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Acid β-Glucosidase 1 Counteracts p38δ-dependent Induction of Interleukin-6

*POSSIBLE ROLE FOR CERAMIDE AS AN ANTI-INFLAMMATORY LIPID**

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Activation of protein kinase C (PKC) by the phorbol ester (phorbol 12-myristate 13-acetate) induces ceramide formation through the salvage pathway involving, in part, acid β-glucosidase 1 (GBA1), which cleaves glucosylceramide to ceramide. Here, we examine the role of the GBA1-ceramide pathway, in regulating a pro-inflammatory pathway initiated by PKC and leading to activation of p38 and induction of interleukin 6 (IL-6). Inhibition of ceramide formation by fumonisin B1 or down-regulation of PKCδ potentiated PMA-induced activation of p38 in human breast cancer MCF-7 cells. Similarly, knockdown of GBA1 by small interfering RNAs or pharmacological inhibition of GBA1 promoted further activation of p38 after PMA treatment, implicating the GBA1-ceramide pathway in the termination of p38 activation. Knockdown of GBA1 also evoked the hyperproduction of IL-6 in response to 4β phorbol 12-myristate 13-acetate. On the other hand, increasing cellular ceramide with cell-permeable ceramide treatment resulted in attenuation of the IL-6 response. Importantly, silencing the δ isoform of the p38 family significantly attenuated the hyperproduction of IL-6. Reciprocally, p38δ overexpression induced IL-6 biosynthesis. Thus, the GBA1-ceramide pathway is suggested to play an important role in terminating p38δ activation responsible for IL-6 biosynthesis. Furthermore, the p38δ isoform was identified as a novel and predominant target of ceramide signaling as well as a regulator of IL-6 biosynthesis.

The lysosomal enzyme acid β-glucosidase 1 (GBA1)[2] cleaves the β-glycosidic linkage of glucosylceramide to generate glucose and ceramide (1). Glucosylceramide serves as a major precursor for complex glycosphingolipids, and the catalytic action of GBA1 plays a key role in the constitutive catabolism of most of glycosphingolipids (2–4). In fact, a severe deficiency of GBA1 activity causes Gaucher disease that results in the aberrant accumulation of glucosylceramide (4, 5). All sphingolipids including glucosylceramide contain the long-chain sphingoid bases (sphingosine) most of which are salvaged for forming ceramide (2). This pathway is referred to as the "salvage pathway" (2, 6).

Recently, our studies (7–9) implicated protein kinase C (PKC) as an upstream regulator of the sphingoid base salvage pathway resulting in ceramide synthesis. Particularly, the δ isoenzyme of PKCs was revealed to play a key role in phorbol 12-myristate 13-acetate (PMA)-induced salvage of ceramide formation in which acid sphingomyelinase is involved (8). More recently, our results also implicate GBA1 in the PKCδ-dependent formation of ceramide (75).

Ceramide has emerged as a bioactive lipid that mediates a variety of cellular responses, including regulation of cell growth, differentiation, and stress responses (10). Extensive studies have partially uncovered the molecular mechanisms of ceramide action. Ceramide-activated protein phosphatases (CAPPs) are identified as candidate direct mediators of ceramide action and are composed of two types of serine/threonine protein phosphatases (PP1 and PP2A) (11–13). Recently, we showed that ceramide formed from the salvage pathway accelerates inactivation of p38 through the action of CAPPs (9). In light of the studies mentioned above, we wondered if the salvage pathway and either GBA or acid sphingomyelinase are involved in regulating the dephosphorylation of p38 and whether this is critical for regulating inflammatory responses.

In the present study, evidence is provided for a role of the GBA1-ceramide pathway (GBA1-dependent ceramide formation through the salvage pathway) in inducing dephosphorylation of p38 MAP kinase. Evidence is also presented implicating the GBA1/ceramide salvage pathway in countering the production of interleukin-6 (IL-6) in response to (pro)-inflammatory cytokines. Additionally, the results specifically implicate the poorly studied δ isoform of p38 MAP kinase as the main target of ceramide action. The implications of these

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[2] The abbreviations used are: GBA, acid β-glycosidase; C_6-ceramide, *N*-hexanoyl-D-erythro-sphingosine; C_{16}-ceramide, *N*-palmitoyl-D-erythro-sphingosine; ERK1/2, extracellular signal-regulated kinase 1/2; IL-6, interleukin-6; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; Q-RT-PCR, quantitative real-time PCR; SCR, scrambled sequence; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay.

TABLE 1
Sequence of siRNAs used in this study

siRNA	Sense/antisense	5′ → 3′
p38δ-a	Sense	CCC UUU CAG UCC GAG AUC Utt
	Antisense	AGA UCU CGG ACU GAA AGG Gtc
p38δ-b	Sense	AGA UCU CGG ACU GAA AGG Gtc
	Antisense	GCU GUA GGG ACU CAU CUU Gtt
p38γ	Sense	AGC UCA UGA AAC AUG AGA Att
	Antisense	UUC UCA UGU UUC AUG AGC Utg
GBA2	Sense	CCC AAU UGG GUG CGU AAC Utt
	Antisense	AGU UAC GCA CCC AAU UGG Gtc

TABLE 2
Sequences of primers used for Q-RT-PCR

	Primers	5′ → 3′
Human IL-6	Forward	TGC GTC CGT AGT TTC CTT CT
	Reverse	GCC TCA GAC ATC TCC AGT CC
Human p38α	Forward	AAC CTG TCT CCA GTG GGC TCT
	Reverse	CGT AAC CCC GTT TTT GTG TCA
Human p38β	Forward	CAC CCA GCC CTG AGG TTC T
	Reverse	AGA TGC TGC TCA GGT CCT TCT
Human p38γ	Forward	CGC CTC CGG CTG AGT TT
	Reverse	GCT TGC ATT GGT CAG GAT AGA
Human p38δ	Forward	TGC TCG GCC ATC GAC AA
	Reverse	TGG CGA AGA TCT CGG ACT GA
Human β-actin	Forward	TCC TCC CTG GAG AAG AGC TA
	Reverse	CCA GAC AGC ACT GTG TTG GC

results in regulated sphingolipid metabolism, signal transduction, Gaucher disease, inflammation, and cancer are discussed.

EXPERIMENTAL PROCEDURES

Materials—Phospho/active p38 antibodies and purified PP2A proteins were purchased from Promega (Madison, WI). Antibodies specific for phospho/active ERK1/2 were from Cell Signaling Technology. PMA and SB202190 were from Calbiochem (La Jolla, CA). Halt™ Phosphatase Inhibitor Mixture was from Pierce (Rockford, IL). Rabbit polyclonal antibodies for p38α were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies or rabbit polyclonal antibodies for p38δ were from BD Bioscience or R&D, respectively. Peroxidase-conjugated antibodies for mouse IgG or rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Enhanced chemiluminescence kit was from Thermo Scientific. Conduritol B epoxide and recombinant active human p38δ were from BIOMOL. Recombinant His_6-tagged human PP1c-γ proteins were prepared as described in Jones and Hannun (14). pcDNA3.0 FLAG-tagged human p38δ expression vectors (15) were a kind gift from Dr. Jiahuai Han (The Scripps Research Institute, La Jolla, CA).

Cell Culture—MCF-7 or A549 cells were grown in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with L-glutamine and 10% fetal bovine serum, respectively. Cells were maintained at less than 80% confluence under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, 37 °C).

Transfection with Small Interfering RNA (siRNA)—Cells (1×10^5 cells/60-mm dish) were transfected with 5 nM double-stranded siRNAs with scrambled sequence (SCR), or sequences from GBA1, GBA2, acid sphingomyelinase, p38γ, and/or p38δ using Oligofectamine (Invitrogen) according to the manufacturer's instructions. After 48 h, transfection reagents were washed out, and cells were stimulated with 25 ng/ml tumor necrosis factor-α (TNF-α) or 100 nM PMA in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Table 1 shows sequences of siRNAs used, except sequences of siRNAs for SCR and acid sphingomyelinase shown in previous studies (7–9), and sequences of GBA1 siRNAs are shown in the accompanying article (75).

Transfection with Human p38δ Expression Vector—MCF-7 cells (1×10^5 cells/35-mm dish) were transfected with the indicated amounts of pcDNA3.0 human p38δ tagged with FLAG using Effectene (Qiagen) according to the manufacturer's instructions. After 24 h, transfection reagents were washed out, and cells were stimulated with 100 nM PMA for 12 h in RPMI 1640 supplemented with 10% fetal bovine serum.

Western Blotting—Cells were washed three times with PBS supplemented with Halt Phosphatase Inhibitor Mixture and then lysed using Laemmli buffer. The protein samples (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis (4–20% gradient gels or 10% gels). 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 with rat polyclonal antibodies for GBA1 (1 to 1,500), phospho-p38 (1 to 1,000), or p38α (1 to 1,000), antibodies for p38δ (1 to 1,000) in PBS-T containing 5% nonfat dried milk. The blots were washed with PBS-T and incubated with secondary antibody conjugated with horseradish peroxidase in PBS-T containing 5% nonfat dried milk. Detection was performed using enhanced chemiluminescence reagent.

Quantitative Real-time PCR (Q-RT-PCR)—One μg of total RNA, isolated using an RNA isolation kit (Qiagen), was used in reverse transcription reactions as described. The resulting total cDNA was then used in the Q-RT-PCR to measure the mRNA levels. The β-actin mRNA was used as an internal reference control to normalize relative levels of mRNA expression, and expression of each transcript is presented as arbitrary units. Sequences of primers used for Q-RT-PCR are shown in Table 2.

ELISA of Human IL-6—Levels of IL-6 in culture supernatants were measured using commercially available ELISA (Quantikine; R&D Systems). IL-6 production was normalized to proteins extracted from adherent cells.

p38δ Dephosphorylation in a Reconstitution System—Recombinant phosphorylated/active human p38δ (0.1 μg) was incubated with recombinant PP1c-γ (1 μl/assay) or PP2Ac (0.5 units/assay) at 30 °C for 15 min in the buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 100 μM $MnCl_2$. The phospho-p38δ was detected by Western blotting using phospho-p38 antibodies.

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

RESULTS

Differential Roles of PKCs in p38 Activation in MCF-7 Cells—PKC activation promotes the salvage pathway of ceramide formation, and in turn ceramide-dependent activation of CAPPs accelerates dephosphorylation/inactivation of p38 (7, 9). Moreover, PKCδ was identified as a predominant kinase for promoting the salvage pathway of ceramide formation (8). In light of these previous studies, PKCδ was presumed to promote p38

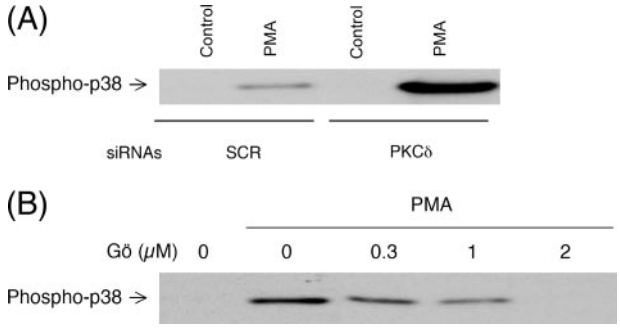

FIGURE 1. **Differential roles of PKCs in p38 modulation in MCF-7 cells.** *A*, MCF-7 cells were transfected with 5 nM siRNAs of SCR or PKCδ for 48 h and then stimulated with 100 nM PMA for 0.5 h. *B*, MCF-7 cells were treated with the indicated concentration of Gö6976 (Gö) for 30 min and then stimulated with 100 nM PMA for 0.5 h. Whole cell lysates were prepared and subjected to immunoblot analysis with antibodies for phospho-p38. Equal amounts of protein were loaded in each lane.

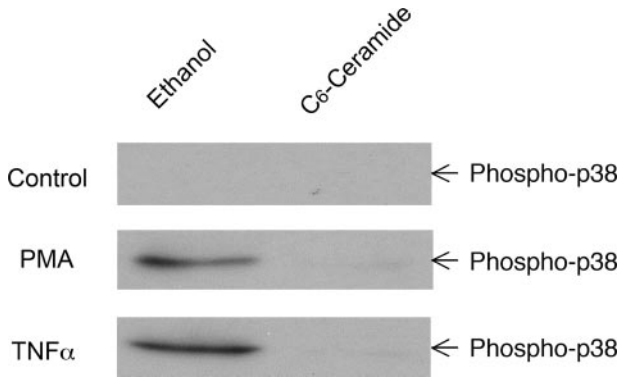

FIGURE 2. **Effects of ceramide on p38 phosphorylation induced by PMA or TNF-α in A549 cells.** A549 cells were treated with 20 μM C_6-ceramide for 2 h and then stimulated with 100 nM PMA or 25 ng/ml TNF-α for 30 min. Whole cell lysates were prepared and subjected to immunoblot analysis with antibodies for phospho-p38. Equal amounts of protein were loaded in each lane.

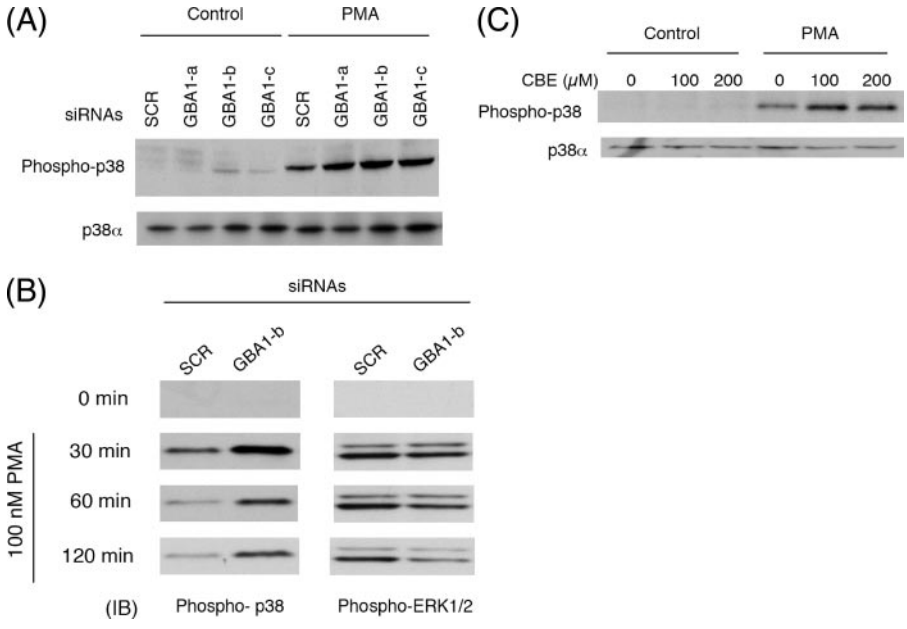

FIGURE 3. **Effects of knockdown of GBA1 and of conduritol B epoxide on p38 activation.** MCF-7 cells were transfected with 5 nM siRNAs of each of three individual sequences separately for 48 h and then stimulated with 100 nM PMA for 1 h (*A*) or the indicated periods (*B*). MCF-7 cells were pretreated with 100 or 200 μM conduritol B epoxide (*CBE*) for 2 h and then stimulated with 100 nM PMA for 1 h (*C*). Whole cell lysates were prepared and subjected to immunoblot analysis with antibodies specific for p38α (*A* and *C*), phospho-p38 (*A–C*), and phospho-ERK (*B*). Equal amounts of protein were loaded in each lane.

dephosphorylation/inactivation by generating a CAPP-mediated ceramide signal. Therefore, it became important to determine whether PKCδ plays a key role in (counter)-regulating p38 phosphorylation. Indeed, knockdown of PKCδ augmented activation of p38 upon PMA treatment (Fig. 1*A*). On the other hand, employment of the specific inhibitor (Gö6976) (16) for classical PKCs completely abolished PMA-induced phosphorylation of p38 (Fig. 1*B*). Thus, classical PKCs mediate the phosphorylation of p38 in response to PMA, whereas the novel PKCδ plays an opposing role (dephosphorylation of p38).

These finding are consistent with the effects of inhibition of ceramide formation by fumonisin B1 or by knockdown of LASS5/CerS5 on p38 phosphorylation (9). Therefore, the direct effects of cell-permeable ceramides on PMA- or TNF-α-induced phosphorylation of p38 were evaluated. Treatment with C_6-ceramide suppressed p38 activation following stimulation of A549 cells with PMA or TNF-α (Fig. 2). These results, taken together with previously published work showing that PKCδ is critical for PMA-induced ceramide formation, are consistent with a pathway in which the PKCδ isoform promotes the generation of ceramide yielding a ceramide signal that induces p38 dephosphorylation.

Involvement of GBA1 in Regulation of Phosphorylation of the p38 MAP Kinase—GBA1 participates in PKCδ-activated salvage pathway of ceramide formation (75). To investigate whether GBA1-dependent ceramide formation contributes to p38 dephosphorylation, the effects of GBA1 knockdown on PMA action were tested. Knockdown of GBA1 by any of three siRNAs led to increased phosphorylation of p38 relative to SCR treatment after 60 min of PMA stimulation (Fig. 3*A*). Moreover, compared with SCR-treated cells, PMA-induced activation of p38 was not only facilitated, but also prolonged over the 2-h time course studied in GBA1 knockdown cells (Fig. 3*B*). On the other hand, silencing GBA1 did not affect the induction of ERK phosphorylation in response to PMA, demonstrating a specificity of the GBA1-ceramide pathway toward p38. In addition to gene silencing, a pharmacological approach using an irreversible GBA1 inhibitor, conduritol B epoxide (17, 18), was employed, and the effects on p38 were determined. Treatment with the pharmacological inhibitor significantly enhanced the phosphorylation of p38 in response to PMA (Fig. 3*C*), supporting the results obtained with GBA1 knockdown (Fig. 3, *A* and *B*). Likewise, knockdown of acid sphingomyelinase also resulted in hyperphosphorylation of p38 upon PMA treatment (data not shown). Taken together, the results show that GBA1 plays a role in termination/attenuation of p38 signaling.

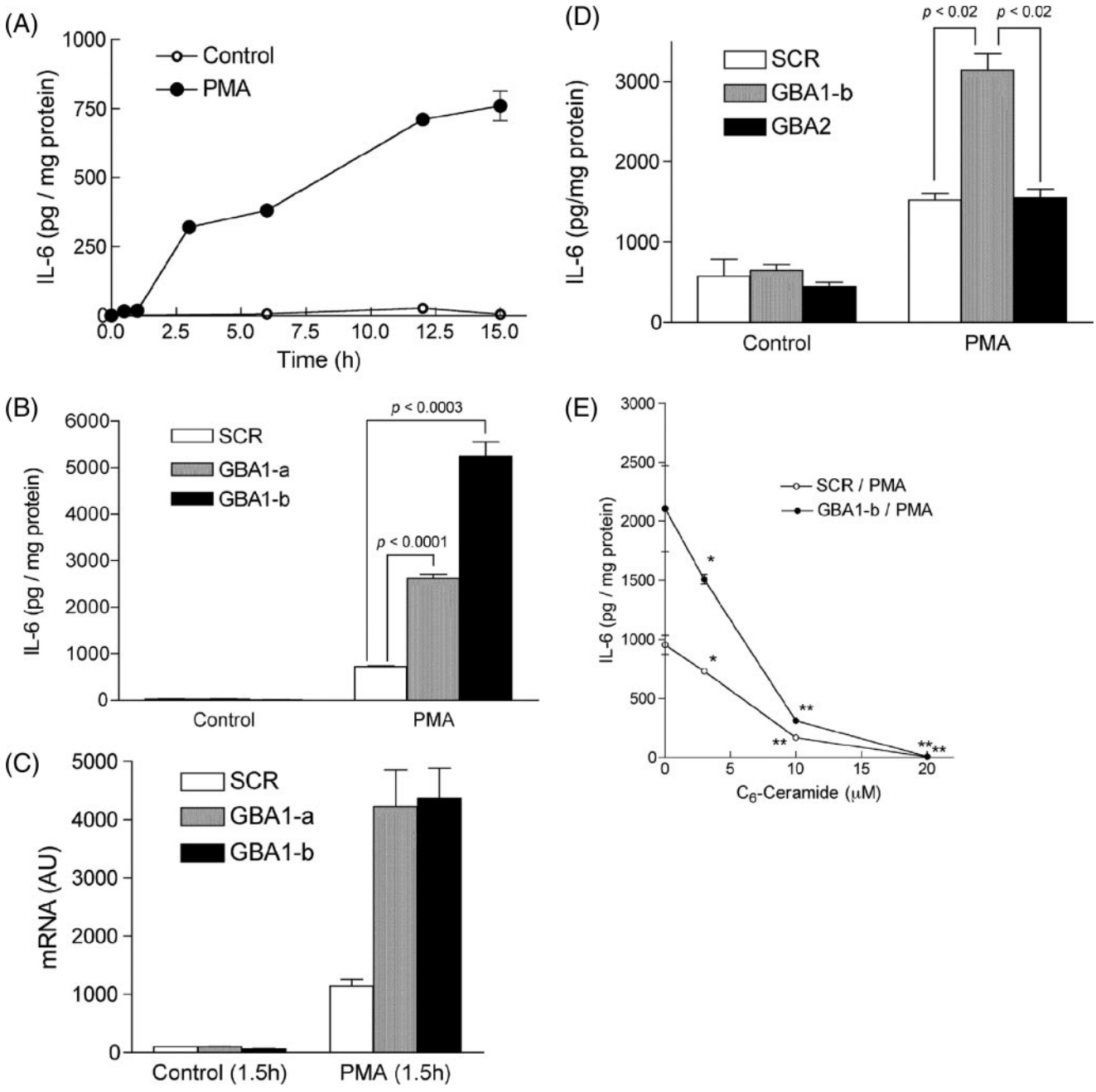

FIGURE 4. **Role for ceramide in PMA-induced generation of IL-6.** MCF-7 cells were stimulated with 100 nM PMA for the indicated periods, and then levels of IL-6 in culture supernatants were measured using the ELISA system (*A*). MCF-7 cells were transfected with the indicated siRNAs (SCR, *open column*; GBA1-a, *gray-filled column*; GBA1-b, *filled column*) for 48 h and then stimulated with 100 nM PMA for 3 (*C*) or 12 h (*B*). *B*, levels of IL-6 in culture supernatants were measured using the ELISA system. The data represent mean ± S.E. of 10 values. *C*, cellular RNAs were extracted and subjected to the Q-RT-PCR as described under "Experimental Procedures." Levels of IL-6 mRNA were expressed as arbitrary units (*AU*). The data represent means of two values, and each *bar* is given with the range. *D*, MCF-7 cells were transfected with 5 nM siRNAs of the indicated siRNAs (SCR, *open column*; GBA2, *gray-filled column*; GBA1-b, *filled column*) for 48 h and then stimulated with 100 nM PMA for 12 h. Levels of IL-6 in culture supernatants were measured using ELISA. *E*, MCF-7 transfected with 5 nM siRNAs (SCR, *open circle*; GBA1-b, *closed circle*) were stimulated with 100 nM PMA for 12 h following treatment with vehicles (0.1% ethanol) or the indicated concentrations of C_6-ceramide for 30 min. Levels of IL-6 in culture supernatants were measured using an ELISA. The data represent mean ± S.E. of two to three values.

Regulation of Pro-inflammatory Cytokine Production by GBA1—To determine the functional consequences of regulation of GBA1, we evaluated whether GBA1 exerted an inhibitory effect on IL-6 because the pro-inflammatory p38 kinase has been suggested to modulate the formation of IL-6 (19–23). MCF-7 cells produced IL-6 in a time-dependent manner in response to PMA activation (Fig. 4*A*). Inhibition of ceramide synthesis by GBA1 knockdown with two individual sequences significantly enhanced IL-6 generation induced by PMA (Fig. 4*B*). The production of IL-6 was confirmed to closely correspond to induction of IL-6 mRNA (Fig. 4*C*). On the other hand, knockdown of GBA2 (24, 25), a neutral glucocerebrosidase, had no effects on IL-6 biosynthesis (Fig. 4*D*). Reciprocally, a cell-permeable ceramide, C_6-ceramide, exerted an inhibitory effect on IL-6 formation in SCR- or GBA1 siRNA-treated MCF-7 cells in a dose-dependent manner (Fig. 4*E*), suggesting a role of cer-

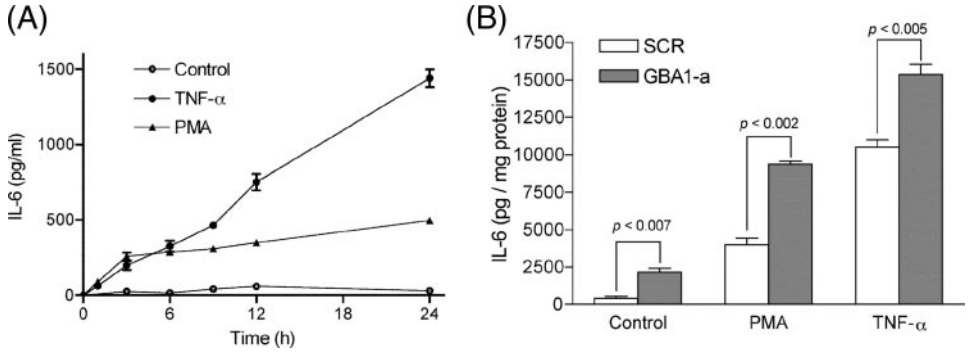

FIGURE 5. **Effects of knockdown of GBA1 by siRNAs on TNF-α or PMA-induced generation of IL-6 in A549 cells.** A549 cells were stimulated with 25 ng/ml TNF-α or 100 nM PMA for the indicated periods (*A*). A549 cells transfected with 5 nM siRNAs of SCR or GBA1-a for 48 h and then stimulated with 25 ng/ml TNF-α or 100 nM PMA for 14 h (*B*). Levels of IL-6 in culture supernatants were measured using an ELISA. The data represent mean ± S.E. of four values.

amide in a negative feedback that prevents cells from producing IL-6. Collectively, these results demonstrate a critical role for GBA1 and GBA1-derived ceramide in down-regulation/attenuation of IL-6 production.

To determine whether these results can be replicated in other cell lines, we examined whether GBA1 inhibits IL-6 biosynthesis in human lung epithelial adenocarcinoma cells (A549 cells), a model employed for the study of pro-inflammatory pathways (26). Treatment of A549 cells with PMA stimulated IL-6 formation in a time-dependent manner up to 24 h (Fig. 5*A*). GBA1 knockdown increased constitutive biosynthesis of IL-6 and facilitated the generation of IL-6 induced by PMA (Fig. 5*B*).

The use of A549 cells also allowed us to determine the pro-inflammatory contribution of GBA1 to a potent inducer of IL-6 biosynthesis, the pro-inflammatory cytokine TNF-α. TNF-α markedly stimulated IL-6 formation up to 24 h (Fig. 5*A*). GBA1 knockdown increased IL-6 induced by TNF-α by 1.4-fold at 14 h (Fig. 5*B*). Thus, GBA1 also plays an important role in attenuating IL-6 formation in response to TNF-α.

A Specific Role for p38δ in Regulating IL-6—The family of p38 MAP kinases (21, 22, 27, 28) consists of four members (α, β, γ, and δ), which differ in their tissue distribution, substrate specificities, and sensitivities to chemical inhibitors such as SB203580 and SB202190, both of which inhibit p38α and p38β (29). Among the main p38 isoforms, p38α is thus far the best characterized and is thought to be primarily responsible for regulating inflammatory responses (27). Thus, we investigated which p38 isoenzyme is regulated by the ceramide signal to mediate IL-6 hyperproduction. The effects of SB202190 were examined in GBA1-knockdown cells. Without the inhibitor, GBA1 knockdown dramatically induced IL-6 production (*first set of bars*, Fig. 6*A*). Pretreatment of SCR-treated MCF-7 cells with 10 μM SB202190 had a small effect on the production of IL-6 induced by PMA, and the inhibitor (10 μM) slightly inhibited IL-6 generation enhanced by GBA1 knockdown upon PMA treatment (Fig. 6*A*). Thus, neither p38α nor p38β appear to play a predominant role in the overproduction of IL-6 induced by PMA in cells defective in GBA1. As shown in Table 3, mRNA expression of p38γ was lower than that of other p38 isoenzymes, and p38γ knockdown had no effect on IL-6 formation in GBA1-knocked down cells (data not shown), ruling out p38γ as a potential target for ceramide signaling.

The δ isoform of p38 is ubiquitously expressed in mammalian cells (30, 31). The isoform expression was detected at the protein (Fig. 6*B*) as well as the mRNA (Fig. 6*D* and Table 3) level in MCF-7 cells. To investigate if p38δ is a specific target for the GBA1 ceramide pathway, siRNA specific for p38δ was employed. Treatment of MCF-7 cells with p38δ siRNA or a combination of p38δ siRNA and GBA1 siRNA significantly and specifically knocked down p38δ, or p38δ and GBA1, respectively (Fig. 6*B*). Using these siRNAs, the effects of p38δ knockdown on IL-6 generation were assessed in GBA1-knocked down cells. Loss of p38δ *per se* did not affect IL-6 generation induced by PMA. As previously, knockdown of GBA1 induced a significant increase of IL-6 production in response to PMA. Importantly, knockdown of p38δ significantly and largely abolished the enhancement of PMA-induced IL-6 formation by GBA1 knockdown (Fig. 6*C*). The release of IL-6 closely corresponded to induction of IL-6 mRNA (Fig. 6*D*). These effects of p38δ knockdown on IL-6 were also confirmed by another siRNA sequence (p38δ-b; shown in Table 1) for p38δ (data not shown).

Moreover, quantitative analyses were performed to determine the contribution of the p38 isoform in enhancing PMA-induced generation of IL-6 in GBA1-knocked down cells. Results from Fig. 6, *A* and *C*, were expressed as the percentage of inhibition by SB202190 or p38δ siRNA treatment in PMA-induced IL-6 formation of GBA1-silenced cells (Fig. 6*E*). Treatment with 10 μM SB202190 or p38δ siRNA treatment inhibited IL-6 formation by 33.0 ± 6.3 or 62.8 ± 9.0%, respectively. These results demonstrate that p38δ siRNA treatment significantly suppressed IL-6 formation more effectively than SB202190 treatment (Fig. 6*E*). Taken together, p38δ appears to predominantly mediate the effects of loss of GBA1 on IL-6 production.

Next, we investigated the contribution of p38δ to the total phospho-p38 detected with immunoblotting by using p38δ siRNA. Silencing p38δ significantly abrogated phosphorylation of p38 induced by PMA in GBA1-knocked down cells (Fig. 7*A*). Thus, p38δ appears to be the main p38 isoform acting downstream of GBA1 loss, inducing p38 phosphorylation and increasing IL-6.

To examine the possible molecular interaction of CAPPs with p38δ, a reconstitution system was employed. Purified PP2Ac, but not PP1c-γ, was able to directly dephosphorylate p38δ proteins (Fig. 7*B*), suggesting that PP2Ac may directly dephosphorylate p38δ.

So far, p38δ-dependent regulation of IL-6 has not been appreciated. Transcriptional factors such as AP1 and/or C/EBPs are believed to mediate IL-6 expression (32, 33), and p38δ is implicated in activation of those transcriptional factors

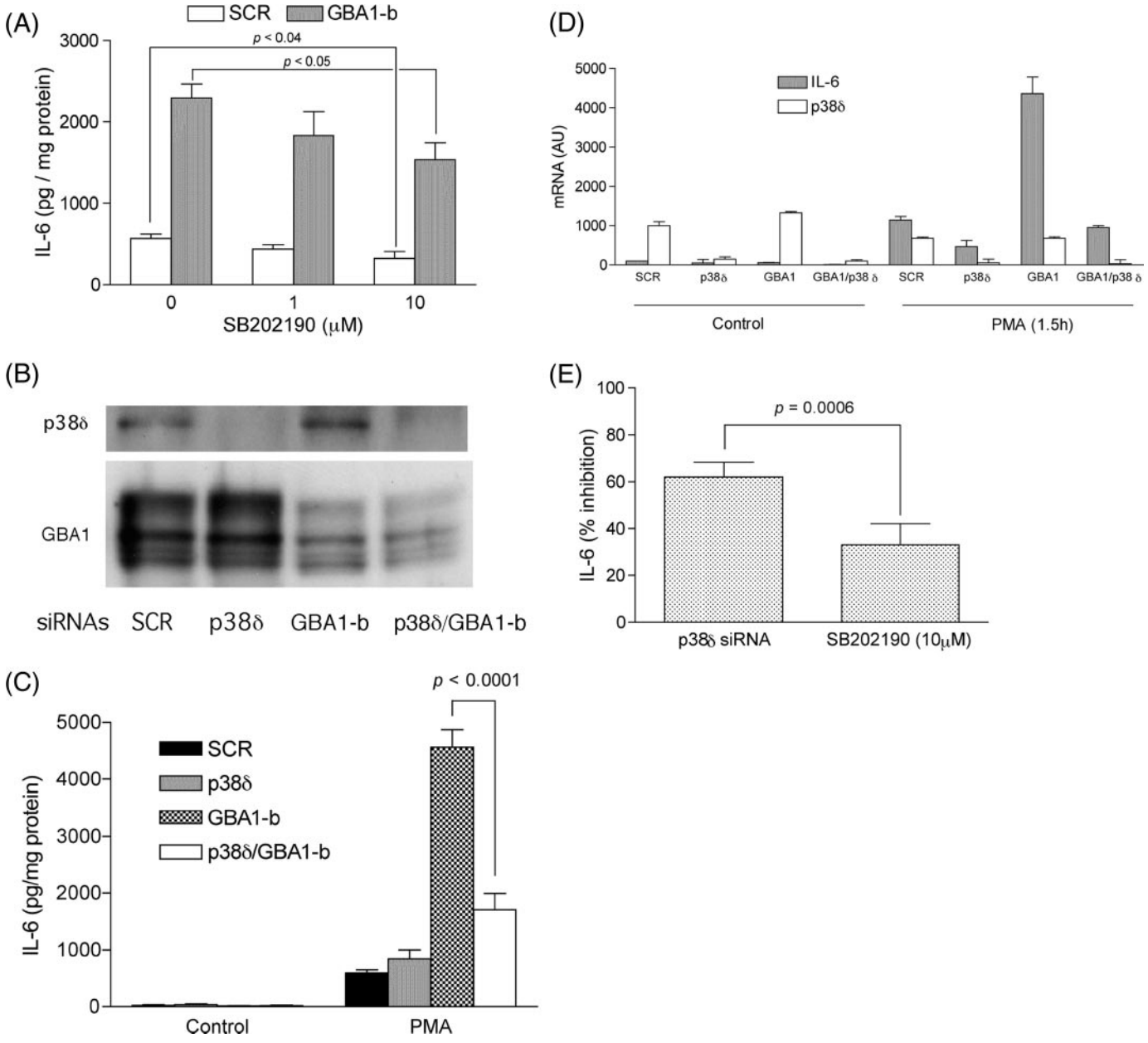

FIGURE 6. **Involvement of p38δ in the production of IL-6 in GBA1-silenced cells.** *A*, MCF-7 cells were transfected with 5 nM SCR (*open column*) or GBA1 (*gray-filled column*) siRNAs for 48 h following pre-treatment with the indicated concentration of SB202190 for 30 min prior to stimulating cells with or without 100 nM PMA for 12 h. Levels of IL-6 in culture supernatants were measured using an ELISA. The data represent mean ± S.E. of six values. *B*, MCF-7 cells were transfected with 5 nM SCR, GBA1-b, p38δ-a, or GBA1-b and p38δ-a for 48 h, and then the effectiveness of siRNA treatment was assessed by immunoblot analysis with antibodies specific for p38δ or GBA1. The transfected cells were further stimulated with 100 nM PMA for 12 (*C*) and 1.5 h (*D*). Levels of IL-6 in culture supernatants (*C*), and mRNA levels of IL-6 (*gray-filled column*) and p38δ (*open column*) (*D*) were measured. The data regarding IL-6 (*C*) or IL-6 mRNA (*D*) represent mean ± S.E. of six values or mean of two values ± the range, respectively. *E*, inhibition of IL-6 generation by 10 μM SB202190 or p38δ siRNA in PMA stimulation of GBA1-silenced cells is expressed as the percentage relative to dimethyl sulfoxide or SCR treatment in PMA stimulation of GBA1-silenced cells, respectively. The values are calculated from the data shown in *A* and *C*. The data represent mean ± S.E. of at least four values.

TABLE 3

Individual mRNA levels of p38 isoenzymes in MCF-7 cells or mouse peritoneal macrophages (MPMs)

Each mRNA of p38 isoforms was measured by real time Q-RT-PCR as described under "Experimental Procedures." Levels of mRNAs were expressed as arbitrary units.

	MCF-7	MPM
p38α	100	100
p38β	9.8	2.9
p38γ	1.1	0.004
p38δ	59.5	0.7

(34, 35). To establish the ability of p38δ to induce IL-6 formation, we determined if p38δ overexpression up-regulates IL-6 biosynthesis. Transfection of MCF-7 cells with human p38δ expression vectors significantly increased IL-6 in a manner that was dependent on the quantity of vector DNA compared with those of cells transfected with 3 μg of empty vector DNA (Fig. 8). Moreover, the overexpression also facilitated IL-6 biosynthesis in PMA-treated cells. Thus, p38δ is sufficient to induce IL-6 and is necessary for mediating the effects of suppressing the GBA1-ceramide pathway.

DISCUSSION

The results from this study implicate GBA1 in the attenuation of p38 phosphorylation/activation. Functionally, the GBA1-ceramide pathway is suggested to play a key role in feedback regulation of IL-6 biosynthesis. This finding has implications for the pro-inflammatory roles of IL-6 and its possible involvement in inflammation, cancer, and the pathogenesis of Gaucher disease, the latter arising from decreased activity of GBA1. Interestingly, the results also implicate the poorly studied δ isoform of p38 MAP kinases as the key downstream mediator of the GBA1-ceramide pathway in the regulation of IL-6 induction.

Overall, the results reveal that PKCδ induces the formation of ceramide responsible for counteracting p38 activation by promoting activation of CAPPs, whereas classical PKCs are likely to mediate p38 activation (Fig. 1). Therefore, two contradictory pathways are proposed to regulate p38 (Fig. 9): (i) PKCδ-dependent negative regulation of p38, and (ii) classical PKC-dependent positive regulation of p38. The PKCδ pathway is only revealed following its inhibition or the downstream inactivation of the salvage pathway (loss of PKC or down-regulation of GBA1).

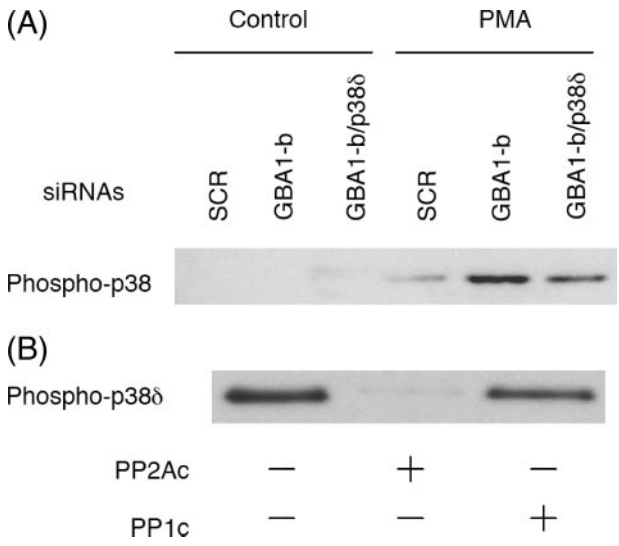

FIGURE 7. **Regulation of p38δ in GBA1-knocked down cells.** *A*, MCF-7 cells were transfected with 5 nM SCR, GBA1-b, or GBA1-b and p38δ-a for 48 h, and then stimulated with 100 nM PMA for 30 min. Equal amounts of protein for phospho-p38 immunoblot analysis were loaded in each lane. *B*, recombinant phosphorylated/active p38δ proteins were incubated with recombinant human PP1c-γ isoform or PP2Ac. p38δ phosphorylation was detected by Western blotting using phospho-p38 antibodies.

The major finding from this study centers on the role of GBA1 in regulating p38. We previously showed that ceramide (likely C_{16}-ceramide) formed from the salvage pathway accelerates dephosphorylation/inactivation of p38 by activating CAPPs (9). In the present study, either down-regulation of GBA1 activity by siRNA or inhibition with conduritol B epoxide enhanced p38 phosphorylation/activation induced by PMA in MCF-7 cells (Fig. 3), which is consistent with the results obtained by inhibition of ceramide synthesis with fumonisin B1 (9). Reciprocally, C_6-ceramide treatment significantly abolished p38 phosphorylation induced by inflammation inducers (PMA and TNF-α) (Fig. 2). Thus, upon treatment with PMA, GBA1 suppression is thought to prevent the generation of ceramide, which causes p38 dephosphorylation mediated by CAPPs, leading to hyperphosphorylation of p38. These results strongly suggest that the GBA1-dependent salvage pathway of ceramide formation is critical in feedback inhibition of p38 (Fig. 9).

Functionally, the results show that the feedback regulation of p38 by the GBA1-ceramide pathway leads to suppression of IL-6 induction. GBA1 knockdown resulted in overproduction of IL-6 in cellular responses of MCF-7 (Fig. 4) and A549 calls (Fig. 5), suggesting a novel role for GBA1 ceramide in regulation of IL-6 biosynthesis. Consistently, inhibition of GBA1 by conduritol B epoxide is reported to display an increase in IL-6 mRNAs (36). In contrast, increasing cellular ceramide by treating with exogenous ceramide (C_6-ceramide) blocked PMA-induced formation of IL-6 despite GBA1 silencing (Fig. 4*E*). Moreover, in mouse peritoneal macrophages, C_8-ceramide was also shown to have an inhibitory effect on IL-6 (37). Collectively, ceramide is thus suggested to exert an inhibitory effect on IL-6 induction, and GBA1 can serve as a controller for generating the relevant ceramide signal.

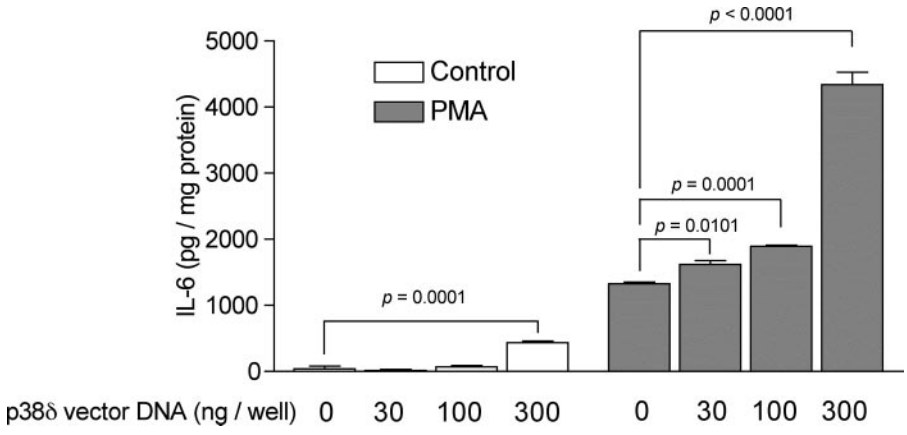

FIGURE 8. **Effects of p38δ overexpression on IL-6 biosynthesis in MCF-7 cells.** MCF-7 cells were transfected with the indicated amounts of human p38δ-FLAG pcDNA3.0 vectors or 300 ng of DNA of empty vectors shown as "0" of human p38δ-FLAG pcDNA3.0 vectors (300 ng) followed by treatment with or without 100 nM PMA for 12 h. Levels of IL-6 in culture supernatants were measured using an ELISA. The data represent mean ± S.E. of three values.

Another major conclusion of the present study was the identification of p38δ as the predominant contributor to the overproduction of IL-6 in MCF-7 cells and that the other p38 isoenzymes p38α and p38β play only a minor role in IL-6 induction sensitive to GBA1-dependent ceramide signal (Fig. 6). Thus, p38δ is proposed to be a key downstream target of the GBA1-dependent salvage pathway of ceramide formation for regulation of IL-6 biosynthesis.

Previous studies suggested PP1c isoforms in mediating the ceramide effect on counteracting p38 phosphorylation (9), whereas in this

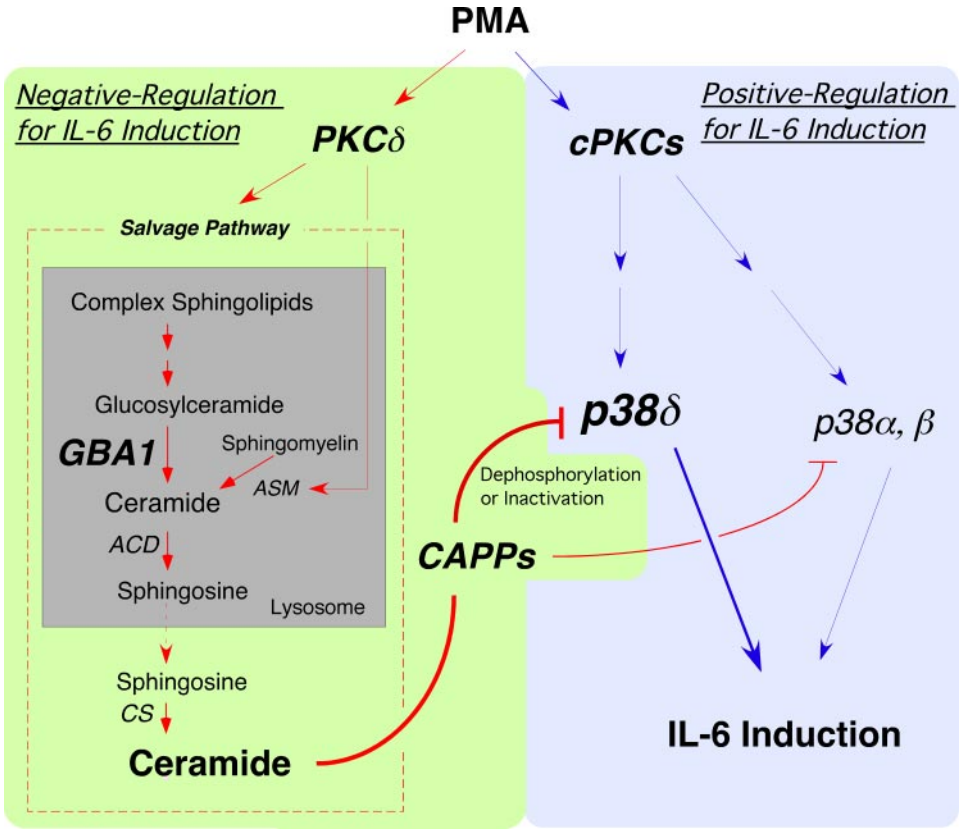

FIGURE 9. **Negative regulation of IL-6 induction by the salvage pathway of ceramide formation.** *Red* or *blue lines* indicate the pathway of negative or positive regulation for IL-6 induction, respectively. *ACD*, acid ceramidase; *ASM*, acid sphingomyelinase; *cPKC*, classical PKC; *CS*, ceramide synthase.

study, PP2Ac, but not PP1c-γ, efficiently dephosphorylated p38δ *in vitro* (Fig. 7*B*). Both PP1c and PP2Ac serve as CAPPs; however, they may likely have distinct targets. For example, PP1c might target the upstream kinases of p38, whereas PP2Ac may serve as a direct protein phosphatase for p38δ.

p38δ has been implicated in the expression of vascular endothelial growth factor, matrix metalloproteinase 1 and 13, which are involved in tumor invasion and angiogenesis (38, 39); however, among the various p38 isoforms, p38δ is a poorly studied isoform relative to p38α and p38γ. In addition, IL-6 is also implicated as a factor important in breast cancer progression and metastasis (40, 41). Potentially, the identification of IL-6 as a target for p38δ (Figs. 7 and 8) further suggests a role for this isoform of p38 MAP kinases as a possible regulator of tumor inflammation, metastasis, and growth.

Taken together the above results suggest an anti-inflammatory function for ceramide. p38 is involved in inflammation by up-regulating expression of many of the inflammation-related protein, such as matrix metalloproteinases, cyclooxygenase-2, TNF-α, and IL-6 (20, 22, 29, 42, 43). Inhibitors of p38 (mostly α and β isoforms) have been developed and proposed for treatment of patients with inflammation-related diseases such as Crohn disease and rheumatoid arthritis (29). In some studies employing cell models, ceramide treatment was shown to suppress p38 activation in cellular responses to lipopolysaccharide (37, 44, 45), IgE receptor cross-linking (46), or PMA (9), which are implicated in inflammation. In light of these emerging findings, it is conceivable that cellular ceramide functions as an anti-inflammatory lipid mediator by suppressing p38 activation. Moreover, it is noted that the liposomal delivery of C_6-ceramide inhibited murine corneal inflammation *in vivo* (45). Possibly, inactivation of p38 is involved in ceramide inhibition of corneal inflammation.

In contrast, in some studies on ceramide-mediated apoptosis, treatment with C_2-ceramide induced weak and sustained activation of p38 leading to apoptosis (47–49). On the other hand, our studies demonstrate that endogenous ceramide C_{16}-ceramide is predominately involved in p38 inactivation (9). Exogenous C_6-ceramide is subject to deacylation for generating longer chain ceramides such as C_{16}-ceramide through the salvage pathway, whereas C_2-ceramide is defective (6, 50, 51). Therefore, the metabolic fates of short chain ceramides may determine the p38 responses, with C_6-ceramide better mimicking the salvage pathway.

The findings in this study also have implications for our understanding of phorbol ester action and the roles of PKC, especially in inflammation. Phorbol esters including PMA are natural, plant-derived organic compounds isolated from *Croton tiglium* and are suggested to account for the inflammatory responses to these plants (52–54). Extensive studies on PKC using phorbol esters (55, 56) have advanced our understanding of the roles of PKCs in the pathophysiology of inflammation-related diseases that involve a variety of cellular responses including the formation and release of matrix metalloproteinases, eicosanoids, and pro-inflammatory cytokines (57–59). Moreover, several studies have revealed an involvement of p38 in those responses (21, 27). Our studies using phorbol esters along with pharmacological and genetic approaches suggest the possibility that PKCδ, in contradistinction from classical PKCs, acts as an anti-inflammatory protein kinase by accelerating inactivation of p38 in a manner dependent on ceramide formation (Fig. 9).

Engagement of TNF-α receptors induces multiple signaling-inducing pro-inflammatory responses. TNF-α is also known as a ceramide inducer in various types of cells including mesangial cells (60), HL-60 (61), fibroblasts (62), and MCF-7 (63). In A549 cells, TNF-α treatment was shown to activate PKCδ (26). Therefore, TNF-induced activation of PKCδ might stimulate the GBA1-ceramide pathway. Importantly, loss of GBA1 augmented IL-6 induction by TNF-α (Fig. 5). Those results suggest that GBA1 also plays a role in counteracting pro-inflammatory response to TNF-α.

Finally, the results from this study raise interesting and potentially significant implications for understanding the

pathophysiology of Gaucher disease. Among the sphingolipidoses (4), Gaucher disease is the most common disorder, and it is caused by a severe deficiency of GBA1 activity resulting in the aberrant accumulation of glucosylceramide (64). The pathogenesis of Gaucher disease appears to extend beyond simple accumulation of glucosylceramide and seems to involve aberrant accumulation/activation of macrophages and induction of inflammatory responses (36, 64–66). In serum of patients with Gaucher disease, IL-6 is increased (64, 67, 68), but the mechanisms of this increase are unknown and its implication in pathogenesis still remains to be defined. Potentially, the elevation of serum IL-6 could explain the osteolytic lesions (69, 70) and the high incidence of multiple myeloma (71, 72) in patients with Gaucher disease, because the cytokine is an osteolytic factor (73) and a major growth factor for multiple myeloma (74). In light of those results, impairments of a GBA1-dependent ceramide may play a role in cellular responses of patients with Gaucher disease leading to possible pathogenic production of IL-6.

In conclusion, the current results identify GBA1 as a novel regulated enzyme. GBA1 is shown to play a significant role in the salvage pathway of ceramide formation in PKC-mediated cellular responses, and the GBA1-ceramide pathway is shown to function to counteract phosphorylation of p38δ (Fig. 9). Moreover, the results implicate, for the first time, p38δ as a regulator of IL-6. These results have implications for the functions of GBA1 and ceramide in inflammation and tumor promotion, and for a possible role for p38δ in Gaucher disease.

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