Involvement of Acid β-Glucosidase 1 in the Salvage Pathway of Ceramide Formation*

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Activation of protein kinase C (PKC) promotes the salvage pathway of ceramide formation, and acid sphingomyelinas has been implicated, in part, in providing substrate for this pathway (Zeidan, Y. H., and Hannun, Y. A. (2007) J. Biol. Chem. 282, 11549–11561). In the present study, we examined whether acid β-glucosidase 1 (GBA1), which hydrolyzes glucosylceramide to form lysosomal ceramide, was involved in PKC-regulated formation of ceramide from recycled sphingosine. Glucosylceramide levels declined after treatment of MCF-7 cells with a potent PKC activator, phorbol 12-myristate 13-acetate (PMA). Silencing GBA1 by small interfering RNAs significantly attenuated acid glucocerebrosidase activity and decreased PMA-induced formation of ceramide by 50%. Silencing GBA1 blocked PMA-induced degradation of glucosylceramide and generation of sphingosine, the source for ceramide biosynthesis. Reciprocally, forced expression of GBA1 increased ceramide levels. These observations indicate that GBA1 activation can generate the source (sphingosine) for PMA-induced formation of ceramide through the salvage pathway. Next, the role of PKCδ, a direct effector of PMA, in the formation of ceramide was determined. By attenuating expression of PKCδ, cells failed to trigger PMA-induced alterations in levels of ceramide, sphingomyelin, and glucosylceramide. Thus, PKCδ activation is suggested to stimulate the degradation of both sphingomyelin and glucosylceramide leading to the salvage pathway of ceramide formation. Collectively, GBA1 is identified as a novel source of regulated formation of ceramide, and PKCδ is an upstream regulator of this pathway.

Sphingolipids are abundant components of cellular membranes, many of which are emerging as bioactive lipid mediators thought to play crucial roles in cellular responses (1, 2). Ceramide, a central sphingolipid, serves as the main precursor for various sphingolipids, including glycosphingolipids, gangliosides, and sphingomyelin. Regulation of formation of ceramide has been demonstrated through the action of three major pathways: the de novo pathway (3, 4), the sphingomyelinase pathway (5), and the salvage pathway (6–8). The latter plays an important role in constitutive sphingolipid turnover by salvaging long-chain sphingoid bases (sphingosine and dihydro sphingosine) that serve as sphingolipid backbones for ceramide and dihydroceramide as well as all complex sphingolipids (Fig. 1A).

Metabolically, ceramide is also formed from degradation of glycosphingolipids (Fig. 1A) usually in acidic compartments, the lysosomes and/or late endosomes (9). The stepwise hydrolysis of complex glycosphingolipids eventually results in the formation of glucosylceramide, which in turn is converted to ceramide by the action of acid β-glucosidase 1 (GBA1) (9, 10). Severe defects in GBA1 activity cause Gaucher disease, which is associated with aberrant accumulation of the lipid substrates (10–14). On the other hand, sphingomyelin is cleaved by acid sphingomyelinase to also form ceramide (15, 16). Either process results in the generation of lysosomal ceramide that can then be deacylated by acid ceramidase (17), releasing sphingosine that may escape the lysosome (18). The released sphingosine may become a substrate for either sphingosine kinases or ceramide synthases, forming sphingosine 1-phosphate or ceramide, respectively (3, 19–21).

In a related line of investigation, our studies (20, 22, 23) have begun to implicate protein kinase Cs (PKCs) as upstream regulators of the sphingoid base salvage pathway resulting in ceramide synthesis. Activation of PKCs by the phorbol ester (PMA) was shown to stimulate the salvage pathway resulting in increases in ceramide. All the induced ceramide was inhibited by pretreatment with a ceramide synthase inhibitor, fumonisin B1, but not by myriocin, thus negating acute activation of the de novo pathway and establishing a role for ceramide synthesis (20, 23). Moreover, labeling studies also implicated the salvage pathway because PMA induced turnover of steady state-labeled sphingolipids but did not affect de novo labeled ceramide in pulse-chase experiments.

Moreover, PKCδ, among PKC isoforms, was identified as an upstream molecule for the activation of acid sphingomyelinase in the salvage pathway (22). Interestingly, the PKCδ isoform

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2 The abbreviations used are: GBA1, acid β-glucosidase 1; C16-ceramide, N-palmitoyl-d-erythro-sphingosine; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; SCR, scrambled sequence; siRNA, small interference RNA; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)).
induced the phosphorylation of acid sphingomyelinase at serine 508, leading to its activation and consequent formation of ceramide. The activation of acid sphingomyelinase appeared to contribute to ~50% of the salvage pathway-induced increase in ceramide (28) (also, see Fig. 4C). This raised the possibility that distinct routes of ceramide metabolism may account for the remainder of ceramide generation. In this study, we investigated glucocerebrosidase GBA1 as a candidate for one of the other routes accounting for PKC-regulated salvage pathway of ceramide formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peroxidase-conjugated antibodies for rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence kit was from Thermo Scientific. NBD-C12-ceramide was from Avanti Polar Lipids. pSPORT GBA1 expression vectors were from ATCC.

**Cell Culture**—MCF-7 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, and further cultured for 12 h. The cells were stimulated with 100 nM PMA for the indicated periods. Lipids were extracted (24) and separated by TLC on Silica Gel G plates using chloroform/methanol/H2O (110: 40:6, v/v/v) as the development system. The area corresponding to ceramide, cerebroside, composed of glucosylceramide and galactosylceramide, or sphingomyelin was scraped off, and the radioactivity measured by liquid scintillation counting. Over 85% of extracted [3H]cerebrosides were degraded by treatment with Cerezyme (Genzyme Corporation), recombinant human GBA1 (data not shown). Moreover, separation of [3H]glucosylceramide and [3H]galactosylceramide by borate-impregnated TLC plates also demonstrated that [3H]glucosylceramide comprises over 95% of [3H]cerebrosides. Thus, in MCF-7 cells, glucosylceramides are thought to mostly be composed of glucocerebrosides.

**Lipid Measurement by High-performance Liquid Chromatography-Tandem Mass Spectrometry**—Analysis of ceramide species in lipid extract was performed by high-performance liquid chromatography-tandem mass spectrometry as described in Bielawski et al. (25). Monohexosylceramides were measured by the modified method above. Amounts of monohexosylceramide were expressed as the total of species with various carbon chains (C14, C16, C18, C18:1, C20, C20:1, C22, C22:1, C24, C24:1, C26, and C26:1).

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

**RESULTS**

Glucosylceramide is a central backbone for most complex glycosphingolipids, and degradation of glucosylceramide is crucial for forming lysosomal ceramide and its catabolic product sphingosine. The latter is constitutively salvaged for generating ceramide in a ceramide synthase-dependent manner (Fig. 1A). Our previous observations indicated that activation of PKC by PMA in MCF-7 cells causes selective accumulation of C16-ceramide by promoting the salvage pathway in which ceramide synthases including LASS5/CerS5 contribute to acylation of recycled sphingosine (20, 23). As shown in Fig. 1B, PMA induced accumulation of 269 pmol/mg of ceramides, of which 241 pmol/mg was due to selective accumulation of C16-ceramide. Importantly, inhibition of ceramide synthase by fumonisin B1 (100 μM) attenuated most of the ceramide induction by PMA. Because acid sphingomyelinase appeared to contribute to only 50% of the accumulated C16-ceramide (Fig. 4C), it became important to determine whether GBA1, a lysosomal glucocerebrosidase, participates in the PKC-activated salvage pathway of ceramide formation. To address the possible involvement of GBA1, glucosylceramide was at first measured by metabolic labeling. MCF-7 cells labeled with [3H]sphin-
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(A) The scheme of the sphingosine salvage pathway of ceramide formation and inhibition of PMA induction of ceramide by fumonisin B1. A, the scheme of the sphingosine salvage pathway of ceramide formation. B, previously published data as to effects of fumonisin B1 on ceramide mass profiles (23) are re-plotted as a PMA induction of ceramide. In brief, MCF-7 cells were pretreated with or without 100 μM fumonisin B1 for 2 h followed by treatment with 100 nM PMA for 1 h. Lipids were extracted, and then the levels of ceramide species were determined by high-performance liquid chromatography-tandem mass spectrometry. Results are expressed as sum of ceramide species (C14-, C18-, C18:1-, C20-, and C24-ceramide), respectively. The data represent mean ± S.E. of three to five values.

(B) Effects of PMA on glucosylceramide levels. MCF-7 cells labeled with [3H]sphingosine were stimulated for the indicated periods, and then lipids were extracted and separated by TLC. The results were expressed as the percentage of control (0 min). At the three time points (15, 30, and 60 min), decreases in [3H]glucosylceramide levels were statistically significant relative to no treatment (0 min). *, p = 0.0213; **, p = 0.0242; ***, p = 0.0395.

gosine were employed to assess whether PKC activation induced the degradation of glucosylceramide. Compared with un-stimulated cells (control), [3H]glucosylceramide declined modestly but significantly (10–15%) after PMA treatment up to 1 h (Fig. 2). This degradation of glucosylceramide possibly resulted from enhancement of acid glucocerebrosidase activation.

To investigate specific involvement of GBA1 in PKC-dependent formation of ceramide through the salvage pathway, specific siRNAs were employed to knockdown GBA1. At first, the effectiveness of all three individual sequences designed (GBA1-a, GBA1-b, and GBA1-c; Fig. 3A) was evaluated. As shown in Fig. 3B, treatment of MCF-7 cells with three distinct GBA1 siRNAs dramatically reduced the activity of acid glucocerebrosidase compared with treatment with SCR siRNA. Concomitant with the activity of acid glucocerebrosidase, protein expression of GBA1 was decreased with GBA1 siRNA treatments (Fig. 3C), implying that GBA1 represents most of the acid glucocerebrosidase activity. Thus, all designed sequences of GBA1 siRNA were confirmed to significantly induce knock-down of GBA1 expression levels as well as acid glucocerebrosidase activities.

Next, we evaluated the effects of GBA1 knockdown on PMA-induced formation of ceramide. None of the siRNAs for GBA1 had significant effects on basal levels of C16-ceramide compared with SCR treatment, whereas GBA1 knockdown by each three individual siRNAs consistently and significantly inhibited PMA-induced generation of C16-ceramide by ~50% relative to SCR treatment (Fig. 4A). As to other ceramide species, GBA1 knockdown had no effect on levels of ceramide species (C14-, C18-, C18:1-, C20-, and C24-ceramide) and decreased basal levels of C24:1-ceramide (Fig. 4B). In addition to GBA1, knockdown of acid sphingomyelinase also inhibited PMA increases of C16-ceramide by ~50% (Fig. 4C). This finding is consistent with our previous observations (22). Therefore, GBA1 is proposed to also contribute to induction of C16-ceramide independently of acid sphingomyelinase after PMA treatment.

In a reciprocal approach, MCF-7 cells were transfected with GBA1 expression vectors. Forced expression of GBA1 significantly increased the basal levels of ceramide and PMA-induced formation of ceramide (Fig. 5). These results provide further support to the notion that GBA1-dependent degradation of glucosylceramide contributes to basal and PKC-regulated ceramide levels.

To test whether GBA1 activity specifically contributes to the degradation of glucosylceramide, effects of GBA1 knockdown were evaluated by measuring radiolabeled glucosylceramide (Fig. 6A) or the mass of monohexosylceramide species (Fig. 6B). GBA1 knockdown impaired PMA-induced formation of [3H]ceramide by 50% (Fig. 6A). In contrast, GBA1 knockdown induced a 10% increase in [3H]glucosylceramide with PMA treatment, which suggests enhanced catabolism of complex sphingolipids to glucosylceramide after PMA treatment (Fig. 6A). It should also be noted that GBA1 siRNA treatment did not influence PMA-induced hydrolysis of [3H]sphingomyelin (Fig. 6A). These data not only rule out effects of GBA1 knockdown on acid sphingomyelinase activation, but also imply specific involvement of GBA1 in a significant component of the PMA-
induced formation of ceramide. Reciprocally, the measurement by high-performance liquid chromatography-tandem mass spectrometry also indicates significant decreases in monohexosylceramides after PMA treatment, whereas GBA1-silenced cells failed to induce significant degradation of monohexosylceramides upon PMA treatment (Fig. 6B). These data indicate that PMA promotes degradation of glucosylceramide by GBA1.

Lysosomal ceramide formed from glucosylceramide is converted to sphingosine that can then serve as a source for generating ceramide. Previously, we showed that PMA induced elevation of the sphingosine levels (20). In this study, GBA1 knockdown significantly suppressed the elevation of free sphingosine after PMA treatment to a level of $\frac{1}{2}$ (Fig. 6C). These data indicate that PKC activation by PMA promotes GBA1 degradation of glucosylceramide to sphingosine as an additional source of ceramide formation from the salvage pathway.

PMA treatment stimulates activation of classical and novel PKCs promoting the salvage pathway of ceramide formation dependent on acid sphingomyelinase or GBA1. Because specific knockdown of PKC$\delta$ isosform without influencing the protein expression of other PKC isoforms (PKC-$\alpha$, $\theta$, $\zeta$, and $\xi$) completely blocked PKC$\delta$-induced formation of ceramide, PKC$\delta$ was implicated as a key enzyme in promoting the salvage pathway (22). To test whether PKC$\delta$ controls GBA1 activation, effects of PKC$\delta$ knockdown on sphingolipid metabolisms were examined (Fig. 7). Silencing PKC$\delta$ significantly abrogated the degradation of glucosylceramide in PMA-treated cells. PMA treatment in SCR-transfected cells induced an increase in $[^{3}H]$ceramide that corresponds to 5% of total radioactivity (in all metabolites), and the magnitude of this change was closely equivalent to the sum of the loss of radioactivities of sphingomyelin and glucosylceramide combined. Importantly, PMA-induced changes in levels of sphingolipids including ceramide and sphingomyelin failed to occur in PKC$\delta$-silenced cells. These data suggest that PKC$\delta$ is the predominant PKC isoenzyme that regulates the generation of ceramide in the salvage pathway.

**DISCUSSION**

The findings from this study reveal, for the first time, that GBA1 plays an important role in the regulated formation of ceramide through the sphingosine salvage pathway. Furthermore, PKC$\delta$ was identified as the likely key regulator in the GBA1-dependent salvage pathway of ceramide formation. These novel findings on regulation of GBA1 could also contribute to further understanding the significance of the enzyme and its involvement in a variety of cellular responses.

Activation of PKCs by PMA promotes the salvage pathway of ceramide formation in which GBA1 contributes to sphingosine salvage by generating lysosomal ceramide. A lysosomal enzyme, GBA1 hydrolyzes glucosylceramide to generate lysosomal ceramide. Subsequently, acid ceramidase converts lysosomal ceramide to sphingosine that is recycled to form ceramide through the action of ceramide synthases. The major
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FIGURE 4. Effects of knockdown of lysosomal enzymes on the generation of ceramide after PMA treatment. A, MCF-7 cells were transfected with 5 nM siRNAs of each of four individual sequences (SCR, GBA1-a, GBA1-b, and GBA1-c) for 48 h and then stimulated with 100 nM PMA for 1 h. Lipids were extracted, and then the levels of the C16-ceramide species were determined by high-performance liquid chromatography-tandem mass spectrometry. The data represent mean ± S.E. of three to nine values. B, MCF-7 cells were transfected with 5 nM siRNAs of SCR or GBA1-a (GBA1) for 48 h and then stimulated with 100 nM PMA for 1 h. Lipids were extracted, and then the levels of individual ceramide species were determined by high-performance liquid chromatography-tandem mass spectrometry. The data represent mean ± S.E. of three to five values. C14-Cer, C16-ceramide; C14:0-Cer, C16-ceramide; C18:0-Cer, C16-ceramide; C18:1-Cer, C16-ceramide; C20:0-Cer, C16-ceramide; C24:1-Cer, C24-ceramide. C14:0-Cer, C16-ceramide; C18:0-Cer, C16-ceramide; C18:1-Cer, C16-ceramide; C20:0-Cer, C16-ceramide; C24:1-Cer, C24-ceramide. C, MCF-7 cells were transfected with 5 nM siRNAs of SCR, acid sphingomyelinase (ASM), or GBA1-a (GBA1) for 48 h following stimulation with (PMA) or without (Control) 100 nM PMA for 1 h. Lipids were extracted, and then the levels of ceramide species were determined by high-performance liquid chromatography-tandem mass spectrometry. Levels of C16-ceramide are shown. The data represent mean ± S.E. of four to five values. Significant changes from SCR-transfected cells treated with PMA are shown in A-C (*, p < 0.02; **, p < 0.05; ***, p < 0.01).

FIGURE 5. Effects of GBA1 overexpression on ceramide formation. MCF-7 cells (1 × 10^6 cells/35-mm dish) were transfected with the indicated amounts of pSPORT GBA1 expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 6 h, cells were further labeled with 0.5 μCi/ml [3H]sphingosine for 18 h. Labeled cells were stimulated with (PMA) or without (Control) 100 nM PMA for 1 h, and then lipids were extracted and separated by TLC. The area corresponding to ceramide was scraped off, and the radioactivity measured by liquid scintillation counting. The data represent mean ± S.E. of three values. *, p < 0.05 versus non-GBA1 overexpression cells (Control); **, p < 0.001 versus non-GBA1 overexpression cells treated with PMA.

Conclusion to emerge from this study is the recognition of a novel role for GBA1 as a mechanism for the PKC-dependent formation of salvaged ceramide. This conclusion is supported by the following lines of evidence: (i) down-regulation of GBA1 attenuated PMA-induced formation of C16-ceramide, which is the major ceramide species formed from the salvage pathway in MCF-7 cells (Fig. 4); (ii) overexpression of GBA1 enhanced PMA-induced ceramide formation (Fig. 5); (iii) PMA treatment significantly decreased levels of glucosylceramide, and this was diminished by the down-regulation of GBA1 (Figs. 2 and 6A); and (iv) GBA1 knock-down abrogated PMA-induced formation of sphingosine (Fig. 6C). This is also supported by our studies (20) showing that PMA-induced ceramide was inhibited by fumonisin B1 (Fig. 1B) and not by myricin, thus ruling out regulation of the de novo pathway and implicating reacylation of sphingosine in the formation of ceramide. This was further supported by results with down-regulation of LASS5/CerS5, which also attenuated PMA-induced ceramide accumulation (23). Therefore, GBA1 is suggested to provide lysosomal ceramides for PMA-stimulated reacylation of sphingosine in the salvage pathway of ceramide formation.

The results from this and previous studies (22) also implicate PKCδ as the major isofrom involved in mediating the effects of PMA on the salvage pathway. Thus, knockdown of PKCδ was previously shown to eliminate nearly all the ceramide formed in response to PMA, and in this study it nearly abolished PMA-induced changes in ceramide, glucosylceramide, and sphingomyelin (Fig. 7). Thus, PKCδ functions upstream of GBA1 (this study) and acid sphingomyelinase (22) in the salvage pathway. This connection of PKCδ and GBA1/ceramide is of particular interest because PKCδ has emerged as the major isofrom of PKC involved in mediating stress responses and apoptosis responses also associated with ceramide (26, 27).

The results showed that GBA1 appeared to contribute ~50% of the induced ceramide (Fig. 4) although the mass levels of glucosylceramide are significantly less than those of sphingomyelin. This most likely reflects both the relative metabolic flux through the acid sphingomyelinase and GBA1 pathways and implicating reacylation of both sphingomyelin and glucosylceramide (and/or more com-
plex sphingolipids) (6–8, 28–30). In MCF-7 cells, both acid sphingomyelinase and GBA1 appear to contribute almost equally to the generated C16-ceramide. The results raise interesting questions about the potential mechanisms by which PKC regulates GBA1 and/or flux through GBA1. In cellular response to PMA, PKCβ was shown to activate acid sphingomyelinase, leading to ceramide formation (22). In contrast, the activity of acid glucocerebrosidase/GBA1 was not altered in response to PMA treatment (data not shown). In light of PMA-induced reduction of glucosylceramide (Figs. 2, 6, and 7), the results suggest either allosteric activation of GBA1 or increased access of GBA1 to its substrate glucosylceramide. One possibility of allosteric regulation would involve saposin C (9, 31, 32), a known cofactor for GBA1 that enhances interaction of GBA1 with substrate (32), such that PKC activation may facilitate the dynamic association of saposin C and GBA1. PKCβ is likely to act upstream of GBA1; however, the specific mechanism remains to be identified.

On the other hand, in addition to lysosomal GBA1, neutral glucocerebrosidases have been cloned and characterized as GBA2 (33, 34) and Klotho-related protein (35), and they differ in a number of characteristics from GBA1, such as sensitivity to conduritol B epoxide, optimal pH, and cellular localization. In light of these distinct characteristics, neutral glucocerebrosidases are unlikely to play a role in lysosomal catabolism of glucosylceramide followed by generating lysosomal ceramide. The results from this study implicate GBA1 and not neutral cerebrosidases; however, these additional isoenzymes may also be subject to other mechanisms of regulation.

Ceramide is emerging as a mediator for apoptosis (1). Sumitomo et al. (26) demonstrated that PKCβ activation by chemotherapeutic reagents (etoposide and paclitaxel) mediates apoptosis of tumor cells by inducing the generation of ceramide that can be inhibited by fumonisin B1, thus possibly involving the salvage pathway. Considering that PKCβ promotes GBA1-dependent formation of ceramide, GBA1 may possibly contribute to ceramide-mediated apoptosis induced by chemotherapeutic reagents. Interestingly, Swanton et al. (36) proposed that GBA1 affects the mitotic arrest induced by paclitaxel. In light of those studies, GBA1 is a possible regulator in cell arrest or apoptosis. Thus, PKCβ, which is known as a pro-apoptotic kinase (27), may be a potential upstream regulator for the GBA1-dependent salvage pathway in apoptotic responses. In our hands, MCF-7 cells failed to undergo significant apoptosis following PMA treatment, and therefore, the role of GBA1 was not discerned. However, it would be of interest to examine whether the PKCβ-GBA1 pathway plays a role in PMA-induced apoptosis of other cell types such as LNCaP cells (37).

On the other hand, PMA is also known as an inducer of inflammation (38, 39). In MCF-7 cells, PMA activation of PKCs triggers a number of pro-inflammatory responses such as induction of cyclooxygenase-2 (40), matrix metalloproteinases (41) and interleukin-6 (42), and activation of pro-inflammatory mitogen-activated protein kinase p38 (23). In the accompanying manuscript (43), we provide evidence for a role for the
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FIGURE 7. Effects of loss of PKCδ on sphingolipid metabolism. MCF-7 cells were transfected with 5 μg siRNAs of SCR (open column) or PKCδ (black-filled column) for 48 h, and then labeled with 0.5 μCi/ml [3H]sphingosine for 12 h. Cells were stimulated with 100 nM PMA for 1 h, and then lipids were extracted and separated by TLC. The area corresponding to glucosylceramide (GC), sphingomyelin (SM), and ceramide (CER) was scraped off, and the radioactivity measured by liquid scintillation counting. The results are expressed as PMA-induced changes in sphingolipid radioactivity. The data represent mean ± S.E. of seven to nine values.

PKCδ-GBA1-ceramide pathway in the regulation of p38-dependent pro-inflammatory responses.

In conclusion, the current results identify GBA1 as a novel source of regulated formation of ceramide. GBA1 is shown to play a significant role in the salvage pathway of ceramide formation in a PMA-mediated pathway.

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