Activation of Acid Sphingomyelinase by Protein Kinase Cσ-mediated Phosphorylation

Youssef H. Zeidan and Yusuf A. Hannun

From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

Although important for cellular stress signaling pathways, the molecular mechanisms of acid sphingomyelinase (ASMase) activation remain poorly understood. Previous studies showed that treatment of MCF-7 mammary carcinoma cells with the potent protein kinase C (PKC) agonist, phorbol 12-myristate 13-acetate (PMA), induces a transient drop in sphingomyelin concomitant with an increase in cellular ceramide levels (Becker, K. P., Kitatani, K., Idkowiak-Baldys, J., Bielawski, J., and Hannun, Y. A. (2005) J. Biol. Chem. 280, 2606–2612). Here we show that PMA selectively activates ASMase and that ASMase accounts for the majority of PMA-induced ceramide. Pharmacologic inhibition and RNA interference experiments indicated that the novel PKC, PKCσ, is required for ASMase activation. Immunoprecipitation experiments revealed the formation of a novel PKCσ-ASMase complex after PMA stimulation, and PKCσ was able to phosphorylate ASMase in vitro and in cells. Using site-directed mutagenesis, we identify serine 508 as the key residue phosphorylated in response to PMA. Phosphorylation of Ser508 proved to be an indispensable step for ASMase activation and membrane translocation in response to PMA. The relevance of the proposed mechanism of ASMase regulation is further validated in a model of UV radiation. UV radiation also induced phosphorylation of ASMase at serine 508. Moreover, when transiently overexpressed, ASMaseS508A blocked the ceramide formation after PMA treatment, suggesting a dominant negative function for this mutant. Taken together, these results establish a novel direct biochemical mechanism for ASMase activation in which PKCσ serves as a key upstream kinase.

Hydrolysis of membrane sphingomyelin (SM)2 constitutes a major route for ceramide formation in mammalian systems. In contrast to the multistep de novo pathway of ceramide biosynthesis, breakdown of SM is catalyzed by a single class of enzymes, the sphingomyelinases (SMases). Currently, the NCBI database contains five entries for human SMases that are grouped according to their optimal pH as acid (ASMase or SMPD1), neutral (NMase or SMPD2, -3, and -4), and alkaline sphingomyelinases (Alk-SMase or ENPP7).

Acid sphingomyelinase (ASMase) was the first SMase to be purified and cloned (2–4). Although encoded by the same gene and mRNA, two forms of the enzyme have been described: an endolysosomal form (or Zn2+-independent form) and a secretory form (or Zn2+-dependent form) (5). Post-translational protein glycosylation plays an important role in both trafficking and stability of ASMase within the lysosomal milieu (6, 7). In addition, cysteine 629 situated toward the C terminus of ASMase has been proposed as an important residue for regulation of the enzyme such that modification (mutation or deletion) of this residue results in 5-fold activation of the enzyme (8).

In humans, mutation of the SMPD1 gene results in Niemann-Pick disease, an autosomal recessive neurovisceral disease of high lethality. The severity of the Niemann-Pick disease phenotype correlates well with the deficiency in the in vitro ASMase activity, which has become a standard test for classification of patients with the disease. Indeed, the knock-out mouse in the SMPD1 gene recapitulates the Niemann-Pick disease phenotype as described by multiple investigators (9, 10).

Biologically, ASMase has been implicated in a variety of physiologic and pathophysiologic processes (reviewed in Refs. 11–14). In particular, ASMase has been shown to be activated in response to various stress stimuli including 1) ligation of death receptors (tumor necrosis factor-α, CD95, and TRAIL), 2) radiation (UV-C and ionizing radiation), 3) chemotherapeutic agents (cispaltin, doxorubicin, paclitaxel, and histone deacetylase inhibitors), 4) viral, bacterial, and parasitic pathogens (rhinoviruses, Neisseria gonorrhoea, Staphylococcus aureus, Pseudomonas aeruginosa, and Cryptosporidium parvum), and 5) cytokines (e.g. IL-1β). Evidence from studies conducted in cell culture and animal models suggested that activation of ASMase by the above agents is a key step during cellular differentiation, growth arrest, apoptosis, and immune defense mechanisms. Although most of these studies describe rapid activation of the enzyme (most likely via a post-translational modification), transcriptional change is yet another regulator of ASMase activity. For instance, differentiation of monocytes to macrophages requires up-regulation of the enzyme at a transcriptional level (15). Transcription factors SP1 and AP-2...
mediate the ASMase response during monocytic differentiation. Later studies demonstrated that AP-2 also up-regulates ASMase in leukemia cells treated with retinoic acid (16). More recently, ASMase was implicated in maintenance of calcium homeostasis in mouse and cell culture models (17, 18). In particular, cerebellar deficits in the Niemann-Pick mouse were reported to be associated with defects in the calcium transport machinery in the endoplasmic reticulum.

Thus, although the scientific literature is replete with reports implicating ASMase in various cellular responses, the biochemical and molecular mechanisms of ASMase regulation remain unclear. Interestingly, recent studies suggest regulation of ASMase by redox mechanisms. For instance, the death ligand TRAIL signals ASMase activation through reactive oxygen species production (19). Additional mechanisms proposed to regulate ASMase enzymatic activity include interaction with lysosomal anionic lipids (bismonoacylglycerophosphate) and sphingolipid activator protein SAP-C (20). Nevertheless, the proximal mechanisms involved in direct regulation of ASMase remain undefined.

In a previous study, we identified a novel mechanism of regulation of ceramide formation by protein kinase C (PKC) through activation of the salvage pathway (1). In this model, treatment of MCF-7 breast cancer cells with PMA, a potent PKC agonist, resulted in a significant increase in cellular ceramide concomitant with a drop in sphingomyelin levels. Interestingly, this response was blocked upon treatment with the ceramide synthase inhibitor, fumonisin B1, but not an inhibitor of serine palmitoyltransferase, mirocin, thus ruling out involvement of the de novo pathway.

In this study, we demonstrate that phorbol esters induce activation of ASMase through PKCθ-dependent phosphorylation. Using site-directed mutagenesis, it is shown that PMA induces phosphorylation of ASMase at Ser508, and this phosphorylation is indispensable for activation of the enzyme. Moreover, this phosphorylation event appears to be required for translocation of ASMase to the plasma membrane. The significance of Ser508 phosphorylation and the role of PKCθ-ASMase association are further confirmed in the context of agonist-driven ASMase activation induced by UV radiation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture material, including RPMI medium, fetal bovine serum (FBS), phosphate-free medium, and dialyzed serum, were from Invitrogen. Bovine sphingomyelin, phosphatidylserine, and diacylglycerol were from Avanti polar lipids (Alabaster, AL). [choline-methyl-14C]Sphingomyelin was kindly provided by Dr. Alicja Bielawska (Medical University of South Carolina, Charleston, SC). Antibodies against PKCθ (polyclonal) and LAMP-1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies against the V5 epitope and GFP were from Invitrogen. Anti-phosphoserine (polyclonal) was from Zymed Laboratories Inc. (San Francisco, CA). Purified ASMase was a kind gift from Dr. Gary Smith (Glaxosmithkline). The PKC antibody sampler kit was purchased from BD Biosciences. The endoglycosidase H digestion kit and all other materials were from Sigma.

**Cell Lines and Culture Conditions**—MCF-7 cells were originally purchased from ATCC (Manassas, VA). Cells were maintained in RPMI 1640 supplemented with 10% FBS at 37 °C in a 5% CO2 incubator. Where indicated, cells were shifted to a phosphate-free medium. Testing for the presence of mycoplasma infections was performed routinely on a monthly basis.

**UV Irradiation**—MCF-7 cells grown on 10-cm dishes were coated with a thin layer of phosphate-buffered saline prior to irradiation. Radiation was performed using a GS Gene Linker UV chamber (Bio-Rad), which emits UV-C light (λavg = 254 nm) at a dose of 50 J/m2. Cells were then reincubated with RPMI medium and then collected for analysis at the indicated time(s) postirradiation.

**ASMase Peptide and Antiserum Production**—Development of ASMase antiserum was conducted at the Sigma Genosys facility. The ASMase peptide sequence was analyzed using the PGCGene program. The program selects regions of a protein that are candidates for raising antibodies, based on antigenicity, flexibility, and β-turn. Based on this analysis, the following peptide sequence was chosen and synthesized: CVGELQAAEDRGDKV (15 mer, residues 361–374). This sequence received the following scores: antigenicity = 2.5; flexibility = 1.064; β-turn = 2.4. The peptide was conjugated with keyhole limpet hemocyanin before immunization of two rabbits. The third bleed was affinity-purified, and specificity of the antibody was verified by enzyme-linked immunosorbent assay, Western blotting, and immunofluorescence.

**Site-directed Mutagenesis**—Mutations of the serine residues of ASMase were performed by PCR cloning using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The primers used for the S to A mutation were as follows: S149A, 5’-AGGTGGAGAGCCGCA GTGCTGAG-3’; S231A, 5’-GTGCCGGCGGTGTGCTGGCT GGC-3’; S248A, 5’-CTGGGCGAATACGGCAAGTGTGACCTGC-3’; S508A, 5’-TACTCCAGGAGCCTGCAGTGCTGAG-3’; S508E, 5’-TACTCCAGGAGCGACACGTGTGTC-3’. All transformants were sequenced to verify the integrity of mutations.

**Immunoprecipitation and Western Blotting**—Transfections were performed according to standard protocols using Effectene (Qiagen). After 24 h, cells were treated as indicated in the figure legends. Cells were then lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 5 mM sodium fluoride, 1.75 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerol phosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM microtuscin. Homogenates were centrifuged at 10,000 × g for 10 min. The supernatants were used for immunoprecipitation of ASMase via a monoclonal V5 antibody (1 μg/ml). Western blot analysis was carried using similar procedures as described previously (21).

**SMase Assays**—In vitro enzymatic assays for acid and neutral sphingomyelinases were performed using [choline-methyl-14C]Sphingomyelin. The assays were performed as previously reported (21, 22).

**RNA Interference**—Gene silencing of human ASMase and PKCθ was performed essentially according to a standard protocol (21). Sequence-specific small interfering RNA reagents
were purchased from Qiagen (Valencia, CA). The sequences of the sense and antisense small interfering RNAs are shown in Table 1. The specificity of the RNAi was verified by sequence comparison with the human genome data base using the NIH Blast program.

In Vitro Kinase Assays—In vitro phosphorylation reactions were performed as follows: 6 µg of purified ASMase were incubated with 25 ng of recombinant PKCδ (Calbiochem) at 30°C in a kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, 0.1 mM EGTA, 0.1 mM ATP), 0.5 µCi of [γ-32P]ATP, and where indicated, the buffer was supplemented with lipid-detergent mixed micelles consisting of 100 µg/ml phosphatidylserine and 10 µg/ml diacylglycerol sonicated in 0.3% Triton X-100 solution. At the indicated time points, reactions were terminated by the addition of SDS sample buffer, and samples were boiled for 5 min. Samples were electrophoresed by SDS-PAGE, and then the gels were dried. ASMase phosphorylation was detected by autoradiography.

In Vivo Labeling of ASMase—MCF-7 cells plated in 10-cm dishes (250 × 103 cells/plate) were transiently transfected with V5-ASMase. Cells were incubated in phosphate-free RPMI medium supplemented with 5% dialyzed FBS for 1 h. Then cells were shifted to the labeling medium containing 0.2 mCi/ml [32P]-orthophosphate and incubated for an additional 4 h. Treatment with either PMA or Me6SO was performed in phosphate-free medium for 1 h. ASMase was immunoprecipitated via the V5 epitope and loaded on SDS-PAGE. Incorporation of 32P into ASMase was detected by autoradiography after gel drying.

Confocal Microscopy—Approximately 5 × 105 cells were seeded to a 2-cm poly-L-lysine-coated confocal plate (MatTek Corp.). After treatment, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with methanol for 5 min. Blocking was performed in 2.5% FBS solution. Primary antibodies were diluted in a solution of 1.5% FBS, 0.15% saponin and incubated for 3 h. The samples were then washed with 1.5% FBS solution three times. This was followed by incubation with secondary antibodies for 1 h at room temperature. Samples were stored at 4°C until image acquisition. Images were acquired using a Zeiss laser-scanning confocal microscope (LSM 510). Excitation wavelengths 488, 543, and 633 were used. Images were acquired at the equatorial plane of monolayer cells guided by DRAQ5 (Alexis) nuclear stain. For in vivo imaging, cells were fitted in a special chamber under controlled temperature and CO2 settings.

Mass Spectroscopy for Ceramide and Sphingomyelin—Sphingolipid analysis was performed using electrospray ionization/tandem mass spectrometry on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a multiple reaction-monitoring positive ionization mode. This method has been recently described (23).

Plasmid Constructs and Overexpression—Original ASMase cDNA was a kind gift from Dr. Ed Schuchman (Mount Sinai School of Medicine). The cDNA (2.37 kb) encoding human ASMase was excised from the original PSVK3 plasmid using EcoRI and EcoRV restriction enzymes. After amplification by conventional PCR, the cDNA was ligated into pEF6/V5-HisTOPO vector (Invitrogen). The V5 epitope is situated at the carboxyl terminus of ASMase. Complementary DNA for PKCδ was ligated into pEGFP-C3 vector (Clontech). The restriction sites used were Xhol and KpnI. The GFP epitope is situated at the amino terminus of PKCδ. The integrity of the new plasmids was verified by DNA sequencing. For transient overexpression experiments, endotoxin-free plasmids were transfected into MCF-7 cells using Effectene (Qiagen) according to the manufacturer’s recommendations.

Real Time PCR Analysis (Reverse Transcription-PCR/Quantitative PCR)—DNA-free messenger RNA from MCF-7 cells was isolated using the Qiagen minikit for mRNA extraction. Complementary DNA was synthesized from 5 µg of total RNA using the reverse transcriptase kit from Promega (Madison, WI). Real time reverse transcription-PCR was performed on an iCycler system from Bio-Rad. The sample real time reverse transcription-PCR reaction volume was 25 µl, including 12.5 µl of SYBR Green PCR reagents (Qiagen, Valencia, CA), 7.5 µl of cDNA template, and 5 µl of the diluted forward and reverse primers (3 µM). First steps of reverse transcription-PCR were 2 min at 50°C followed by a 10-min hold at 95°C. Cycles (n = 40) consisted of a 15-s melt at 95°C, followed by a 1-min annealing/extension at 60°C. The final step was a 60°C incubation for 1 min. Reactions were performed in triplicate. The threshold for cycle of threshold (Ct) analysis of all samples was set at 0.15 relative fluorescence units. The data were normalized to levels of an internal control gene, β-actin. The reverse transcription primers used in this study were as follows: β-actin (forward, 5′-ATTGGCAATGACCGGTTC-3′; reverse, 5′-GGTAGTTTCCGTGGATGCCACA-3′) and ASMase (forward, 5′-TGGCTCTATGAAGCGATGGC-3′; reverse, 5′-TTGAGAGAGATGAGGCAGAGAC).

Deglycosylation with Endoglycosidase H—Treatment of immunoprecipitated ASMase with endoglycosidase H (Sigma) was performed according to the instructions of the manufacturer. Briefly, samples were boiled in denaturation buffer containing 50 mM β-mercaptoethanol and 0.1% SDS for 5 min. Samples were cooled on ice for another 5 min. Then endoglycosidase was added (0.01 units/sample), and the reaction was carried at 37°C for 12 h in a reaction buffer consisting of 50 mM sodium phosphate, pH 5.5.

Statistical Analysis—Mann-Whitney or Student’s t tests were performed between control and treated states and/or between treatment and treatment plus RNAi-mediated inhibition states in a minimum of three independent experiments. A p value of 0.05 or less is considered as statistically significant and marked in the figures with an asterisk.

RESULTS

PMA Induces Activation and Membrane Translocation of ASMase—Previously, we reported that PMA treatment induces an elevation in ceramide levels that persisted after blocking the de novo pathway with myriocin but was inhibited by fumonisin B1, suggesting operation of the salvage pathway (resynthesis of ceramide from sphingosine derived from the breakdown of complex sphingolipids but not from de novo synthesis) (1). The concomitant drop in sphingomyelin levels induced by PMA treatment, therefore, prompted us to investigate the potential involvement of SMases. As shown in Fig. 1A, treatment of
MCF-7 cells with 100 nM PMA resulted in transient activation of ASMase, with the maximum in vitro activity detected after 60 min (Fig. 1A). Since diacylglycerol has been suggested to activate ASMase directly (24), the effect of direct addition of PMA into the enzyme reaction buffer (post-cell lysis) was evaluated; however, PMA failed to activate ASMase directly (data not shown), suggesting that the stimulation process was indirect. Moreover, no change in total cellular neutral SMase activity was detected within 60 min of PMA treatment (Fig. 1A).

Next, we examined whether activation of ASMase was associated with a change in its subcellular localization. For these studies, a novel polyclonal antibody that recognizes ASMase was employed. After affinity purification of third bleed serum, the specificity of the antibody was tested. Western blot analysis of liver homogenates derived from WT and ASMase/H11002/H11002 mice revealed that the new antibody detected the 72-kDa form of ASMase (supplemental Fig. 1A). Moreover, the antibody proved useful for immunofluorescence applications, as indicated by colocalization of its signal with the lysosomal marker LAMP-1 (lysosomal-associated membrane protein 1) (supplemental Fig. 1B).

As expected, under basal conditions, most of cellular ASMase was concentrated within acidic compartments, as evidenced by colocalization with the acidophilic dye lysotracker red. There was no change in ASMase staining acutely after PMA treatment (5 min). However, confocal microscopy imaging revealed translocation of ASMase from endolysosomes to the plasma membrane after sustained PMA treatment (100 nM, 60 min) (Fig. 1B). Therefore, these data demonstrate that the stimulatory effect of PMA on ASMase is associated with relocation of the enzyme to the plasma membrane.
Mechanism of Acid Sphingomyelinase Activation

Requirement for ASMase in PMA-induced Ceramide Formation—The contribution of ASMase to the observed accumulation of ceramide following PMA treatment was consequently investigated. Although tricyclic amines (and other closely related compounds) have been routinely used to block ASMase, our recent data showed that the action of these compounds is not restricted to ASMase (21). Therefore, knockdown of ASMase was attempted using two different RNAi sequences. Real time PCR analysis was employed to analyze levels of ASMase mRNA (with β-actin as internal control) 48 h after transfection of two different RNAi sequences. As shown in Fig. 2A, successful knockdown of ASMase mRNA was achieved with one of the two RNAi sequences (sequence 1) at a concentration as low as 5 nM. Knockdown of ASMase was further confirmed by in vitro ASMase enzymatic assay. Transfection of sequence 1 (5 nM) resulted in about 70% reduction in total ASMase activity observed at 48 and 72 h post-transfection (Fig. 2B). Therefore, cells were transfected with either scrambled (SCR) or ASMase RNAi (5 nM for 48 h) prior to treatment with PMA. Mass spectrometric analysis showed a doubling in total cellular ceramide levels in response to PMA in cells pretreated with SCR sequence. This response was significantly blocked in cells where ASMase was knocked down (Fig. 2C). Taken together, these results suggest that ASMase is required for PMA-induced ceramide formation in the salvage pathway.

Role of PKCζ in Activation of ASMase—Phorbol esters signal primarily through proteins with C1 domains, of which the classical and novel PKCs are the most studied. Therefore, to investigate the possible involvement of PKCs in ASMase activation, a panel of pharmacologic inhibitors that block different classes of PKCs was employed. In vitro SMase assays showed that pretreatment with either bisindolelammide, an inhibitor of classical and novel PKCs, or rottlerin, an inhibitor of novel PKCζ, blocked the ASMase activation in response to PMA. However, the classical PKC inhibitor GO 6976 did not significantly affect ASMase activity (Fig. 3A). Based on the above results and on published studies on the role of PKCζ in mediating stress responses, we hypothesized that the novel PKCζ may mediate activation of ASMase. RNAi knockdown of PKCζ was therefore employed. Transfection of a specific PKCζ RNAi sequence (Table 1) at a dose of 5 nM for 48 h resulted in substantial down-regulation of PKCζ at the protein level without influen-

Figure 3. Involvement of PKCζ in ASMase activation. A, MCF-7 cells were pretreated with the PKC inhibitor bisindolelammide (Bis) (1 μM), classical PKC inhibitor GO 6976 (3 μM), or the novel PKC inhibitor rottlerin (10 μM) for 1 h. Then cells were stimulated with PMA for an additional 1 h prior to cell collection and lysis.

SMase assay was conducted in an acidic buffer (pH 4.5), and results are normalized to protein levels. B, knockdown of PKCζ using RNA interference. Cells were transfected with a 5 nM concentration of either SCR or PKCζ-specific RNAi for 48 h. Cells were collected and lysed, and 30 μg of lysates from each sample were loaded to 10% SDS gels. PKCζ, α, δ, ε, and ζ were detected at 78, 75, 79, 90, and 68 kDa, respectively, using specific polyclonal and monoclonal antibodies. C, effect of PKCζ knockdown on ASMase activity. After transfection of either SCR or PKCζ RNAi sequences (48 h, 5 nM), cells were treated with PMA (100 nM, 1 h). After cell lysis, 100 μg of each lysate were used for determination of ASMase activity. D, cells were treated as indicated in C. After Bligh and Dyer lipid extraction, ceramide levels were determined by mass spectroscopy. Results are normalized to total phospholipids. Shown are averages of three independent measurements of SMase activity and ceramide levels ± S.E. (p < 0.05). ns, not significant. E, cells were double transfected with V5-ASMase and PKCζ RNAi (or SCR) for 48 h. After treatment with either PMA (100 nM) or MeSO4 for 48 h, cells were stained with a V5 monoclonal antibody (green channel). Nuclei were visualized via DRAQ-5 nuclear stain (red). Results shown are representative of images obtained in three independent experiments. CN, control.
ing total cellular levels of other detected PKC isoforms (Fig. 3B). Interestingly, knockdown of PKCδ blocked activation of ASMase after PMA treatment but had no effect on basal enzymatic activity (Fig. 3C). In further support of these results, mass spectrometric analysis revealed a proportional drop in ceramide levels after PKCδ knockdown (Fig. 3D). Additionally, knockdown of PKCδ blocked the PMA-induced ASMase translocation to the plasma membrane (Fig. 3E). Therefore, these results indicate that PKCδ lies upstream of ASMase activation.

Early Translocation of PKCδ to the Plasma Membrane Followed by Partial Localization to the Lysosome—Similar to most other members of the PKC family, translocation of PKCδ from the cytosol to the plasma membrane is a hallmark of its activation. However, it is worth mentioning that additional sites of PKCδ translocation have been recently reported, including mitochondria (25, 26), Golgi (27), and the nuclear envelope (28, 29). Using confocal microscopy imaging of live cells, time-dependent changes in the subcellular localization of PKCδ were examined after PMA treatment. To that end, MCF-7 cells were transiently transfected with GFP-tagged PKCδ and labeled with the acidophilic dye lysotracker red. Initially (t = 0), PKCδ adopted a diffuse cytosolic pattern, whereas lysotracker red concentrated mainly within compartments of different sizes, consistent with endolysosomes. These compartments were not only undergoing fission and fusion but also in continuous contact with the plasma membrane (see supplemental material for movie). As expected and within 1 min of PMA treatment, PKCδ translocated to the plasma membrane along with clearing of the GFP signal from the cytosol (Fig. 4). In some instances, translocation of PKCδ was seen as early as 30 s after PMA addition. This localization pattern persisted up to 15 min. By 20 min, PKCδ was seen to (a) internalize within endocytic compartments that colocalized with lysotracker red and (b) adopt a concomitant perinuclear distribution (Fig. 4 and supplemental data). It should be also noted that the intensity of the lysotracker red signal was preserved up to 1 h, suggesting that PMA treatment does not profoundly influence lysosomal integrity. Therefore, the above results raised the possibility that in addition to the plasma membrane, PMA treatment induces redistribution of a portion of PKCδ to ASMase proximal sites, such as endolysosomes.

Formation of a PKCδ-ASMase Complex upon PMA Treatment—Given that PMA treatment induced ASMase activation as well as PKCδ relocation to endolysosomes within comparable time frames, it became important to determine whether there is direct or indirect protein-protein interaction between PKCδ and ASMase. To that end, ASMase was immunoprecipitated from lysates of cells treated with either Me₂SO or PMA. The isolated immune complexes were then tested for the presence of PKCδ using a polyclonal antibody. Although PKCδ was absent (or below detection level) in complexes derived from cells treated with a short course of PMA (5 min), PKCδ commmunoprecipitated with ASMase after 60 min of PMA treatment (Fig. 5A), corresponding to the time frame in which maximal ASMase activation was detected. These results were further corroborated by transient overexpression of PKCδ (GFP-tagged) and ASMase (V5-tagged). Association of PKCδ and ASMase was again observed to be dependent on PMA stimulation (Fig. 5B). The subcellular site of PKCδ-ASMase association was further examined by immunofluorescence. Consistent with the previous result (Fig. 4, third panel), within 30 min of PMA stimulation, ASMase and PKCδ co-stained in a perinuclear subset of lysosomes, and only minimal ASMase translocation was observed (Fig. 5C). Taken together, these results suggest that sustained stimulation of PKCδ with PMA induces its translocation to endolysosomes and further association with ASMase.

Phosphorylation of ASMase in Vitro by PKCδ—The above results prompted us to investigate whether ASMase is a potential substrate for PKCδ. To that end, an in vitro phosphorylation assay was performed using recombinant PKCδ and ASMase proteins. The purity of the recombinant proteins was evaluated by Coomassie Blue gel staining, which revealed only the major bands for ASMase at 72 kDa and PKCδ at 78 kDa (data not

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**TABLE 1**

| Target protein | RNAi target sequence (21 bases) |
|----------------|---------------------------------|
| hASMase (sequence1) | AAC TCC TTT GGA TGG GCC TGG |
| hASMase (sequence2) | AAG GAC ACA TCG CAT AGT GCC |
| PKCδ | AAC GAC AAG ATC ATC GGC AGA |

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**FIGURE 4.** PMA induces early plasma membrane translocation of PKCδ followed by lysosomal internalization. Cells were plated on 2-cm glass bottom dishes (5 × 10⁴ cells/dish). After transient transfection with GFP-PKCδ, cells were incubated for 24 h for overexpression. Lysotracker red dye was added 30 min prior to PMA stimulation. Cells were fitted inside an in vivo imaging chamber with controlled CO₂ and temperature settings. Images were acquired at the nuclear plane at a frequency of 4 scans/min over a time frame of 30 min. Shown are representative images of the in vivo imaging experiment with the corresponding time after PMA stimulation indicated. The complete movie is provided as supplemental data. Scale bar, 20 μm.
PKCδ Phosphorylates ASMase at Ser<sup>508</sup>—Based on the data above, it became important to investigate whether ASMase is regulated by phosphorylation and to determine if there are specific phosphorylation sites that respond to PKCδ. The human ASMase primary sequence was analyzed using the NetPhos phosphorylation prediction algorithm. Four potential serine phosphorylation residues were found to be conserved in mouse, rat, and Caenorhabditis elegans homologs of ASMase. Interestingly, two of these sites were also reported to be mutated in Niemann-Pick disease patients (30). To investigate phosphorylation of ASMase at these sites, a site-directed mutagenesis approach was employed, where each of the candidate serines was mutated to alanine. After transient transfection into MCF-7 cells, the activity, expression, and localization of all four mutants were determined and compared with wild type (WT) ASMase. In vitro SMase assays and Western blot analysis revealed similar basal activities and degree of expression among the mutant and WT ASMases (Fig. 7A). Furthermore, an endolysosomal staining pattern was observed for these mutants and confirmed by partial colocalization with LAMP-1 (Fig. 7B).

Next, the response of the four Ser<sup>3</sup>→Ala mutants to agonist-driven activation of ASMase was examined. Three of the mutants exhibited a response comparable with that of WT ASMase upon stimulation with PMA (100 nM, 1 h). In contrast, mutation of serine 508 to alanine abrogated PMA-induced ASMase activation (Fig. 8A). Serine phosphorylation of WT and the four Ser<sup>3</sup>→Ala ASMase mutants was studied next. As shown in Fig. 8B, although PMA treatment induced serine phosphorylation of WT and three of the mutants, ASMase<sup>S508A</sup> failed to show any change (Fig. 8B). Interestingly, a basal phosphoserine signal could be detected and remained unchanged in all mutants.

FIGURE 5. Association of ASMase and PKCδ after PMA treatment. A, immunoprecipitation (IP) of endogenous ASMase and PKCδ. Cells grown on 10-cm plates were treated with either vehicle (Me2SO) or PMA (100 nM) for 5 or 60 min. After cell lysis, ASMase was immunoprecipitated overnight from the different samples using a homemade polyclonal antibody. Representative samples of the immune complexes and supernatants were loaded on 10% SDS gels. The presence of PKCδ was checked by Western blotting. B, immunoprecipitation of overexpressed PKCδ and ASMase. MCF-7 cells transiently overexpressing PKCδ and ASMase were treated with PMA (100 nM) for 1 h. PKCδ (via GFP tag) or ASMase (via V5 epitope) was immunoprecipitated, separated on SDS-PAGE, and subjected to Western blot for V5-ASMase or GFP-PKCδ. Total cellular lysates were also separated by SDS-PAGE and analyzed for levels of ASMase and PKCδ overexpression (bottom). Blots shown are representative of those obtained in three independent experiments. C, cells were plated on 2-cm glass bottom dishes (5 × 10<sup>4</sup> cells/dish). After treatment with either PMA (100 nM) or Me2SO (DMSO) for 30 min, cells were co-stained using polyclonal antibodies for ASMase (red channel) and PKCδ (green channel), as described under “Experimental Procedures.” Results shown are representative of images obtained in at least three independent experiments.
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**FIGURE 6. In vitro phosphorylation of recombinant ASMase by PKCδ.** In vitro phosphorylation of purified ASMase (72 kDa) with human recombinant PKCδ was carried out as described under “Experimental Procedures.” The time courses of the phosphorylation of ASMase were detected by autoradiography after 48 h of film exposure. The experiment was performed either in the absence (top) or presence of PKC lipid activators (diacylglycerol (10 μg/ml) and PS (100 μg/ml)) (bottom). The same experiment was performed twice with similar results obtained.

detected by autoradiography for both WT and ASMaseS508A that masked any differences in peptide phosphorylation. This signal has been examined previously and found to be due to phosphomannose glycosylation of asparagine residues of ASMase (7). Therefore, digestion with endoglycosidase H was employed to remove this phosphomannose. Indeed, endoglycosidase H abrogated this signal (Fig. 3C), supporting the previous findings that the 32P labeling was preferentially taking place at mannose 6-phosphate (7). A concomitant decrease in molecular mass of about 10 kDa was also detected. In line with previous studies, basal phosphorylation of the ASMase peptide core was still observed after deglycosylation (31). Importantly, the addition of PMA induced a significant increase in the phosphorylation of WT ASMase but not ASMaseS508A (Fig. 8C). Moreover, ClustalW analysis of ASMase sequence revealed that Ser508, located within the catalytic domain, is conserved in human, mouse, and monkey homologs (Fig. 8D). Therefore, these results corroborate the phosphorylation of ASMase in response to PMA and provide strong evidence that Ser508 is involved in this phosphorylation.

Next, the role of PKCδ in PMA-induced ASMase phosphorylation was investigated. Knockdown of PKCδ was successfully achieved (more than 80%) via PKCδ-specific RNAi oligonucleotides. Phosphorylation of ASMase in response to PMA was determined 48 h after transfection of either PKCδ RNAi or control sequence (SCR). As shown in Fig. 8E, loss of PKCδ impeded the ability of PMA to induce ASMase serine phosphorylation. Therefore, these findings provide genetic evidence for the requirement of PKCδ in the ASMase response to PMA.

**UV Light Triggers the PKCδ-ASMase Pathway**—Based on the above biochemical studies, it became important to determine if the phosphorylation of Ser508 is required for stress-induced activation of ASMase. To this end, MCF-7 cells overexpressing WT ASMase or ASMaseS508A were exposed to UV-C light irradiation (50 J/m²), a previously established ASMase stimulant (32–34). Phosphorylation of ASMase was examined 10 min after UV exposure, a time where maximal ASMase activity was detected. In line with previous results, basal serine phosphorylation could be detected in untreated cells overexpressing WT ASMase and ASMaseS508A. However, enhanced serine phosphorylation, comparable with the one previously observed with PMA treatment (positive control), was detected in WT ASMase but not ASMaseS508A overexpressor following UV exposure (Fig. 9). These results demonstrate that phosphorylation of ASMase at Ser508 is induced by stress stimuli as represented by UV light.

**ASMaseS508A Acts as a Dominant Negative ASMase**—Several recent reports described that activation of ASMase is associated with its translocation from the endolysosomal compartment to the plasma membrane (35). Since the above results indicated a similar translocation process after PMA treatment, it became important to investigate whether ASMase translocation to the plasma membrane is dependent on Ser508 phosphorylation. To that end, MCF-7 cells plated on poly-1-lysine-coated confocal dishes were transiently transfected with either WT or S508A ASMase. Although a similar distribution pattern was observed under basal conditions, both PMA and UV light stimulation induced membrane association of only WT and not ASMaseS508A (Fig. 10A). Therefore, these findings suggest that phosphorylation of Ser508 is also required for ASMase translocation to the plasma membrane.

The significance of Ser508 phosphorylation in the response of MCF-7 cells to PMA was further pursued. Cells transiently overexpressing either WT or ASMaseS508A were treated with PMA (1 h). After lipid extraction, samples were subjected to mass spectrometric analysis of different ceramide species. No major differences in the different ceramides were seen under basal conditions. However, upon PMA treatment, a major increase in C16-ceramide and a minor one in C24-ceramide were detected only in WT- and not ASMaseS508A-overexpressing cells (Fig. 10B). Therefore, these results demonstrate that phosphorylation of Ser508 is required for agonist-induced activation of ASMase and subsequent ceramide generation.

**DISCUSSION**

ASMase is a well-characterized lipid phospholipase with suggested roles in cellular stress responses, yet its biochemical and molecular mechanisms of regulation remain largely unknown. The results from the present study show that activation of ASMase proceeds through phosphorylation at Ser508. In addition to its activation, translocation of ASMase from the endolysosomal compartment to the plasma membrane was found to be dependent on this phosphorylation step. The study also indicates that PKCδ is an important kinase that could function upstream of ASMase in cell signaling pathways.

A major conclusion from this study relates to the role of ASMase in the regulated salvage pathway of ceramide accumu-
It was shown previously that the ceramide response to PMA stimulation requires ceramide synthase activity (inhibited by fumonisin B1) but not de novo synthesis (not inhibited by myriocin). Using a metabolic labeling approach, the same study indicated that the ceramide response originated from breakdown of complex sphingolipids and not from de novo synthesis (1). Therefore, these results indicated the operation of the “salvage pathway,” whereby complex sphingolipids are broken down to ceramide and then to sphingosine, which is then reacylated to ceramide.

In preliminary results, it was found that PMA stimulated breakdown of SM; therefore, it became important to determine the enzyme responsible for the initiation of the salvage pathway. The results from this study implicate ASMase in the ceramide response to PMA. This is supported by two lines of evidence. First, RNAi targeting of ASMase significantly attenuated ceramide accumulation in response to PMA. Second, the dominant negative ASMaseS508A mutant also inhibited the PMA response. Therefore, in addition to the classical de novo and SM breakdown pathways, these results highlight a third “hybrid” salvage pathway of ceramide formation.

Interestingly, this salvage pathway, by combining ASMase activation with fumonisin-inhibitable ceramide formation, may explain previously seemingly contradictory results. For example, the chemotherapeutic agents fenretinide and anandamide were shown to induce ceramide production through activation of ASMase (36, 37). However, other reports showed that ceramide generated by these agents can be blocked by fumonisin B1 treatment (38, 39). In some cases, there were different results reported for the same cell line. Although radiation of MOLT-4 cells induced breakdown of sphingomyelin to ceramide through activation of ASMase, it was shown in a recent study that this ceramide response can be blocked by fumonisin in the same cell line (40, 41). Also, a previous study showed an important role for ceramide synthase in the PMA response of LNCaP prostate cancer cells (42); however, the effects on SM turnover were not investigated.
The effects of PKC agonists on sphingolipid metabolism have been studied previously. In GH3 pituitary cells, it was shown that diacylglycerol but not phorbol esters directly trigger SM breakdown independent of PKC (24). However, using the same metabolic labeling approach with radioactive sphingomyelin as in the aforementioned study, Tettamanti et al. (43) came to a different conclusion. The authors found that treatment of the human neuroblastoma cell line SH-SY5Y with PMA induces SM breakdown, a critical step in the differentiation of this cell line. In agreement with the latter study, we previously reported that stimulation of the MCF-7 breast cancer cell line with PMA triggered the sphingomyelin breakdown pathway (1). In addition...

FIGURE 8. Role of Ser\(^{508}\) in ASMase activation. Cells transiently overexpressing WT ASMase or the indicated Ser \(\rightarrow\) Ala mutants were treated with either vehicle (Me\(_2\)SO) or PMA for 1 h. A, lysates (100 \(\mu\)g each) were used for the ASMase enzymatic assay described under "Experimental Procedures." B, ASMase was immunoprecipitated (IP) via V5 epitope from each sample. Immunoprecipitates were electrophoresed by SDS-PAGE and probed using anti-phosphoserine antibody. C, in vivo phosphorylation of WT ASMase and ASMase\(^{508A}\). Cells transiently transfected with either WT ASMase or ASMase\(^{508A}\) were labeled with \(^{32}\)P orthophosphate for 1 h, followed by treatment with PMA for 1 h. After immunoprecipitation with a V5 monoclonal antibody, ASMase was deglycosylated by overnight incubation with endoglycosidase H (Endo H) (except the first lane sample). D, ClustalW algorithm sequence alignment of ASMase sequences from different organisms in the region of Ser\(^{508}\) of human ASMase. E, cells overexpressing WT-ASMase were transfected with PKC\(_\delta\) RNAi or SCR (5 \(\mu\)M) for 48 h. ASMase was immunoprecipitated 10 min after treatment with PMA (100 \(\mu\)M, 1 h), using a V5 monoclonal antibody. Serine phosphorylation was evaluated by immunoblotting using a phosphoserine polyclonal antibody. Data are from one of three independent experiments with similar results (\(p < 0.05\)). CN, control.

FIGURE 9. Role of Ser\(^{508}\) in UV light-induced ASMase activation. MCF-7 cells overexpressing WT-ASMase or ASMase\(^{508A}\) were seeded in 100-mm dishes (5 \(\times\) 10\(^4\) cells/plate). ASMase was immunoprecipitated (IP) 10 min after exposure to UV light or treatment with PMA (100 nm, 1 h), using a V5 monoclonal antibody. Serine phosphorylation was evaluated by immunoblotting using a phosphoserine polyclonal antibody. Blots shown are representative of at least three independent experiments. CN, control.

FIGURE 10. ASMase\(^{508A}\) displays dominant negative properties. A, ASMase\(^{508A}\) fails to translocate to the plasma membrane. Cells transiently transfected with WT ASMase or ASMase\(^{508A}\) were analyzed by confocal microscopy prior to and after treatment with PMA for 1 h or 10 min after exposure to UV light (\(\lambda = 254\) nm, 50 J/m\(^2\)). ASMase was stained using a V5 antibody (green channel), and nuclei were visualized using a nuclear dye (red channel). Scale bar, 5 \(\mu\)m. B, overexpression of ASMase\(^{508A}\) blocks ceramide formation after PMA treatment. Cells transiently overexpressing WT ASMase or ASMase S508A were treated with either Me\(_2\)SO or PMA for 1 h. Lipids were extracted according to the Bligh and Dyer method, and ceramide species were analyzed by mass spectroscopy. Ceramide measurements are expressed as mean \(\pm\) S.E. from three experiments (\(p < 0.05\)). CN, control.
revealed that phosphorylation of ASMase at Ser508 is essential for activation of ASMase. Furthermore, the phosphorylated form of ASMase (p-ASMase) is consequently targeted to the plasma membrane, where it hydrolyzes membrane SM to ceramide. This process seemed to require the integrity of vesicular trafficking in addition to microtubules and actin cytoskeleton. Hence, it is tempting to speculate that phosphorylation of ASMase facilitates its interaction with membrane transport and/or docking machinery. This hypothesis is further supported by several studies documenting a central role for PKCδ in stress responses elicited by UV radiation and Fas ligand (45–47). However, further studies are required in order to identify specific mechanisms by which the phosphorylated form of ASMase translocates to the plasma membrane.

Although determining the exact mechanism by which PKCδ interacts with ASMase requires further study, it is clear that this kinase is critical for ASMase activation at least in response to PMA and UV light. This conclusion may begin to tie in previous results on stress signaling involving both PKCδ and ASMase. First, generation of reactive oxygen species has been recently shown to be an obligatory step for ASMase activation by UV radiation and TRAIL (19, 33). Interestingly, there are several reports highlighting the role of PKCδ both as a key target and effector during oxidative stress (48). Second, the PKCδ−/− mouse shows a phenotype of suppressed apoptosis in response to tumor necrosis factor-α, UV, and γ-radiation (49). Importantly, the aforementioned stress agents signal ceramide generation primarily through ASMase activation; this raises the question of whether the PKCδ−/− mouse has a defective sphingomyelin/ceramide stress signaling pathway. Although the results shown in this study indicate that PKCδ can associate and phosphorylate ASMase in cells and in vitro, we cannot rule out the potential involvement of other kinases at this point.

The identification of key substrates for PKCδ has emerged as an important goal in deciphering mechanisms of action of this kinase. Although activation of PKCδ has been associated with growth arrest and in some cases initiation of an apoptotic cascade, only few PKCδ substrates have been identified. For instance, PKCδ-dependent phosphorylation of phospholipid scramblase 1 (plasma membrane) (50), phospholipid scramblase 3 (mitochondrion) (51), and LaminB (nucleus) (52) are critical events during certain apoptotic cascades. Given the involvement of PKCδ in apoptotic pathways, perhaps it is not surprising that ASMase, which in turn is critical in several cellular stress responses, is a substrate and an effector of PKCδ.

The novel PKCδ and the sphingolipid ceramide are well established as intermediates in signal transduction pathways, and recent studies have begun to explore the cross-talk between the two (see commentary by Grant and Spiegel (53)). Saito and co-workers (27) showed that ceramide induces translocation of PKCδ to the Golgi apparatus in HeLa cells. In a later study, the authors reported that ceramide activation of Src kinase induces phosphorylation of PKCδ inside the Golgi and that this process is indispensable for ceramide-mediated apoptosis (54). Furthermore, de novo synthesis of ceramide caused cytochrome c release by targeting PKCδ to the mitochondrion in prostate cancer cells treated with DNA-damaging agents (55). The current results further extend this area of research to show that PKCδ can also function upstream of ceramide generation through regulation of ASMase. With this in mind, one can hypothesize the existence of a positive feedback loop between activation of PKCδ and downstream ceramide formation.
Mechanism of Acid Sphingomyelinase Activation

The results from this study also raise intriguing questions as to the subcellular site(s) of PKCδ and ASMase interaction. In addition to the plasma membrane, PKCδ has been reported to relocate to other compartments, such as the mitochondrion, the Golgi complex, and the nuclear envelope (48). For the first time, we show that sustained activation of PKCδ, which induces an early plasma membrane association, is followed by its relocation to endolysosomes. It is more likely that the lysosomal pool of PKCδ rather than the plasma membrane one is critical for ASMase activation. First, the course of activation and translocation of both PKCδ and ASMase appears to favor initial interaction prior to translocation of ASMase to the plasma membrane. Second, inhibition of phosphorylation of ASMase in the S508A mutant abrogated translocation to the plasma membrane, demonstrating that regulation by PKCδ precedes this translocation and is required for it. Finally, topologically, a protein-protein interaction at the plasma membrane seems unfavorable, since ASMase and PKCδ are expected to reside on opposite membrane leaflets (external and internal, respectively). However, membrane fusion and formation of multivesicular endolysosomal structures may allow for interaction of PKCδ and ASMase (Fig. 11).

How does Ser508 phosphorylation induce ASMase activation? Although answering this question is rather difficult in the current absence of a crystal structure for ASMase, three possibilities are expected to reside on opposite membrane leaflets (external and internal, respectively). However, membrane fusion and formation of multivesicular endolysosomal structures may allow for interaction of PKCδ and ASMase (Fig. 11).

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