Differential Effects of Sphingomyelin Hydrolysis and Resynthesis on the Activation of NF-κB in Normal and SV40-transformed Human Fibroblasts*

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The precise role of ceramide in NF-κB signaling remains unclear. The recent observation of differential sphingomyelin synthase (SMS) activity in normal (low SMS) *versus* SV40-transformed (high SMS) WI38 human lung fibroblasts provides an opportunity to assess the involvement of ceramide and SMS in NF-κB activation. Treatment of normal WI38 fibroblasts with bacterial sphingomyelinase resulted in a 4-fold elevation of ceramide and blocked NF-κB activation by serum stimulation. Such inhibition was not observed in SV40-transformed fibroblasts. Under regular growth conditions, after sphingomyelinase was washed out, normal WI38 did not show SM re-synthesis nor NF-κB activation. In SV40-WI38, on the other hand, sphingomyelinase washout induced resynthesis of SM due to the action of SMS on ceramide generated at the plasma membrane. NF-κB activation correlated with SM resynthesis. This activation was abrogated by D609, which inhibited SM resynthesis but not the initial formation of ceramide. The differential activity of SMS may explain the effects of ceramide in NF-κB signaling: in the absence of significant SMS activity, ceramide inhibits NF-κB, whereas with high SMS, the conversion of the ceramide signal to a diacylglycerol signal by the action of SMS stimulates NF-κB. These results also suggest a role for SMS in regulating NF-κB.

The sphingolipid metabolite ceramide has garnered attention for its potential role in mediating or regulating many cellular effects, including such diverse processes as cell growth and differentiation, cell cycle arrest, cellular senescence, and apoptosis (for review, see Refs. 1–3). In particular, ceramide has been associated with many of the effects of the cytokine TNFα[1] (4–6). One important action of TNFα involves the expression of specific genes via activation of the NF-κB/Rel family of transcription factors (Refs. 7 and 8; for review, see Ref. 9).

[1] The abbreviations used are: TNF, tumor necrosis factor; PC-PLC, phosphatidylcholine-specific phospholipase C; DAG, diacylglycerol; bSMase, bacterial sphingomyelinase; PMA, phorbol 12-myristate 13-acetate; SM, sphingomyelin; SMS, SM synthase; MEM, minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

Before TNFα stimulation, inactive dimers of NF-κB subunits remain bound by I-κB in the cytosol. Upon TNFα stimulation, I-κB undergoes phosphorylation, ubiquitination, and degradation, allowing free NF-κB to translocate to the nucleus and stimulate transcription of its appropriate target genes (10, 11).

Some proteins such as Traf2 have been shown to associate with the receptors for TNFα and possibly mediate TNF-induced activation of NF-κB (12). In addition, TNFα has been suggested to activate phosphatidylcholine-specific phospholipase C (PC-PLC), and the resultant diacylglycerol (DAG) functions as an activator of protein kinase C (13). Protein kinase C may contribute to NF-κB activation by anti-neoplastic agents (14), and the ζ isoform of protein kinase C has also been proposed as a specific activator of NF-κB (15).

TNFα is also known to induce sphingomyelin hydrolysis and concomitant ceramide generation (1), and there have been many attempts to couple ceramide to the activation of NF-κB. Some studies have suggested that ceramide plays an essential role in NF-κB activation (16, 17), and in one proposed scheme, it has been suggested that DAG generated from PC-PLC causes activation of acid sphingomyelinase, and the resultant ceramide induces activation of NF-κB (13, 18).

Evidence supporting a role for ceramide pathways in the activation of NF-κB has come primarily from the following observations. 1) The addition of bacterial sphingomyelinase (bSMase) to some (16) but not all (19) cells results in activation of NF-κB, and 2) D609, a putative inhibitor of PC-PLC, inhibits activation of NF-κB (13). However, the existence of membrane PC-PLC and the specificity of D609 have not been demonstrated at a biochemical level. In fact, the use of D609 as an inhibitor of a mammalian PC-PLC-like activity is based solely on *in vitro* studies of the effects of D609 on a bacterial PC-PLC (31); no such evidence has been provided for direct effects of D609 on a mammalian PC-PLC.

Other studies have found acid SMase and ceramide to have no effect or even exert a negative effect on NF-κB (5, 19–23). In certain cell lines, TNFα activates NF-κB without ceramide generation (20–22). Studies using cell-permeable analogs of ceramide or bSMase showed that these manipulations could mimic apoptosis or activation of c-Jun NH₂-terminal kinase but had no effect on NF-κB (5, 20, 23). Also, fibroblasts derived from Niemann-Pick patients, which lack acid SMase (24), still show activation of NF-κB by TNFα (21), thus refuting a necessary role for this enzyme in activation of NF-κB. A recent study showed that ceramide inhibits the activation of NF-κB by PMA (19).

In recent studies, we have observed that mammalian sphingomyelin synthase (SMS) demonstrates many of the properties that had been ascribed to the putative PC-PLC, including in-

hibition by D609 both in intact cells and in cellular membrane preparations (25). These observations raised the possibility that SMS may account for some of the cellular effects that had been attributed to PC-PLC. In particular, we wondered whether SMS might be involved in activation of NF-κB and, thus, may explain some of the conflicting results on the possible roles of ceramide. SMS utilizes as substrates ceramide and phosphatidylcholine to produce sphingomyelin and diacylglycerol, "converting" a sphingolipid bioeffector into a glycerolipid messenger. With each lipid's own unique profile of biological activity, any shift in levels between the two lipids could result in very divergent signaling outcomes.

Moreover we had shown that WI38 human lung fibroblasts have low or undetectable plasma membrane-related SMS activity, whereas the SV40-transformed WI38 demonstrated high SMS activity: in the wild type cells, ceramide generated at the plasma membrane persisted over several hours, with no detectable resynthesis of SM. In contrast, ceramide generated at the plasma membrane of SV40-transformed cells was re-incorporated into SM over 6 h, indicating the presence of a distinct SMS in these cells. We exploited this difference between the two cell lines to investigate the role of SMS in activation of NF-κB by endogenous ceramide. The results indicate a clear increase in NF-κB activation in the presence of high SMS activity not seen in the WI38 cell line or when SMS activity is inhibited by D609. These observations help to clarify the heretofore perplexing effects of ceramide on NF-κB activation and suggest a possible physiologic function of the SMS pathway in regulation of NF-κB.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, MEM, and all MEM solutions were from Life Technologies, Inc. Fetal bovine serum (FBS) was from Summit Technology. bSMase (from *Staphylococcus aureus*) was from Sigma, and D609 was from Calbiochem. TNFα was a kind gift from Dr. Phillip Pekala (East Carolina University). [γ-^{32}P]ATP was from NEN Life Science Products. Poly(dI-dC) and poly(dN6) were purchased from Amersham Pharmacia Biotech. Anti-NF-κB (p65 and p50) monoclonal antibodies were purchased from Santa Cruz Biotechnology, and anti-NF-κB (p65) polyclonal antibodies were purchased from Rockland.

Cell Culture—Both normal and SV40-transformed WI38 human lung fibroblasts were obtained from the National Institute on Aging Cell Repository. Normal WI38 fibroblasts were maintained in Dulbecco's modified Eagle's medium high glucose supplemented with 10% (v/v) FBS and Hepes buffer. WI38 cells were used up to passage 24–26. SV40-transformed fibroblasts were maintained in MEM supplemented with 10% FBS along with MEM solutions of vitamins and essential and non-essential amino acids. Both cell lines were grown at 5% CO_2 at 37 °C.

Ceramide Measurements—Cells were harvested in methanol, and lipids were extracted by the method of Bligh and Dyer (26). The chloroform organic phase was separated into aliquots (in duplicates) and dried down for ceramide and phosphate measurements. Ceramide levels were evaluated using the *Escherichia coli* diacylglycerol kinase assay. Ceramide was quantitated by using external standards and normalized to phosphate content (25).

Metabolic Labeling of Cellular SM and Measurements of [^{3}H]Choline-labeled SM after Treatment with Bacterial SMase—Cells were seeded at a density of 2×10^5 in 100-mm dish. After 2 days, the medium was changed, and the cells were labeled with [methyl-^{3}H]choline chloride (final specific activity: 0.5 μCi/ml) for 60 h. After this time, cells were washed once with phosphate-buffered saline (PBS) and chased for 2 h. After washing with PBS again, cells were treated with 100 milliunits/ml bSMase for 25 min. Then cells were washed twice with PBS, and fresh medium was added to continue the incubation for up to additional 6 h. In some experiments 50 μg/ml D609 (aqueous solution) were added to the medium. After removing the medium, cells were washed and scraped in PBS. The suspensions were centrifuged at 1000 × g for 5 min at 4 °C, and the pellets were stored at −80 °C. SM measurements were performed as indicated previously (25).

Serum Stimulation—Cells were seeded at a density of 2×10^5 cells in a 100-mm dish in 10% FBS-supplemented medium. After 2 days, the medium was removed, the cells were washed with PBS, and finally, medium supplemented with 0.1% FBS was added. After approximately 2.5 days, on the day of the experiment, the medium was replaced with fresh 10% FBS medium with or without 100 milliunits/ml bSMase.

PMA Stimulation—Cells were seeded at a density of 6×10^5 cells in a 100-mm dish in regular growth medium. After 2 days, cells were washed with PBS, and medium supplemented with 0.1% FBS was added (7 ml/plate). After 12 h, cells were treated with PMA (50 nM) in the presence or absence of bSMase (100 milliunits/ml) in a final volume of 8 ml.

bSMase Washout—Cells were seeded at a density of 6×10^5 cells in a 100-mm dish in regular growth medium. After 2 days, the medium was changed (7 ml of fresh medium were added), and after 12 h, cells were treated with 100 milliunits/ml bSMase for 25 min in a final volume of 8 ml of medium/plate (bSMase was added in 1 ml medium/plate). The cells were washed twice with PBS and further incubated in fresh medium (8 ml) for the specified time points. In some experiments, 50 μg/ml D609 (aqueous solution) was added to the bSMase treatment and to the subsequent incubation medium.

Nuclear Extracts—The nuclear extraction procedure was modified from Dignam *et al.* (27), with all procedures performed at 4 °C. After treatments, the medium was removed, and cells washed twice with PBS. The cells were harvested by scraping in PBS and pelleted at 500 × g for 10 min. The pellet was resuspended in 400 μl of lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and incubated for 10 min on ice. Just before centrifugation, 20 μl of 10% Nonidet P-40 were added, and the suspension was triturated 3 times. Nuclei were pelleted by microcentrifugation at 1300 × g for 10 min. The supernatant was removed, and the nuclei were resuspended in 20 μl of extraction buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 25% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The suspension was mixed gently for 30 min and pelleted at 13,000 × g for 15 min. The supernatant was flash-frozen and stored at −80 °C. Protein concentrations were determined using the Bio-Rad assay.

Electrophoretic Mobility Shift Assay—Assays were performed in a final volume of 20 μl using 10 μg of nuclear extract and incubated in HDKE buffer (20 mM Hepes, pH 7.9, 50 mM KCl, 5% (v/v) glycerol, 1 mM EDTA, 5 mM dithiothreitol) containing 1 μg poly(dI-dC), 1 μg poly(dN6), and 10 μg of bovine serum albumin. One μl of radiolabeled oligonucleotide probe (60,000–100,000 cpm) was added to each reaction and incubated at room temperature for 20 min. The reaction was terminated by the addition of 6 μl 15% Ficoll solution containing indicator dyes (bromphenol blue and xylene cyanol). For supershift experiments, 1 μl of antibody was added to appropriate samples and incubated for 30 min on ice before Ficoll termination. Equal amounts (20 μl) of reaction mixture were loaded on a 5% nondenaturing polyacrylamide gel in 90 mM Tris borate, 2 mM EDTA and run at 200 V. Gels were placed onto Whatman filter paper, dried, and autoradiographed.

Oligonucleotides—A synthetic NF-κB consensus oligonucleotide from Promega with the following sequence: 5′-ACTTGAGGGGACTTTC-CCAGGC-3′ was end-labeled using polynucleotide T4 kinase and [γ-^{32}P]ATP and purified on a microspin G25 column.

Immunocytochemistry of NF-κB—For immunocytochemical analysis, wild type or transformed WI38 cells were plated at a density of 2×10^5 cells/100-mm plate that contained 22-mm glass coverslips coated with poly-L-lysine (Sigma).

After treatment, cells were fixed and permeabilized during a 20-min incubation in 4% paraformaldehyde and 0.2% Triton X-100 in PBS, washed, blocked, and treated for 30 min with a rabbit anti-p65 NF-κB antibody (1:200). The cells were then washed and incubated with a fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:100). Nuclei were visualized by a 5-min incubation with Hoechst dye (10 μg/ml bisbenzimide; Roche Molecular Biochemicals). The cells were photographed with a Dage-MTI 100 video camera mounted on a Zeiss Axiovert microscope equipped with fluorescence optics, a using a video digitizer (Snappy Video Snapshot, Play Inc., Rancho Cordova) operated through Adobe Photoshop.

RESULTS

bSMase Treatment Blocks NF-κB Activation Induced by Serum and PMA in Normal WI38 Fibroblasts but Not in SV40-transformed WI38—To investigate the role of endogenous plasma membrane ceramide in the regulation of NF-κB activation, the effect of bSMase was assessed on serum-induced NF-κB translocation. Normal WI38 human lung fibroblasts were withdrawn from serum for approximately 60 h and then
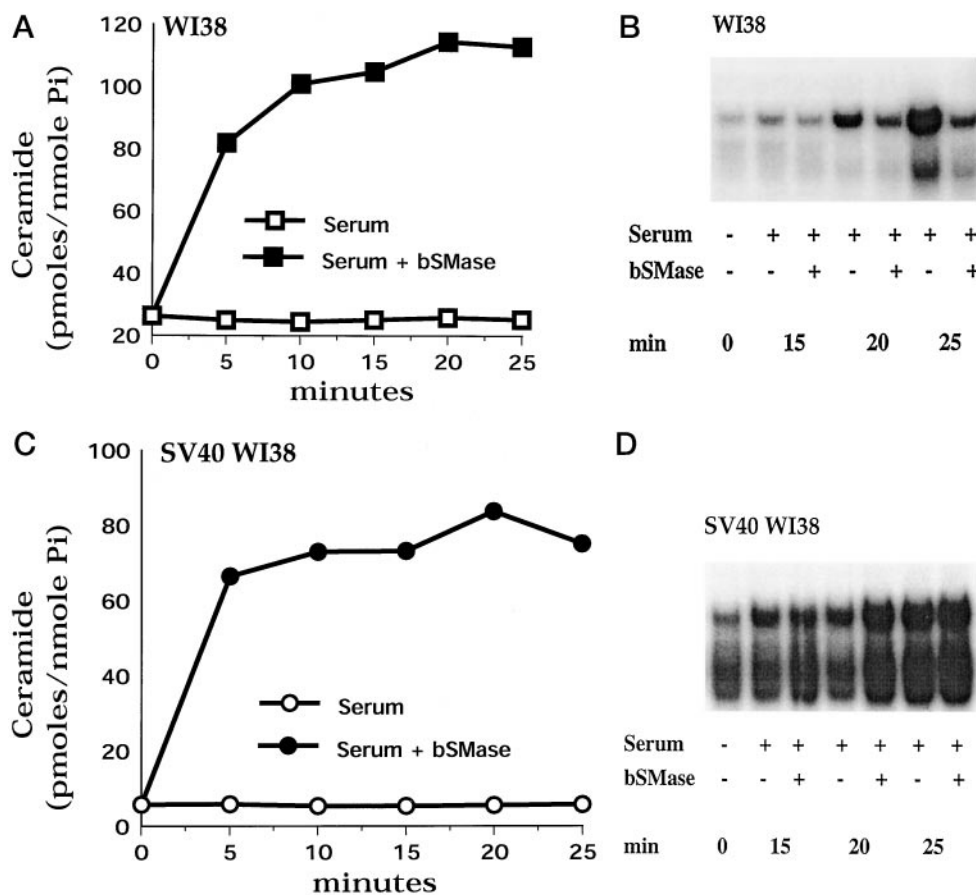
FIG. 1. **Effects of bSMase treatment on ceramide metabolism and NF-κB activation in normal and SV-40 WI38 fibroblasts during acute serum stimulation.** After 60 h of serum withdrawal (0.1% FBS) (zero time), cells were stimulated for 30 min with medium containing 10% FBS in the presence or absence of 100 milliunits/ml bSMase. Ceramide metabolism (*E. coli* DGK assay) (*A* and *C*) and NF-κB translocation (electrophoretic mobility shift assay) (*B* and *D*) were studied in normal (*A* and *B*) and SV40-transformed WI38 (*C* and *D*).

stimulated by the addition of fresh medium containing 10% FBS, in the presence or absence of 100 milliunits/ml bSMase. As shown in Fig. 1*A*, simple serum stimulation of WI38 cells did not induce changes in ceramide levels during 25 min incubation (zero time *versus* 25 min). On the other hand, the presence of bSMase caused a burst of ceramide (110 pmol/nmol P_i *versus* 27 pmol/nmol P_i) that was generated through the hydrolysis of the SM in the outer leaflet of the plasma membrane. When determining the effect of serum stimulation on NF-κB activation as evaluated by electrophoretic mobility shift assay, we found that normal WI38 cells showed a time-dependent activation of the transcription factor, clearly seen after 20 min of stimulation (Fig. 1*B*). Interestingly, the presence of bSMase, although inducing ceramide accumulation, significantly inhibited this activation during the time course (Fig. 1*B*) and was most pronounced at the 25 min time point.

To assess whether the same pattern of activities occurred in SV40-transformed WI38 fibroblasts compared with the normal counterpart, the effect of bSMase treatment during acute serum stimulation was studied in the transformed cells. As shown in Fig. 1*C*, in a manner very similar to the normal WI38, bSMase treatment induced a significant accumulation of ceramide over the incubation period (75 pmol/nmol P_i), whereas simple serum stimulation did not cause any changes in the lipid level. Under these conditions, serum stimulation induced a progressive activation of NF-κB over time (25 min *versus* 15 min), similar to what was observed in the normal fibroblasts (Fig. 1*D*). On the other hand, in the SV40-WI38 cells, the treatment with bSMase was not able to block the serum-induced effect on NF-κB and, if anything, it promoted its activa-

tion (Fig. 1*D*). It is important to note that, as previously observed in this cell line (25) and, as reported by several authors (for a review, see "Discussion" in Zhang *et al.* (32)), treatment with bSMase did not exert any cytotoxic effect or significant morphological changes appreciable by optical microscopy analysis.

To confirm the results obtained on NF-κB activation in the two cell lines under conditions of serum stimulation, we investigated the effect exerted by the treatment with bSMase on phorbol ester-induced NF-κB activation. Cells withdrawn from serum (0.1% FBS-containing medium) for 12 h were stimulated with 50 nM PMA for 30 min in the presence or absence of 100 milliunits/ml bSMase. As shown in Fig. 2, stimulation with PMA caused NF-κB activation in both normal (*left panel*) and SV40-transformed WI38 (*right panel*). When bSMase was added concomitantly with PMA, it blocked almost completely NF-κB activation in normal WI38, whereas it had no inhibitory effect in the SV40-WI38. These results reproduce the effects observed during serum stimulation conditions.

The Differential Activation of NF-κB in Normal and SV40-transformed WI38 after bSMase Treatment Correlates with the Different SM Synthase Activity in the Two Cell Lines—We have recently reported that there is a significant difference in ceramide metabolism between normal and SV40-transformed WI38 (25). In response to treatment with bSMase, the normal and SV40-transformed WI38 show distinct levels and patterns of SMS activity. A 25-min incubation with bSMase resulted in significant SM hydrolysis and ceramide generation in both cell lines. However, following washout of bSMase, only the SV40-transformed cells metabolized the high levels of ceramide gen-
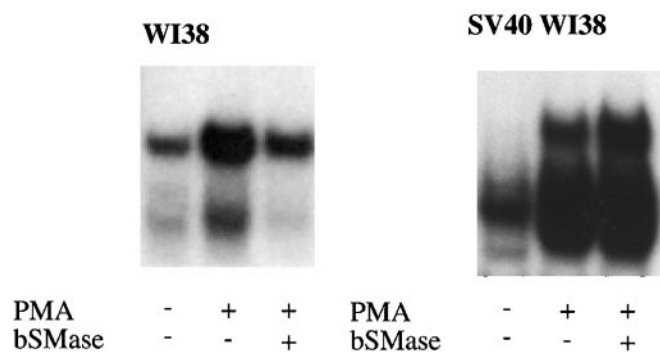
FIG. 2. **Effects of bSMase treatment on PMA-induced NF-κB activation in normal and SV-40-transformed WI38 fibroblasts.** After 12 h of serum withdrawal (0.1% FBS), cells were stimulated with 50 nM PMA for 30 min in the presence or absence of bSMase (100 milliunits/ml). NF-κB activation was evaluated by electrophoretic mobility shift assay in normal (*left panel*) and SV40-transformed (*right panel*) WI38.

erated at the plasma membrane (25). Moreover, the decrease in ceramide levels in the transformed cells was accompanied by an increase in SM (Fig. 3*A*).

In view of these observations, we asked whether the differences in SM and ceramide metabolism between the two cell lines relate to the differences in NF-κB activation. To address this question, both cell lines were treated with bSMase for 25 min in growth medium, washed twice (to remove bSMase), then incubated with fresh medium up to 30 min. In normal cells, after bSMase washout, there was little if any increase over basal activity of NF-κB, as assayed by electrophoretic mobility shift (Fig. 3*B*, *left panel*). However, SV40-transformed cells, with their high SMS activity, showed an increase in NF-κB activation following bSMase treatment (Fig. 3*B*, *right panel*) during the phase of SM resynthesis.

Specificity of NF-κB Activation—Since the NF-κB-DNA binding appeared different between normal and transformed WI38, the specificity of this activation was examined using monoclonal antibodies against the p65 and p50 subunits of the NF-κB heterodimer. In both cell lines, the supershift of NF-κB-specific bands was not seen with the p50 antibody. However, co-incubation with the p65 antibody resulted in similar retardation of the bands in both TNFα- and bSMase-treated extracts (Fig. 4). These bands were competitively washed out by addition of excess cold consensus oligonucleotide. Of note is also the slightly higher basal NF-κB activation in WI38 cells compared with the SV40 fibroblasts (Fig. 4,*first lanes*) (confirmed by immunocytochemistry, see Fig. 5). In the SV40 WI38, the patterns of NF-κB activation induced by bSMase treatment were similar to those seen with TNFα (included as positive control), although bSMase appeared to be significantly less effective.

Immunocytochemical analysis was subsequently carried out to confirm the differences in the activation of NF-κB after bSMase washout in the two cell lines. Cells were fixed 30 min after bSMase washout and probed with an anti-NF-κB p65 antibody. As shown in Fig. 5, SV40-WI38 control cells displayed a diffuse cytosolic NF-κB signal. After bSMase treatment, the immunoreactivity was almost exclusively present in the nucleus, indicating that NF-κB translocated from the cytosol into the nucleus, confirming what was observed by the electrophoretic mobility shift assay. No such NF-κB translocation was seen in normal WI38 cells either under control conditions or after bSMase. However, it should be noted that normal WI38 fibroblasts showed a small basal NF-κB activation seen both by immunocytochemistry and by gel shift assay. Nevertheless, treatment with TNFα caused a strong NF-κB translocation (Fig. 5), indicating that NF-κB activation remained inducible.
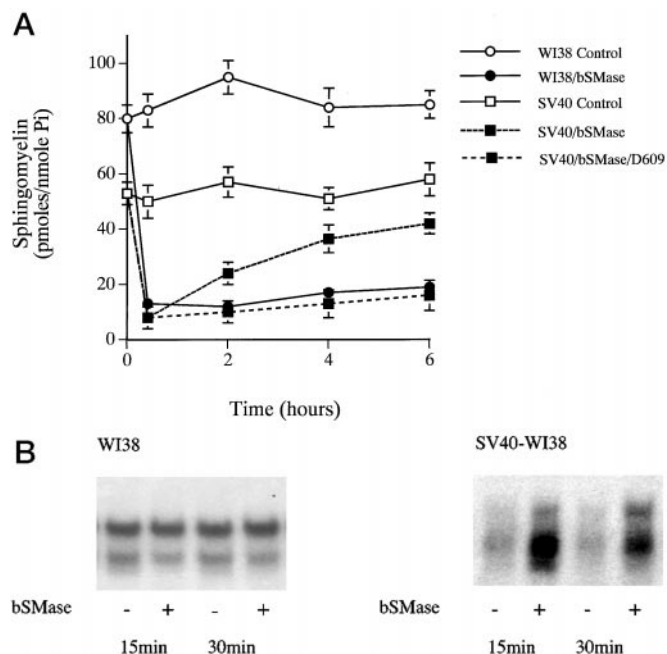

FIG. 3. **NF-κB activity in normal and SV40-WI38 fibroblasts after treatment with bSMase and its washout.** Cells were incubated with or without bSMase (100 milliunits/ml) for 25 min. bSMase was then washed away, new fresh medium was added, and cells were further incubated for the indicated time points. In some experiments, D609 (50 μg/ml) was included after bSMase washout. SM levels throughout the incubation (*A*) and NF-κB activity (*B*) 15 and 30 min after the bSMase washout were determined in normal and SV40-WI38 fibroblasts.

Effects of D609 on SMS and NF-κB Activation—Previous studies have described and utilized D609 as a PC-PLC-specific inhibitor. In the SV40-transformed cell line, we were unable to detect any PC-PLC activity (25). However, we found that the high level of SMS activity in transformed cells was inhibited by D609 (Fig. 3*A*). This provided another opportunity to establish the effect of SMS on NF-κB activation and to distinguish the effects of bSMase due to ceramide formation *per se* from effects due to subsequent metabolism through SMS.

SV40-transformed cells were treated for 25 min with or without bSMase. The cells were then washed to remove the bSMase and then incubated in fresh medium (see Fig. 6*A*). The effect of D609 on NF-κB activation was investigated, and D609 was added during bSMase incubation and after its washout (Fig. 6*A*) to determine the effects of D609 on the induction of ceramide by bSMase and its effects on resynthesis of SM. To verify that the presence of D609 during bSMase treatment did not interfere with the enzymatic activity, ceramide levels were determined. As shown in Fig. 6*B*, D609 had no effect *in vivo* on ceramide generation by bSMase in SV40-transformed cells. In contrast, D609 nearly totally inhibited SM resynthesis (Fig. 3*A*). These results clearly show that D609 inhibits SMS but not the initial ceramide formation. Notably, the robust activation of NF-κB by bSMase at 30 min was virtually eliminated by incubation with D609, and this inhibitory effect remained after a 1-hr incubation (Fig. 6*C*).

Altogether these results demonstrate that the action of D609 is downstream of ceramide generation but upstream of SM resynthesis, consistent with a major effect on SMS.

DISCUSSION

In this study, we demonstrate two distinct effects of bSMase on NF-κB (Fig. 7). First, serum- and PMA-induced activation of NF-κB was inhibited during treatment with bSMase in normal WI38 fibroblasts. This is consistent with a previous study
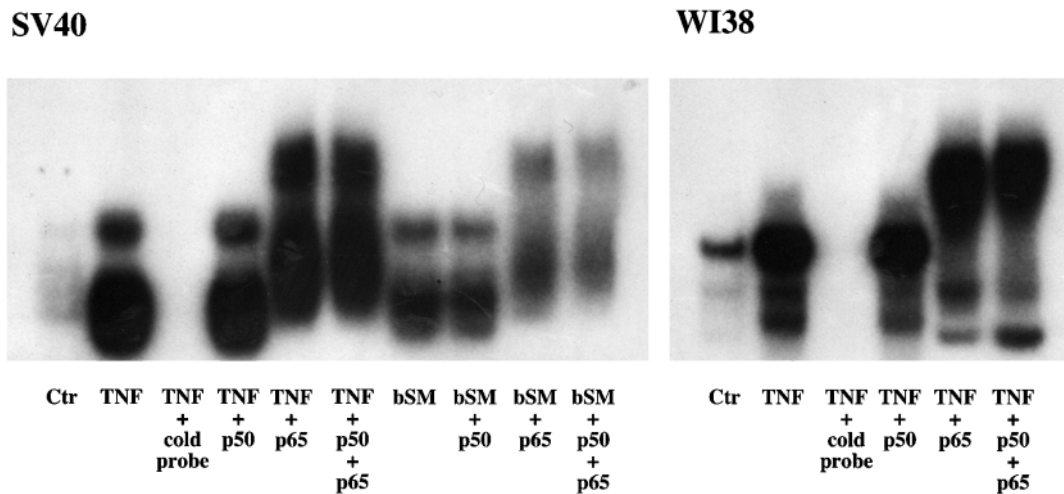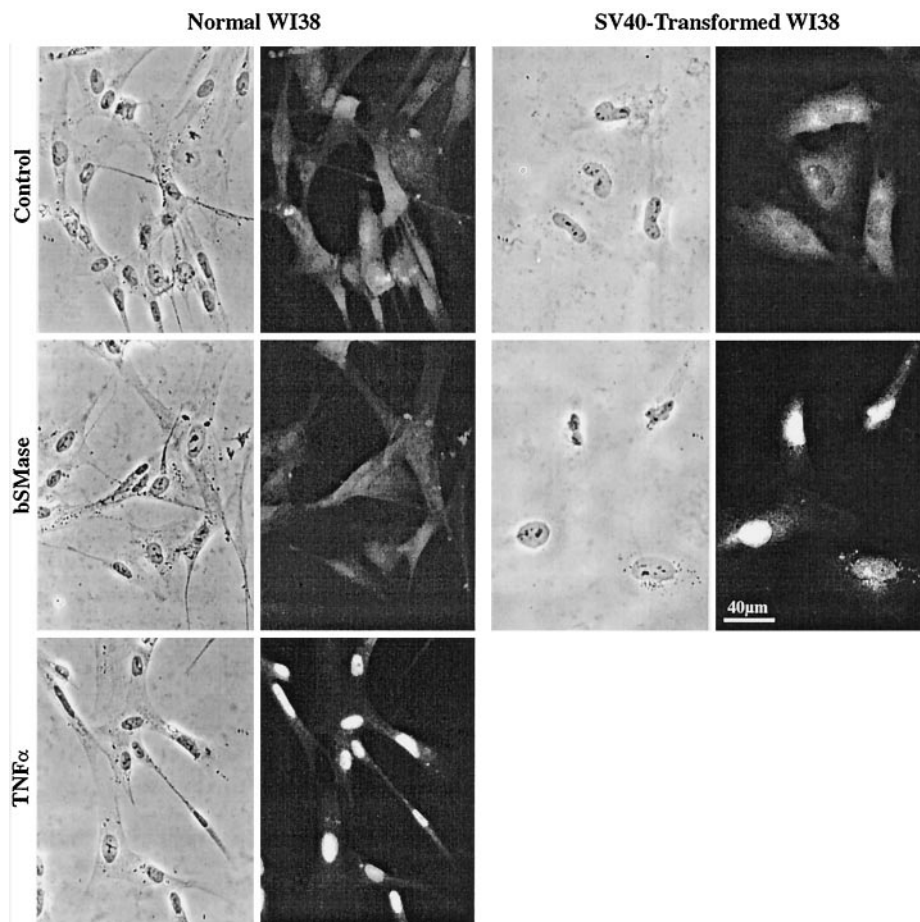
SV40

WI38

Ctr | TNF | TNF + cold probe | TNF + p50 | TNF + p65 | TNF + p50 + p65 | bSM + p50 | bSM + p65 | bSM + p50 | bSM + p50 + p65

Ctr | TNF | TNF + cold probe | TNF + p50 | TNF + p65 | TNF + p50 + p65

FIG. 4. **Specificity of NF-κB activation in normal and SV40-transformed WI38 fibroblasts.** After 30 min of stimulation with TNFα (3 nM) (second through *sixth lanes*) or 30 min after bSMase (100 milliunits/ml) washout (seventh through tenth *lanes*), protein-DNA binding reactions were carried out in the presence of ^{32}P-labeled NF-κB consensus oligonucleotide alone or with excess of unlabeled consensus oligonucleotide (third *lanes*) or monoclonal antibodies against NF-κB p65 and p50 subunits separately or together. *Ctr*, control.

Normal WI38   **SV40-Transformed WI38**

FIG. 5. **Immunocytochemical analysis of NF-κB translocation in normal and SV40-WI38 fibroblasts after bSMase washout.** Wild type or transformed WI38 were grown for 2 days on 22-mm glass coverslips coated with poly-L-lysine. Medium was changed (7 ml/100-mm plate), and after 12 h, 100 milliunits/ml bSMase was added in 1 ml of medium. After 25 min of stimulation, the cells were washed 2 times with PBS, new medium was added, and the cells were incubated for an additional 30 min. When TNFα was used, it was added at 3 nM for 30 min after a mock washout. All cells were fixed and processed for NFkB immunostaining as described under "Experimental Procedures." SV40-transformed cells are particularly flat, and their perimeter can barely be tracked under phase optics, but it is better-defined in the corresponding fluorescent images.

showing that short chain ceramides inhibit activation of NF-κB by PMA but not by TNFα (19). Second, bSMase treatment activated NF-κB in the SV40-transformed line. This activation correlated with high SMS activity in the transformed cells *versus* their normal WI38 counterparts. This activation was prevented by D609.

Although the results with D609 do not conclusively support a role for SMS in the activation of NF-κB, they do rule out a direct role for ceramide in this process because ceramide generation was not inhibited by D609. Moreover, the action of

exogenous ceramides or bSMase in the absence of SMS activity results in inhibition of NF-κB activation. These results suggest that metabolism of ceramide is needed for the activation of NF-κB. We propose that SMS action on ceramide is responsible for NF-κB activation because 1) it is inhibited by D609, 2) its activity is absent in the wild type cells, 3) it is the primary metabolic pathway that acts on ceramide in the transformed cells (for approximately 40 pmol/nmol P_i of resynthesized SM there are 40 pmol/nmol P_i of cleared ceramide), and 4) the production of DAG by SMS or its analog PMA are sufficient to
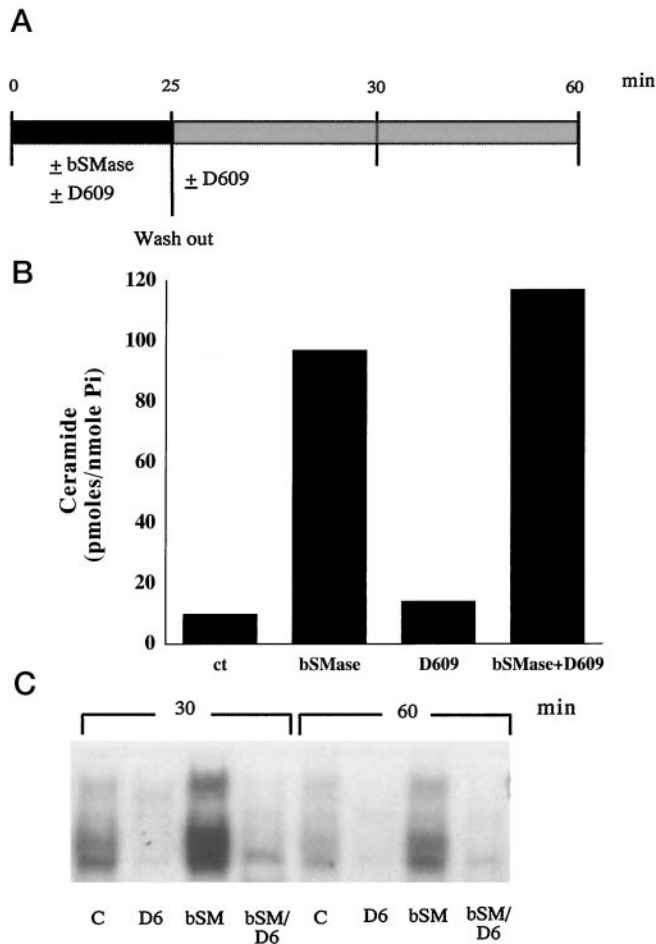
A

B

C

FIG. 6. **Effect of D609 on NF-κB activation in SV40-transformed WI38 fibroblasts after bSMase treatment and washout.** Cells were treated with bSMase (100 milliunits/ml) for 25 min in the presence or absence of D609 (50 μg/ml). After bSMase washout, cells were incubated for an additional 60 min, and D609 was added to the media where D609 was present before the washout (*A*). *B*, after a 25-min incubation in the presence or absence of bSMase (100 milliunits/ml) ± D609, ceramide levels were determined as described under "Experimental Procedures." *C*, NF-κB translocation was evaluated (electrophoretic mobility shift assay) at 30- and 60-min incubations after bSMase washout in the presence or absence of D609.
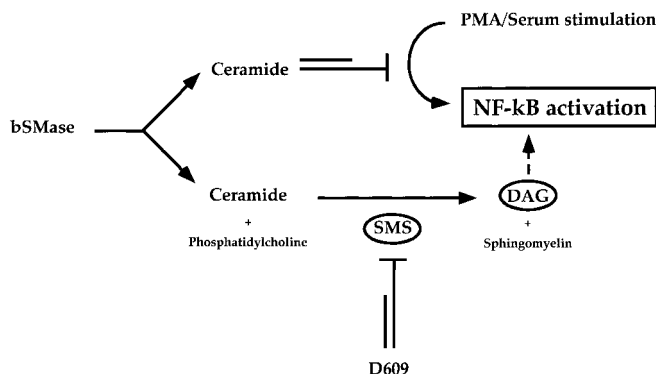

FIG. 7. **Schematic representation of potential roles of ceramide in NF-κB activation.** Following bSMase treatment, the endogenous ceramide may result in two different outcomes in NF-κB signaling, possibly depending on the presence of SMS activity. See text for further discussion and references.

activate NF-κB (Ref. 15 and see Fig. 3). Nevertheless, it should be noted that, in the absence of more specific inhibitors or antisense regulators of SMS, we cannot exclude other metabolites of ceramide as being involved in activation of NF-κB.

However, for this to occur, the following conditions must apply. (i) This metabolite must be sufficient to activate NF-κB, at least in the SV40 cells, (ii) formation of this metabolite or its action must be inhibited by D609, and (iii) this metabolite should be generated in the SV40 cells but not in the WI38 cells. Therefore, based on all the factors in support of SMS and against other metabolites, we favor a role for SMS as the key determinant in the SV40 cells that permits activation of NF-κB following ceramide generation, although additional tools are required to fully substantiate this suggestion.

These results may have multiple implications on the physiologic regulation of NF-κB and the possible role of ceramide in this process. The pleiotropic cytokine TNFα activates NF-κB and induces sphingomyelin hydrolysis with resultant ceramide generation in many cell lines. The possible relationship between these two separate observations has generated much discussion and conflicting bodies of evidence. A review is provided by Gamard *et al.* (19) and by Levade and Jaffrézou (29). Briefly, Schütze *et al.* (13) reported activation of NF-κB in permeabilized Jurkat T cells treated with exogenous SMase and nanomolar concentrations of ceramide. The group also found SMase and ceramide-induced I-κB proteolysis in nuclei-free lysates (30). Other groups utilizing intact cell systems met with varying degrees of success in activating NF-κB via exogenous SMase and ceramide treatment (16, 17). However, several other groups using intact cells reported no activation of NF-κB with SMase or ceramide (5, 19–23). In Jurkat cells, we previously reported that TNFα can activate NF-κB independent of ceramide and that ceramide has an inhibitory effect on NF-κB activation by PMA (19).

The current findings suggest that the discrepancies in the literature may be due to the presence or absence of SMS in the various cell lines utilized in the various studies. Cells with low SMS activity, similar to the normal WI38 fibroblast line, would show no activation of NF-κB by ceramide and possibly a negative feedback of its regulation, whereas those cells with high SMS activity would demonstrate activation. Moreover, the results with D609 further refute the model whereby NF-κB is activated by TNFα through a PC-PLC/acid SMase/ceramide pathway. According to that proposed scenario, ceramide is downstream of the inhibition of PC-PLC by D609, and D609 should inhibit ceramide formation (13, 18). However, in the present study, when ceramide is generated by bSMase, the activation of NF-κB is still inhibited by D609 without effects on ceramide generation. This necessitates a model where ceramide is upstream of the target of D609. This PC-PLC/acid SMase model is also negated by the lack of inhibition of NF-κB stimulation by TNFα in Niemann-Pick fibroblasts (21), thus ruling out a role for acid SMase.

Finally, these results also have three potential implications. First, there are many cellular events that have been attributed to PC-PLC based on studies with D609. These events, such as activation of mitogen-activated protein kinase or the atypical protein kinase C, may need to be reconsidered as potential downstream targets of the SMS pathway or possibly other targets of D609. Second, by potentially monitoring and regulating the effective levels of ceramide and DAG, SMS may assume a key "biostat" role. According to this scenario, high SMS activity can efficiently "convert" a ceramide signal to a DAG one, whereas low activity allows the predominance of the ceramide signal (Fig. 7). Third, SMS may have a physiologic role in regulating NF-κB. It is tempting to speculate that a specific pool of DAG (or subsequent metabolites) formed from PC by the action of SMS may couple many agents to activation of NF-κB.

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Differential Effects of Sphingomyelin Hydrolysis and Resynthesis on the Activation of NF- κB in Normal and SV40-transformed Human Fibroblasts

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