activated protein kinases (ERK1/2, JNK1/2, and p38) followed by dephosphorylation/inactivation. Interestingly, fumonisin B1 (FB1), an inhibitor of the salvage pathway, attenuated loss of phosphorylation of p38, suggesting a role for ceramide in p38 dephosphorylation. This was confirmed by knock-down of longevity-assurance homologue 5, and the generated ceramide modulates the p38 cascade via PP1. Upon PMA treatment, a mitochondria-enriched/purified fraction, phospho-p38 resided in PMA-stimulated mitochondria. Moreover, PMA recruited PP1 catalytic subunits to mitochondria, suggesting activation of PP1 results in an inhibitory effect on p38. To determine which protein phosphatases act in this pathway, specific knock-down of serine/threonine protein phosphatases was performed, and it was observed that knock-down of protein phosphatase 1 (PP1) catalytic subunits significantly increased p38 phosphorylation, suggesting activation of PP1 results in an inhibitory effect on p38.

Several metabolic routes contribute to ceramide formation, and the two best studied involve activation of sphingomyelinas (17, 18) or the de novo pathway (11, 19). Recently, we also described activation of the “salvage pathway,” which re-utilizes long-chain sphingoid bases (sphingosine and sphinganine) formed by degradation of (dihydro)ceramide or complex sphingolipids (15). The results showed that PMA, a protein kinase C (PKC) activator, stimulated the generation of ceramide, which was inhibited by treatment with fumonisin B1 (FB1) (20), a (dihydro)ceramide synthase inhibitor, but not by myriocin (21), an inhibitor of de novo synthesis. This distinguishes the de novo from the salvage pathway and suggests the utility of FB1 (versus myriocin) in elucidating biological functions of the salvage pathway-derived ceramide in PKC-dependent cell responses. Based on such approaches, ceramide formed from the salvage pathway has been shown to be involved in the inhibition of juxtanuclear translocation of PKC-βIII upon PMA treatment, implying a role of ceramide in intracellular signal transduction (15).

At present, several direct target molecules of ceramide action have been identified, including kinase suppressor of Ras (22), PKC-ζ (23), cRaf (24), and ceramide-activated protein phosphatases (CAPPs), the latter composed of the serine/threoniceramide specie, which was suppressed by FB1. Taken together, these data suggest that accumulation of C16-ceramide in mitochondria formed from the protein kinase C-dependent salvage pathway results at least in part from the action of longevity-assurance homologue 5, and the generated ceramide modulates the p38 cascade via PP1.

Sphingolipids have emerged as pleiotropic lipid mediators in various processes, including inflammation, senescence, apoptosis, and stress responses (1–6). The central sphingolipid mediator, ceramide, acts as an intracellular regulator of growth, cell death, differentiation, trafficking, and signaling pathways (1, 4, 7, 8). The levels of ceramide increase in response to various stimuli such as Fas ligand (9, 10), chemotherapeutic drugs (11), inflammatory cytokines (12), oxidative stress (13), heat stress (14), and 4β-phorbol-12-myristate-13-acetate (PMA)2 (15, 16).

The abbreviations used are: PMA, 4β-phorbol 12-myristate 13-acetate; C16-ceramide, N-hexanoyl-o-erythro-sphingosine; CAPP, ceramide-activated protein phosphatase; ERK1/2, extracellular signal-regulated kinase 1/2; FB1, fumonisin B1; JNK1/2, c-Jun N-terminal kinases 1/2; LASS, longevity-assurance homologue; MAM, mitochondria-associated membrane; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; siRNA, small interference RNA; LC, liquid chromatography; MS, mass spectrometry; CMV, cytomegalovirus.

* This work was supported in part by National Institutes of Health Grants CA87584 (to Y. A. H.), AG16583 (to L. M. O.), CA88932 (to B. O.), and GM08716 (to R. W. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., P. O. Box 250509, Charleston, SC 29425. Tel.: 843-792-4321; Fax: 843-792-4322; E-mail: hannun@musc.edu.

‡§ The abbreviations used are: PMA, 4β-phorbol 12-myristate 13-acetate; C16-ceramide, N-hexanoyl-o-erythro-sphingosine; CAPP, ceramide-activated protein phosphatase; ERK1/2, extracellular signal-regulated kinase 1/2; FB1, fumonisin B1; JNK1/2, c-Jun N-terminal kinases 1/2; LASS, longevity-assurance homologue; MAM, mitochondria-associated membrane; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; siRNA, small interference RNA; LC, liquid chromatography; MS, mass spectrometry; CMV, cytomegalovirus.

‡ The abbreviations used are: PMA, 4β-phorbol 12-myristate 13-acetate; C16-ceramide, N-hexanoyl-o-erythro-sphingosine; CAPP, ceramide-activated protein phosphatase; ERK1/2, extracellular signal-regulated kinase 1/2; FB1, fumonisin B1; JNK1/2, c-Jun N-terminal kinases 1/2; LASS, longevity-assurance homologue; MAM, mitochondria-associated membrane; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; siRNA, small interference RNA; LC, liquid chromatography; MS, mass spectrometry; CMV, cytomegalovirus.
phorylation of target substrates through the action of CAPPs has been implicated in cell growth arrest and apoptosis (1, 4, 27). Several substrates have now been described for CAPPs, including Bcl-2 (27), PKC-α (28), c-Jun (29), SR proteins (9), the Rb protein (30), and AKT/protein kinase B (31). Although involvement of ceramide in cell responses has been investigated, further studies are required to understand the detailed mechanisms regarding signal transduction following association of ceramide with the target molecules described above.

The activation of the salvage pathway by PKC suggested possible roles for ceramide in regulating some PKC-mediated responses (15). Activation of PKC results in phosphorylation and regulation of a myriad of substrates (32, 33). Some of the best studied are members of the mitogen-activated protein kinases (MAPKs), which include c-Jun N-terminal kinases 1/2 (JNK1/2), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 (34, 35). Ceramide has been reported to exert various effects on the MAPK cascades (22, 36–43). For example, ceramide has been shown to activate kinase suppressor of Ras (22) and TAK1 (40), which act upstream of the MAPK cascades. In contrast, some studies showed that ceramide down-regulated activation of MAPKs (38, 43). These considerations led us to investigate the role of ceramide, specifically generated from the salvage pathway, in regulating protein phosphorylation/de-phosphorylation of members of the MAPK family.

In this study, we examined specific roles of ceramide formed from the salvage pathway in signal transduction in PMA-stimulated human breast cancer cells (MCF-7). The results demonstrate that 1) PMA activation of the salvage pathway contributes to selective increases in C16-ceramide, which significantly occurred in mitochondria and were at least partly mediated by the activity of longevity-assurance homologue 5 (Lass5), 2) one of the CAPPs, PP1, as well as p38 were relocalized to mitochondria where C16-ceramide was enriched, and 3) this ceramide/PP1 pathway functioned as a negative regulator of the p38 cascade.

**EXPERIMENTAL PROCEDURES**

Reagents—FB1 was purchased from Alexis Corp. (Carlsbad, CA). N-Hexanoyl-n-erythro-sphingosine (C6-ceramide) was from Avanti Polar Lipids Inc. (Alabaster, AL). Rabbit polyclonal antibodies for calnexin, HSP60, and p38, goat polyclonal antibodies for Lamin B, PP2 catalytic subunit (PP2Ac), and isoforms of PP1 catalytic subunit (PP1c), and mouse monoclonal anti-PP1c antibody recognizing all PP1c isoforms (E9) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho/active p38 antibody was from Promega (Madison, WI). Antibodies specific for phospho/active ERK1/2 or JNK1/2 were from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody specific for cytochrome c was from BD Bioscience. TRITC-conjugated antibodies specific for mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor488-conjugated antibodies specific for goat or rabbit IgG were from Molecular Probes, Inc. (Eugene, OR). Enhanced chemiluminescence kit was from Amersham Biosciences (Buckinghamshire, UK). PMA and mouse monoclonal antibody for porin were from Calbiochem. Halt™ phosphatase inhibitor mixture was from Pierce. Mouse FLAG monoclonal antibody and rabbit polyclonal calreticulin antibody were obtained from Sigma. pCMVexSNvneo-LASS5 plasmid was kindly gifted from Dr. S. M. Jazwinski.

Cell Culture—MCF-7 cells were grown in RPMI 1640, supplemented with 1-glutamine and 10% (v/v) fetal bovine serum. Cells were maintained at <80% confluency under standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37 °C).

Subcellular Fractionation by Differential Centrifugation and Mitochondrial Isolation by Continuous Centrifugation Using a Percoll Gradient—Cells were incubated in buffer containing 250 mM sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA, and 0.5 mM phenylmethlysulfonyl fluoride for 30 min on ice. Cells were disrupted by passage through a 25-gauge needle for 5 strokes and then were centrifuged at 1,000 × g for 10 min, 10,000 × g for 10 min, and 100,000 × g for 60 min, for collection of the nuclear fraction, mitochondria-enriched heavy membrane fraction, and light membrane fraction, respectively. These membrane fractions were washed twice with the above buffer, and ceramide levels were measured. To isolate mitochondria, the washed heavy membrane fractions were further loaded onto a 30% Percoll solution and centrifuged at 100,000 × g for 2 h. Each fraction (fraction numbers 1–12) was subjected to immunoblotting with voltage-dependent anion channel (porin) or calnexin to determine fractions of mitochondria or endoplasmic reticulum, respectively.

Western Blotting—Cells were washed three times with ice-cold phosphate-buffered saline (PBS) supplemented with Halt™ phosphatase inhibitor mixture and then lysed using Laemmli buffer. Proteins (10 µg) were subjected to 10% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with PBS/0.1% Tween 20 (PBS-T) containing 5% nonfat dried milk, washed with PBS-T, and incubated for 14 h with primary antibody in PBS-T containing 5% nonfat dried milk. The blots were washed with PBS-T and incubated with secondary antibody in PBS-T containing 5% nonfat dried milk. Detection was performed using enhanced chemiluminescence.

Transfection with Small Interference RNA—Cells (2 × 105 cells/60-mm dish) were transfected with double strand siRNAs using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. After 48 h, transfection reagents were washed out and cells were stimulated with PMA in RPMI 1640 supplemented with 10% fetal bovine serum. Specific siRNAs for PP2Ac-β (sc-36301) and PP1c-β (sc-36295) were from Santa Cruz Biotechnology. The sequences of siRNAs for human longevity-assurance homologue (LASS) family members, LASS1 and LASS5, were AAGTGTCTGTATGCCACGAGT and AAACCGTGTGACTCTGTATT, respectively. The other sequences of siRNAs for scrambled RNA, human PP1c-α, and PP1c-γ were AATTCTCCGAACGTGTCACGT, AAGGCAGTTGGTCACT, and AAGGCGAGTGTGTCAGTC, respectively.

Quantitative Real-time PCR—One µg of total RNA, isolated using an RNA isolation kit (Qiagen), was used in reverse transcription reactions as described (44). The resulting total cDNA was then used in the quantitative real-time PCR to measure the
mRNA levels using TaqMan® gene expression kit (Applied Biosystems) as described by the manufacturer with ABI 7300 Q-PCR system. The mRNA levels of rRNA were used as internal control.

**Transfection with FLAG-tagged LASS5 Expression Vector**—Cells, growing on glass coverslips, were transfected with 1 μg of pCMVexSVneo-LASS5 plasmid DNA using an Effectene transfection kit (Qiagen) according to the manufacturer’s instructions.

**Direct Immunofluorescence**—Cells, growing on glass coverslips, were fixed for 10 min at room temperature with 4% formaldehyde in PBS and washed with PBS. Next, cells were treated for 10 min with 0.1% Triton X-100, washed with PBS, and blocked for 1 h with PBS containing 2% human serum. The primary antibodies for PP1c, Lamin B, HSP60, phospho-p38, calreticulin, FLAG, or cytochrome c were diluted in PBS containing 2% human serum, and incubated for 90 min at room temperature. Samples were washed with PBS, and TRITC- or fluorescein isothiocyanate-conjugated anti-IgG antibodies were applied for 1 h in PBS containing 2% human serum. Confocal laser microscopy was performed using an LSM510 microscope (Carl Zeiss, New York).

**LC/MS**—Analysis of ceramide species in lipid extract was performed by LC/MS as described in Becker et al. (15).

**Statistical Analysis**—Comparison between two groups was carried out by unpaired or paired Student’s t test.

**RESULTS**

**Transient Activation of MAPKs and Ceramide-dependent Inactivation upon PMA Treatment**—Many reports have shown that PMA induces acute activation of MAPKs in a PKC-dependent manner (32, 35, 45); this activation is mostly transient, probably because of the action of various protein phosphatases. To determine the contribution of ceramide selectively derived from the salvage pathway to the regulation of MAPKs, MCF-7 cells were initially treated with PMA for up to 8 h, and then MAPK activation was monitored by immunoblotting with antibodies to the phosphorylated/active forms of JNK1/2, ERK1/2, and p38. As shown in Fig. 1, activation of JNK1/2, ERK1/2, and p38 was observed 30 min after PMA treatment and was transient such that by 2 h there was loss of PMA-induced phosphorylation, indicating action of protein phosphatases. To determine if the ceramide generated in response to PMA participates in the dephosphorylation phase of MAPKs, MCF-7 cells were stimulated with C6-ceramide, which is inhibitable by various protein phosphatase inhibitors. FB1, we next evaluated their participation in PMA-induced ceramide formation and ceramide-dependent dephosphorylation of p38. Specific knock-down of the LASS family members, LASS1 (47) or LASS5 (48, 49), was achieved using siRNAs. Each siRNA treatment was confirmed to decrease the levels of the respective mRNA (Fig. 3A). Knock-down of LASS1 or LASS5 evoked a slight increase in basal levels of phospho-p38. Importantly, phosphorylation of p38 upon PMA treatment was markedly increased at the indicated time points in LASS5 knock-down cells as compared with the use of scrambled RNA or LASS1 siRNA (Fig. 3B). It should be noted that LASS5 knock-down had no effects on either the phosphorylation of ERK1/2 or JNK1/2 (data not shown).

**FIGURE 1. Activation of JNK1/2, ERK1/2, and p38.** MCF-7 cells were stimulated with 100 nM PMA for the indicated periods. Whole cell lysates were prepared and subjected to immunoblot analysis with anti- phospho-JNK1/2, anti-phospho-ERK1/2, and anti-phospho-p38 antibodies. Equal amounts of protein were loaded in each lane. The results are representative of two to three independent experiments.
A Mitochondrial Ceramide/Protein Phosphatase 1 Pathway

FIGURE 3. Knock-down of LASS family members and the effects on p38 activation. A, MCF-7 cells were transfected with 20 nm siRNA for scrambled RNA (open bars), LASS1 (gray-filled bars), or LASS5 (black-filled bars) for 48 h. Each mRNA of LASS1 or LASS5 was measured by real time Q-RT-PCR as described under “Experimental Procedures.” Levels of mRNAs were expressed as arbitrary units. The data represent mean ± S.E. of four values. B, MCF-7 cells transfected with the indicated siRNAs (20 nm) were stimulated with 100 nm PMA for the indicated time. Whole cell lysates were prepared and subjected to immunoblot analysis with antibodies specific for p38 and PP1c. C, MCF-7 cells transfected with 20 nm siRNA for scrambled RNA or LASS5 were stimulated with or without 100 nm PMA for 30 or 60 min. C16-ceramide levels were measured by LC/MS. The data represent averages of values from two independent experiments.

not shown). In addition, the partial silencing of LASS5 resulted in a commensurate partial inhibition of the generation of C16-ceramide after PMA stimulation (Fig. 3C). Taken together, the results suggest that LASS5 is at least in part involved in the salvage pathway and that LASS5-mediated generation of ceramide then acts to enhance dephosphorylation of p38.

Involvement of the PP1 Serine/Threonine Protein Phosphatase in p38 Dephosphorylation—The above results suggested that endogenous ceramide might activate a protein phosphatase in the p38 response. The serine/threonine phosphatases PP1 and PP2A have been implicated as CAPPs in vitro and as mediators of actions of ceramide on protein dephosphorylation in cells (1, 4, 27). To assess their roles in the ceramide effect on p38, individual isoforms of the catalytic subunits of CAPPs were knocked down by treatment with specific siRNA. The specific effectiveness of the knock-down was confirmed by immunoblotting with antibodies for PP1c-α, PP1c-β, PP1c-γ, and PP2Ac (Fig. 4A). Next, the effects of the siRNA on the PMA-induced phosphorylation of the MAPKs were evaluated. Interestingly, knock-down of each of the PP1c isoforms resulted in mild enhancement of basal phosphorylation of p38, with PP1c-β showing the least effect (Fig. 4B). In PMA-stimulated cells, each of PP1c siRNAs displayed a significant elevation in p38 phosphorylation as well (Fig. 4B), implying that PP1c acts as a negative regulator of the p38 pathway. In contrast, knock-down of PP1c-α and PP1c-β did not show much effect on phosphorylation of JNK1/2 or ERK1/2 induced by PMA, but PP1c-γ knock-down showed modest enhancement of phosphorylation of JNK1/2 and ERK1/2. In contradistinction to PP1 isoforms, knock-down of PP2Ac-β did not modulate the effects of PMA on the phosphorylation status of p38 or the other MAPKs (Fig. 4B). Taken together, these results show that PP1c regulates the dephosphorylation of p38 following PMA-induced phosphorylation.

Relocalization of PP1c to Mitochondria in Response to PMA—To investigate the spatial association of PP1c with PMA-induced increases in ceramide, the effects of PMA on the intracellular localization of PP1c were examined. For these studies, PP1c was detected by direct immunofluorescence confocal microscopy using an antibody (E9), which recognizes all three isoforms of PP1c (PP1c-α, PP1c-β, and PP1c-γ), and colocalization was determined using antibodies for Lamin B as a nuclear membrane marker or HSP60 as a mitochondrial marker. As shown in Fig. 5, PP1c was present initially in a diffuse cytosolic pattern, but became relocalized to the perinuclear region in response to 1 h of treatment with PMA. Treatment of cells with PMA caused partial colocalization of PP1c with Lamin B; however, the pattern was such that PP1c localized in a ring like pattern partially overlapping but surrounding Lamin B, suggesting extra-nuclear localization (Fig. 5A). On the other hand, PMA not only induced a significant degree of colocalization of most of PP1c with HSP60, but it also induced a redistribution of HSP60, a mitochondrial matrix protein, into the same perinuclear pattern of PP1c (Fig. 5B). Staining with calreticulin, an endoplasmic reticulum protein, exhibited tubules as well as ring-shape structure around the nucleus, but PMA failed to recruit PP1c to the tubular endoplasmic reticulum (Fig. 5C). Taken together, the results demonstrate dynamic regulation of PP1c localization and association with a perinuclear pool of mitochondria in PMA-stimulated MCF-7 cells.

Ceramide-dependent Relocalization of PP1c—To determine if ceramide formation is involved in the relocalization of PP1c, the effects of FB1 were examined, because under these conditions FB1 specifically inhibited ceramide formation in response to PMA. As shown in Fig. 6A, FB1 treatment significantly diminished the PMA-induced relocalization of PP1c, and the percentages of cells with perinuclear PP1c was reduced from 78 ± 10% to 36 ± 3% with FB1 treatment (Fig. 6B). Taken together, this result indicates that ceramide formation is involved in the relocalization of PP1c. The above results on the ceramide-dependent relocalization of PP1c and dephosphorylation of p38 suggested that p38 may
also localize, at least in part, to mitochondria. Therefore, direct immunofluorescence was performed to determine the intracellular compartment in which active p38 localizes. In contrast to a small amount of phospho-p38 observed in unstimulated cells, PMA induced significant increases in phospho-p38, and the majority of the staining was colocalized with cytochrome c (Fig. 7A). Consistent with the above immunocytochemical analysis, phospho-p38 increased with PMA stimulation in heavy membrane fraction (Fig. 7B). Because the negative regulation of p38 by PP1 is controlled by ceramide synthesis at least in part through the action of LASS5, the localization of this enzyme was also investigated. Direct immunofluorescence was performed in MCF-7 cells transfected with an expression vector of FLAG-tagged LASS5. Consistent with a previous study regarding LASS5 localization (48), FLAG-LASS5 was detected on the nuclear envelope and as a reticular structure in the perinuclear and cytoplasmic regions in unstimulated cells (Fig. 7C). Interestingly, whereas PMA treatment did not affect LASS5 localization, it did induce clustering of mitochondria around the nuclear envelope in close proximity to LASS5 (Fig. 7C).
the absolute levels were significantly lower than those of C16-ceramide (Fig. 8). Interestingly, C16-ceramide in the heavy membrane fraction displayed the highest concentration relative to total protein (Fig. 8B). Moreover, PMA-induced elevation of total ceramide levels in whole cells, and the ceramide in both the light and heavy membrane fractions was diminished by pre-treatment with FB1, an inhibitor of (dihydro)ceramide synthase (Fig. 9A). As shown in Fig. 9B, the specific increases in C16-ceramide in the heavy membrane fraction in response to PMA stimulation were significantly diminished by treatment with FB1. In contrast, PMA had no effects on C24-ceramide levels, and FB1 reduced the basal levels of this ceramide in total homogenate as well as in most fractions. Taken together, PMA caused a specific accumulation of C16-ceramide observed in both light and heavy membranes but not in the nuclear fraction.

To determine ceramide levels in mitochondria specifically, the heavy membrane fraction was subjected to Percoll centrifugation, and ceramide levels in the isolated mitochondria were determined. As expected, the heavy membrane fraction acquired by centrifugation at 10,000 × g contained porin, a protein highly enriched in mitochondria and had some contamination with calnexin, an endoplasmic reticulum protein (Fig. 10A). Further centrifugation of the heavy membrane fractions using a 30% Percoll solution separated mitochondria from endoplasmic reticulum (Fig. 10B). Mitochondria acquired from three fractions (fraction number 8–10) were subjected to LC/MS analysis. As shown in Fig. 10C, PMA induced a signifi-
DISCUSSION

In this study, we demonstrate that the generation of C16-ceramide via the salvage pathway resulted in dephosphorylation of p38 through the action of PP1c isoforms. Moreover, the results revealed that LASS5 at least partly contributed to ceramide synthesis, and in turn LASS5 regulated ceramide- and PP1c-dependent dephosphorylation of p38. This study has implications for the subcellular localization of ceramide action, the role of protein phosphatases, and the emerging functions of the regulated salvage pathway of ceramide generation.

Our recent study demonstrated that the sphingoid base salvage represented the predominant pathway of induction of ceramide synthesis in response to PMA stimulation of MCF-7 cells (15). The salvage pathway most likely operates by salvaging sphingoid bases arising in lysosomes from hydrolysis of complex sphingolipids. Presumably, the sphingoid bases can traverse lysosomes and then re-enter sphingolipid biosynthetic pathways via ceramide synthases, probably LASS family members (52–54).

The results from this study demonstrate that the re-synthesis of ceramide in response to PMA leads to partial accumulation of ceramide in (or close to) mitochondria (e.g. in mitochondria-associated membranes (MAMs)). Thus, FB1-sensitive elevation of C16-ceramide was observed in the mitochondria-enriched heavy membrane fraction (Fig. 9). In addition, C16-ceramide accumulated in the mitochondrial fraction acquired from continuous centrifugation using a Percoll gradient (Fig. 10). Interestingly, ceramide synthase activity (55) has been observed in MAMs where free sphingoid bases are converted to (dihydro)ceramide by (dihydro)ceramide synthases. Moreover, ceramide-enriched mitochondria upon PMA treatment were seen to local-
The subcellular location of ceramide generation is likely to play an important role in dictating its downstream targets and thereby the biological response of the cell to this mediator. As shown in Figs. 2 and 6, inhibition of ceramide accumulation by FB1 reversed the PMA-induced relocalization of PP1 to mitochondria and enhanced p38 activation, strongly indicating the potential of ceramide to mediate PP1 relocalization to mitochondria and subsequent effects on p38. Notably, the ceramide signaling is unlikely to link to the pathways for ERK1/2 or JNK1/2. In addition to pharmacological approaches using FB1 (Fig. 2), inhibition of C16-ceramide synthesis by LASS5 knock down upon PMA treatment resulted in enhancement of p38 phosphorylation (Fig. 3). These approaches targeting (dihydro)ceramide synthases, the LASS family members, strongly suggest an involvement of LASS5 in the ceramide signaling capable of influencing PP1 action and subsequent p38 dephosphorylation.

Interestingly, phorbol esters were shown to stimulate PP1 activity (60), implying an association of PKC activation with PP1 action. Further, a potent inhibitor of CAPPs (both PP1 and PP2A), okadaic acid, has been shown to increase p38 activation (61, 62), which supports the ceramide/PP1-dependent dephosphorylation of p38 demonstrated in this study. Although studies on the involvement of PP1 in the p38 pathway have been restricted because of the lack of a PP1 isoform-specific inhibitor, knock-down by siRNA specific for PP1c isoforms shown in this study (Fig. 4) was able to support specific roles of PP1 isoforms in p38 dephosphorylation. However, further investigation is required for determining if the ceramide/PP1 pathway inactivates p38 directly or indirectly by acting on upstream kinases that regulate p38 phosphorylation.

In this context of mitochondrial action of ceramide, it is noteworthy that multiple studies have demonstrated strong links between ceramide and mitochondria in the regulation of cell death. Selective hydrolysis of a mitochondrial pool of sphingomyelin by bacterial sphingomyelinase targeted to the mitochondrial matrix resulted in apoptosis, whereas production of ceramide in the plasma membrane, endoplasmic reticulum, nucleus, and Golgi apparatus by bacterial sphingomyelinase targeted to these compartments exerted little effect on cell viability (63). Mitochondrial ceramide has also been proposed to play roles in mediating cell responses to UV irradiation (57) or tumor necrosis factor-α (56). Mechanistically, accumulation of mitochondrial ceramide was shown to trigger relocalization of Bax to the mitochondrion and subsequent cell death (56). Moreover, D609 (an inhibitor for sphingomyelin synthase) inhibition of UV-induced mitochondrial ceramide generation significantly prevented both disruption of mitochondrial transmembrane potential and release of cytochrome c from mitochondria, which resulted in suppression of apoptosis (57). Another recent study showed that targeting positively charged...
ceramides to the mitochondrion resulted in reduction of cell viability of HepG2 cell and MCF-7 cells (64). Moreover, mitochondrial ceramides have been shown to not only inhibit mitochondrial respiratory chain complex III (65) but also stimulate mitochondrial ceramides to the mitochondrion resulted in reduction of cell viability of HepG2 cell and MCF-7 cells (64). Moreover, mitochondrial ceramides have been shown to not only inhibit mitochondrial respiratory chain complex III (65) but also stimulate mitochondrial outer membranes to small proteins (66, 67). Thereby, mitochondrial ceramides have been shown to not only inhibit mitochondrial respiratory chain complex III (65) but also stimulate mitochondrial ceramides to potentiate these kinase cascades, exogenous ceramides on the phosphorylation and activation of ERK1/2, negative regulator of p38 though PP1 recruitment to mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PP1 results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways. In summary, the present study provides evidence that ceramide formation is pathway-specific and compartment-specific. In mitochondria, ceramide exhibits the potential to drive PPI to the mitochondria as well as to cause mitochondrial clustering around the nucleus. Activation of PPI results in an inhibitory effect on p38 cascade; thus, demonstrating intricate cross-talk of the PKC, phosphatase, and MAPK pathways.

In the present study, we propose that LASS5 not only contributes to the synthesis of C16-ceramide through the salvage pathway but also subsequently to the ceramide signal resulting in PP1-dependent regulation of p38 (Fig. 11).

Acknowledgments—We extend a special thanks to Drs. Christopher Clarke, Jeffrey A. Jones, and Yi-Te Hsu for critical review. We also thank the Hollings Cancer Center Molecular Imaging Facility and the Lipidomics Core at the Medical University of South Carolina.

REFERENCES
1. Ogretmen, B., and Hannun, Y. A. (2004) Nat. Rev. Cancer 4, 604–616
2. Futerman, A. H., and Hannun, Y. A. (2004) EMBO Rep. 5, 777–782
3. Merrill, A. H., Jr., Schmelz, E. M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, I. J., Riley, R. T., Voss, K. A., and Wang, E. (1997) Toxicol. Appl. Pharmacol. 142, 208–225
4. Hannun, Y. A. (1996) Science 274, 1855–1859
5. Kolesnick, R. N., and Kronke, M. (1998) Annu. Rev. Physiol. 60, 643–665
6. Chalfant, C. E., and Spiegel, S. (2005) J. Cell Biol. 168, 4605–4612
7. Spiegel, S., Foster, D., and Kolesnick, R. (1996) Curr. Opin. Cell Biol. 8, 159–167
8. Kolesnick, R. (2002) J. Clin. Invest. 110, 3–8
9. Chalfant, C. E., Ogretmen, B., Galadari, S., Kroesen, B. J., Pettus, B. J., and Hannun, Y. A. (2001) J. Biol. Chem. 276, 44848–44855
10. Cifone, M. G., De Maria, R., Roncaiola, P., Rippo, M. R., Azuma, M., Lanier, L. L., Santoni, A., and Testi, R. (1994) J. Exp. Med. 180, 1547–1552
11. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., and Kolesnick, R. (1995) Cell 82, 405–414
12. Kim, M. Y., Linardic, C., Obeid, L., and Hannun, Y. (1991) J. Biol. Chem. 266, 484–489
13. Goldkorn, T., Bablan, N., Shannon, M., Chea, V., Matsukuma, K., Gilchrist, D., Wang, H., and Chan, C. (1998) J. Cell Biol. 111, 3209–3220
14. Jenkins, G. M., Cowart, L. A., Signorelli, P., Pettus, B. J., Chalfant, C. E., and Hannun, Y. A. (2002) J. Biol. Chem. 277, 42572–42578
15. Becker, K. P., Kitatani, K., Idkowiak-Baldys, J., Bielawski, J., and Hannun, Y. A. (2005) J. Biol. Chem. 280, 2606–2612
16. Garzotto, M., White-Jones, M., Jiang, Y., Ebleiter, D., Liao, W. C., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. (1998) Cancer Res. 58, 2260–2264
17. Hannun, Y. A. (1994) J. Biol. Chem. 269, 3125–3128
18. Guiblin, E., and Kolesnick, R. (2003) Oncogene 22, 7070–7077
19. Perry, D. K., Carton, J., Shah, A. K., Meredith, F., Uhlinger, D. J., and Hannun, Y. A. (2000) J. Biol. Chem. 275, 9078–9084
20. Merrill, A. H., Jr., Liotta, D. C., and Riley, R. T. (1996) Trends Cell Biol. 6, 218–223
21. Miyake, Y., Kozutsu, Y., Nakamura, S., Fujita, T., and Kawasaki, T. (1995) Biochem. Biophys. Res. Commun. 211, 396–403
22. Zhang, Y., Yao, B., Delikan, S., Bayoumy, S., Lin, X. H., Basu, S., McGinley, M., Chan-Hui, P. Y., Lichenstein, H., and Kolesnick, R. (1997) Cell 89, 63–72
23. Bourbon, N. A., Sun, Y., and Kester, M. (2000) J. Biol. Chem. 275, 35617–35623
24. Huwiler, A., Brunner, J., Hummel, R., Vervoordeldonk, M., Stabel, S., van den Bosch, H., and Pleischlifer, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6959–6963
25. Dobrowsky, R. T., Kamibayashi, C., Mummy, M. C., and Hannun, Y. A. (1993) J. Biol. Chem. 268, 15523–15530
