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Ceramide Activates NFκB by Inducing the Processing of p105*

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The role of ceramide as a second messenger in tumor necrosis factor (TNF)-mediated signal transduction has been much debated. It is supported by recent reports describing an expanding number of potential targets for this lipid, but is opposed by those describing how ceramide is not necessary for many TNF-mediated cellular events. In this paper, we directly compare the effects of the cell-permeable ceramide analogue, *N*-acetylsphingosine (C_2-ceramide), with TNF, on NFκB function, a transcription factor whose activation is central to many TNF-mediated effects. We describe how C_2-ceramide failed to drive κB-linked chloramphenicol acetyltransferase gene expression in either HL60 promyelocytic or Jurkat T lymphoma cells. Furthermore, it had no effect on TNF-mediated transcription of this reporter gene. However, electrophoretic mobility shift analysis following cell stimulation with this ceramide analogue revealed a dose-responsive activation of NFκB, which was not apparent following cell treatment with the inactive dihydro form. Activated complexes from treated cells were shown to contain predominantly the p50 subunit, in contrast to complexes from TNF-treated cells, where both p50 and p65/RelA subunits were present. The specific activation of p50 homodimeric complexes by C_2-ceramide, which are known to lack trans-activating activity, was strongly suggested from these data. Further investigations revealed that C_2-ceramide had only a marginal effect on IκBα degradation but strongly promoted the processing of p105 to its p50 product as revealed by immunoblot analysis. The increase in p50 arising from the processing of its p105 precursor was further established from p105/p50 ratios obtained by scanning densitometric analysis of bands from immunoblots. TNF, on the other hand, stimulated both IκBα degradation and p105 processing, in accordance with previous findings. Furthermore, the effect of TNF on NFκB activation was rapid, whereas C_2-ceramide required an optimal treatment time of 1 h. Interestingly, TNF was found to increase ceramide in cells but only after a 1-h contact time. Our data therefore suggest that ceramide promotes the activation of NFκB complexes that lack transactivating activity by enhanced processing of p105.

Tumor necrosis factor (TNF)[1] is a pleiotropic cytokine that induces a variety of cell type-specific events including proliferation, differentiation, necrosis, and apoptosis (1). It is among a range of agents that have been reported to increase levels of the neutral lipid, ceramide, in cells (2–12). This has led to the proposal that ceramide may be a second messenger for TNF, where cell-permeable analogues of ceramide can mimic several TNF effects, including activation of the transcription factor, NFκB (13–15). Ceramide has also been shown to activate several protein kinases (16–23), a protein phosphatase (24), and the nucleotide exchange protein, Vav (25), and to induce apoptosis (26–30).

NFκB is a member of the Rel family of transcription factors (31). It is activated by a wide range of stimuli, including TNF, interleukin-1, UV irradiation (reviewed in Ref. 32), and chemotherapeutic drugs (12, 33), all of which have been shown to increase ceramide levels in cells (30). The mechanism of NFκB activation has been the subject of much scrutiny. It binds to a discrete nucleotide sequence (5′-GGGACTTCC-3′) in the upstream regions of genes that code for mediators of the immune, acute phase, and inflammatory responses, thereby regulating their expression (32). The activation of NFκB can occur by two mechanisms. The prototypical pathway involves its liberation from an inactive complex with the inhibitor protein, IκB, which resides in the cytosol (31, 32). Several forms of IκB have been identified (34), with particular attention focusing on IκBα. In resting cells, it is complexed to the NFκB heterodimer, typically comprising p50 and p65/RelA subunits. Following cell stimulation, a pathway is activated that culminates in the phosphorylation of IκBα on two serine residues (Ser^{32} and Ser^{36}) (35), which tags it for degradation by the proteosome (36), leading to the nuclear translocation of the NFκB heterodimer. Recently, a pathway activated by TNF involving TRAF-2, an NFκB-inducing kinase, and two novel kinases termed IKK-1 and IKK-2 (or IKK-α and IKK-β) has been described (37–40), with both IKKs phosphorylating IκBα (39, 40). Another less well defined pathway for NFκB activation has also been described (31). The p50 subunit of NFκB is generated following processing from its precursor, p105 (41). Proteolysis results in the removal of an inhibitory C-terminal domain containing seven ankyrin repeats, a motif present in IκB proteins, revealing a nuclear translocation signal for this subunit. Specifically, a 68-amino acid sequence in the C-terminal PEST domain of p105 contains multiple serines that are phosphorylated (42) by an as yet unidentified kinase, prior to proteosome-directed proteolytic degradation (43). Processing of p105 complexed to p65/RelA would allow the heterodimer to translocate into the nucleus in an analogous manner to that which occurs following IκBα proteolysis. In addition, p50 homodimers, which have been shown to be transcriptional repressors (44), would also translocate to the nucleus. Once in the nucleus, p50 could complex with p65/RelA and c-Rel, giving rise to heterodimers with transcriptional potential. p105 processing has been shown to be stimulated by TNF, phorbol esters, and double-stranded RNA

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[1] The abbreviations used are: TNF, tumor necrosis factor; NFκB, nuclear factor κ B; C_2-ceramide, *N*-acetylsphingosine; CAT, chloramphenicol acetyltransferase.

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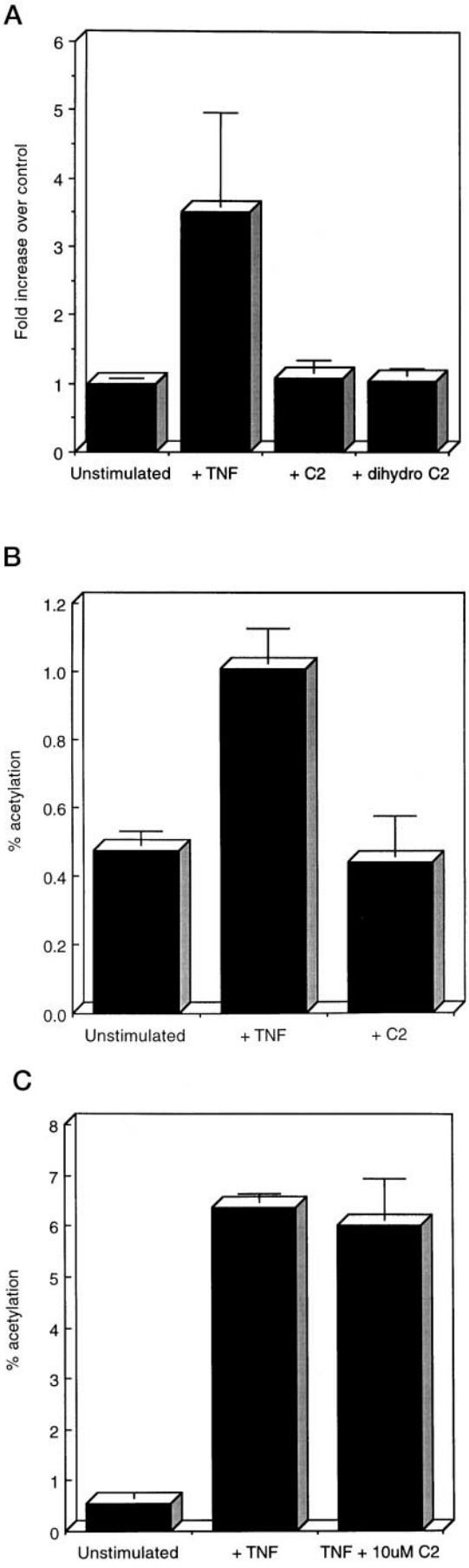

FIG. 1. *A*, C_2-ceramide does not stimulate expression of CAT activity in Jurkat T cells transfected with an NFκB-linked reporter plasmid. Transfected Jurkat T cells (1×10^6/ml) were incubated with TNF (30 ng/ml), C_2-ceramide, or its dihydro form (10 μM) for 24 h. Cell lysates were prepared and assayed for CAT activity as described under "Experimental Procedures." Results are shown as fold increase over unstimulated control and are the means ± S.D. of three separate experiments, each carried out in triplicate. *B*, C_2-ceramide does not stimulate expression of CAT activity in HL60 cells transfected with an NFκB-linked reporter plasmid. Transfected HL60 cells (1×10^6/ml) were incubated with either TNF (30 ng/ml) or C_2-ceramide (10 μM). After 24 h, cell lysates were prepared and assayed for CAT activity as described under "Experimental Procedures." The results are represented as % acetylation and are the means ± S.D. from a single experiment carried out in triplicate, which is representative of three separate experiments. *C*, C_2-ceramide does not potentiate TNF-stimulated expression of CAT activity in Jurkat T cells transfected with an NFκB-linked reporter plasmid. Transfected Jurkat T cells (1×10^6/ml) were incubated with C_2-ceramide (10 μM) for 30 min prior to the addition of TNF (30 ng/ml). After 24 h, cell lysates were prepared and assayed for CAT activity as described under "Experimental Procedures." Results are represented as % acetylation and are the means ± S.D. from a single experiment carried out in triplicate, which is representative of three separate experiments.

(45, 46). The activation of NFκB family members may therefore be regulated by this pathway in parallel with that mediating IκB degradation in response to the same signal.

The mechanism by which ceramide can trigger either of the pathways leading to NFκB activation is unresolved, with conflicting results being presented (47–53). Here, we have compared the effect of a commonly used short chain cell-permeable analogue of ceramide, *N*-acetylsphingosine (C_2-ceramide), with TNF in four independent assays of NFκB function. We have found that although C_2-ceramide can activate NFκB as judged by gel shift analysis, prolonged treatment times are required and only a marginal increase in IκBα degradation is observed. Furthermore, there is no effect on NFκB-linked reporter gene expression. However, C_2-ceramide can induce p105 processing, with NFκB complexes predominantly comprising p50 homodimers. In contrast, TNF-activated complexes contain both p50 homodimeric and p50/p65 heterodimeric forms of NFκB. TNF promotes both IκBα and p105 processing and as expected, induces NFκB-linked gene expression. Our results may therefore help to resolve some of the controversy in this area, in that although ceramide does not appear to be involved in the pathway leading to IκBα phosphorylation and degradation, it may signal p105 processing in response to TNF.

EXPERIMENTAL PROCEDURES

Materials—HL60 and Jurkat T cells (both obtained from the European Collection of Animal Cell Culture, Salisbury, UK) were grown in suspension culture in RPMI 1640 supplemented with 10% fetal calf serum, penicillin/streptomycin (100 units/ml and 100 mg/ml, respectively), and L-glutamate (final concentration, 2 mM), all obtained from Life Technologies, Inc. (Paisley, UK). Recombinant human TNFα was a gift from Zeneca Pharmaceuticals Ltd. (Macclesfield, UK). Poly(dI-dC) was from Pharmacia Biosystems (Milton Keynes, UK), T4 polynucleotide kinase and oligonucleotide containing the consensus sequence (5′-GG GAC TTT CC-3′), corresponding to the κ light chain enhancer motif, were purchased from Promega (Southampton, UK). [γ-^{32}P]ATP (3000 Ci/mmol), ([^{14}C]chloramphenicol (56 mCi/mmol), and ECL reagent were from Amersham Pharmacia Biotech (Aylesbury, UK). Diacylglycerol kinase was from Calbiochem (UK). Rabbit polyclonal antibody preparations to the NFκB subunits, RelA/p65, and p50, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Dr. Jean Imbert (INSERM, Marseille, France) (where indicated). Polyclonal antibodies (41) raised against an N-terminal peptide (residues 2–15) of the p105 protein (which also recognized its proteolysis product, p50, containing this N-terminal sequence) were generously supplied by Dr. Alain Israel (Institut Pasteur, Paris Cedex 15, France), and monoclonal antibodies to the inhibitor protein, IκB-α, were from Dr. Ron Hay (St. Andrews, Scotland). The pCAT™-Promoter plasmid was a gift from Dr. Tim Bird (Immunex Corporation, Seattle, WA). Mutant NFκB oligonucleotide was from Santa Cruz Biotechnology Inc. C_2-dihydroceramide (*N*-acetyldihydrosphingosine) was from Matreya Inc. (Pleasant Gap, PA).

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All other reagents were purchased from Sigma (Poole, Dorset, UK).

Cell Culture—For treatments, cells in late log phase of growth were resuspended in serum-free medium at a concentration of 5×10^6/ml (2.5×10^6/ml for whole cell extracts) and incubated at 37 °C in a humidified atmosphere of 5% CO^2/95% air. Following stimulation (60 min unless otherwise stated), incubations were discontinued by the addition of ice-cold phosphate-buffered saline, and either nuclear or whole cell extracts were prepared as described previously (33). Protein determinations were made using the Bradford assay with bovine albumin as standard.

Transfection Studies—The transactivating potential of activated NFκB complexes was assessed following transfection of cells with a plasmid containing five NFκB consensus sequences upstream of a chloramphenicol acetyltransferase reporter gene. Jurkat T cells were transfected as described previously (33). HL60 cells were transfected by electroporation (54) with modifications; cells were washed with 5 units/ml heparin post-transfection followed by incubation in medium supplemented with 20% serum for 24 h. Following treatment (indicated in figure legends), extracts prepared from harvested cells (1×10^6) were assayed for CAT activity (33). Statistical significance was evaluated by employing Student's *t* test for unpaired data.

Electrophoretic Mobility Shift Assays—Nuclear NFκB was assessed by the electrophoretic mobility shift assay using a 22-base pair oligonucleotide containing the human κ light chain enhancer motif, which had previously been end-labeled with [γ-^{32}P]ATP as described (33). Typically, 2–4 mg of nuclear extract protein was incubated with radiolabeled oligonucleotide (10,000 cpm) at room temperature for 30 min using conditions as described previously (33). NFκB complexes were resolved on 5% acrylamide gels and identified following autoradiography. To identify the subunit components of activated NFκB complexes and the specificity of the binding reaction, supershift analysis and competition studies were carried out as described previously (33), using antibodies described under "Experimental Procedures" to individual NFκB subunit components and mutant/wild type unlabeled NFκB consensus sequence, respectively.

Western Blot Analysis—Equal amounts of whole cell lysate protein (as indicated) were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose, and IκBα or p50/p105 immunoblot analysis was performed as described previously (33). Secondary antibody was used at a dilution of 1:1000 and 1:2000 for IκBα and p50/p105, respectively. The blots were developed by ECL according to the manufacturer's recommendations.

Lipid Studies—Ceramide was quantified by the diacylglycerol kinase assay (10) with modifications as described (33). The level of ceramide in HL60 cells following treatment with TNFα or not (control) was determined by comparison with a standard curve generated with known amounts of ceramide (ceramide type III; Sigma).

RESULTS

Neither C_2-ceramide nor Its Dihydro Form Stimulate κB-driven Gene Expression—In our first experiments with C_2-ceramide, we attempted to mimic the stimulatory effect of TNF on an NFκB-linked reporter gene construct. HL60 or Jurkat were therefore transfected with a gene construct containing five NFκB sites upstream of a CAT reporter gene. Transfected cells were then stimulated with C_2-ceramide or TNF. At 10 μM C_2-ceramide no increase in CAT activity was observed over control values (unstimulated cells) in either Jurkat (Fig. 1*A*) or HL60 cells (Fig. 1*B*). 30 ng/ml TNF, as expected, stimulated NFκB-driven gene expression, causing a 4–6-fold increase over controls in Jurkat T cells. A 2-fold stimulation over control levels was seen in HL60 cells. Co-incubation of Jurkat with both C_2-ceramide and TNF resulted in a similar response to that seen with TNF alone (Fig. 1*C*). These results suggested that treatment of cells with ceramide had no effect on NFκB-linked gene expression.

C_2-ceramide, but Not Its Dihydro Congener, Activates NFκB in Both HL60 and Jurkat T Cells—We next tested whether ceramide could activate NFκB in these cells, as has been reported by others (13–15). C_2-ceramide dose-dependently activated NFκB in HL60 cells, as was demonstrated by the detection of protein-DNA complexes in nuclear extracts from ceramide-treated cells (Fig. 2*A*). This effect was evident from 1 μM and contact times of 1 h were required to see an effect. Stimulation of HL60 cells with both C_2-ceramide and TNF resulted in a modest potentiation of NFκB activation in comparison with that seen with TNF alone (Fig. 2*A*, *lanes 7* and *8*). The dihydro form of C_2-ceramide (which should be ineffective, because it lacks a critical 4,5-*trans* double in the sphingoid base backbone) was inactive (Fig. 2*B*). TNF strongly activated NFκB in both cell types (Fig. 2, *A* and *C*, *lane 7*) and was found to increase ceramide levels in HL60 causing a 2.64 ± 0.29-fold increase over control values after 1 h of stimulation, as shown in Fig. 1*C*. C_2-ceramide also activated NFκB in Jurkat T cells over a similar concentration range effective in HL60 cells and a contact time of 1 h (Fig. 2*D*).

NFκB Complexes Activated by C_2-ceramide Contain Predominantly p50 Subunit—We next examined the NFκB complex activated by C_2-ceramide in more detail. Binding specificity of activated complexes from C_2-ceramide-treated HL60 cells was demonstrated by competition studies. Excess unlabeled oligonucleotide containing the NFκB consensus sequence inhibited the appearance of retarded complexes, whereas a mutant oligonucleotide had no effect at equivalent concentrations (Fig. 3*A*). We then determined the composition of the activated complexes from HL60 cells treated with either C_2-ceramide or TNF. To achieve this supershift analysis was performed using specific antisera to p50 and p65/RelA. The complexes were electrophoresed further than usual to optimize resolution. Fig. 3*B* shows that the complexes activated by TNF differ markedly from those induced by C_2-ceramide (compare *lanes 4* and *1*). TNF induces two main complexes. Treatment of extracts with anti-p65 antiserum inhibited the formation of the upper complex (*lane 5*), whereas anti-p50 antiserum affected both upper and lower complexes (*lane 6*) and caused a further retardation of the DNA probe. In contrast, the C_2-ceramide complex was only marginally affected by the anti-p65 antiserum (*lane 2*). However, this complex was almost completely supershifted by anti-p50 anti-serum (*lane 3*), indicating that p50 was much more prevalent than p65/RelA in the C_2-ceramide-activated NFκB complex, whereas both were present in the TNF-activated complex.

C_2-ceramide Has a Marginal Effect on IκBα Degradation but Promotes p105 Processing in HL60 Cells—HL60 cells treated with C_2-ceramide or TNF at concentrations that resulted in NFκB activation were next examined for degradation of IκBα. A rapid and marked degradation was observed following stimulation with TNF (Fig. 4*A*) as determined by immunoblotting. The presence of a doublet (*lane 2*), prior to complete degradation, was most likely because of the phosphorylation of this protein. IκBα appeared to be completely degraded by 15 min (*lane 3*). Resynthesis restored IκBα levels within 60 min to the level of the unstimulated cells. In contrast, C_2-ceramide treatment resulted in a more marginal degradation of IκBα, complete degradation not being evident at any point (*lanes 6–8*).

We next determined the effect of TNF and C_2-ceramide on p105 and p50 levels in HL60. A time course revealed that treatment of HL60 for 60 min with 2 ng/ml TNF resulted in a modest increase in p50 levels with a concomitant decrease in p105 (Fig. 4*B*, compare *lanes 1* and *4*). A more marked effect was seen with 100 μM C_2-ceramide, where a pronounced degradation of p105 was observed (*lane 8*). This effect can be seen more clearly when the ratio of p105 to p50 is determined for each sample by analyzing the intensity of each band from the immunoblots by densitometry (Fig. 4*C*). This allows one to correct for loading variability between samples and reveals more accurately the precursor (p105)/product (p50) relationship for each sample. A clear increase in p50 with a concomitant decrease in p105 can be seen for both TNF and more particularly for C_2-ceramide (Fig. 4*C*). The effect of ceramide

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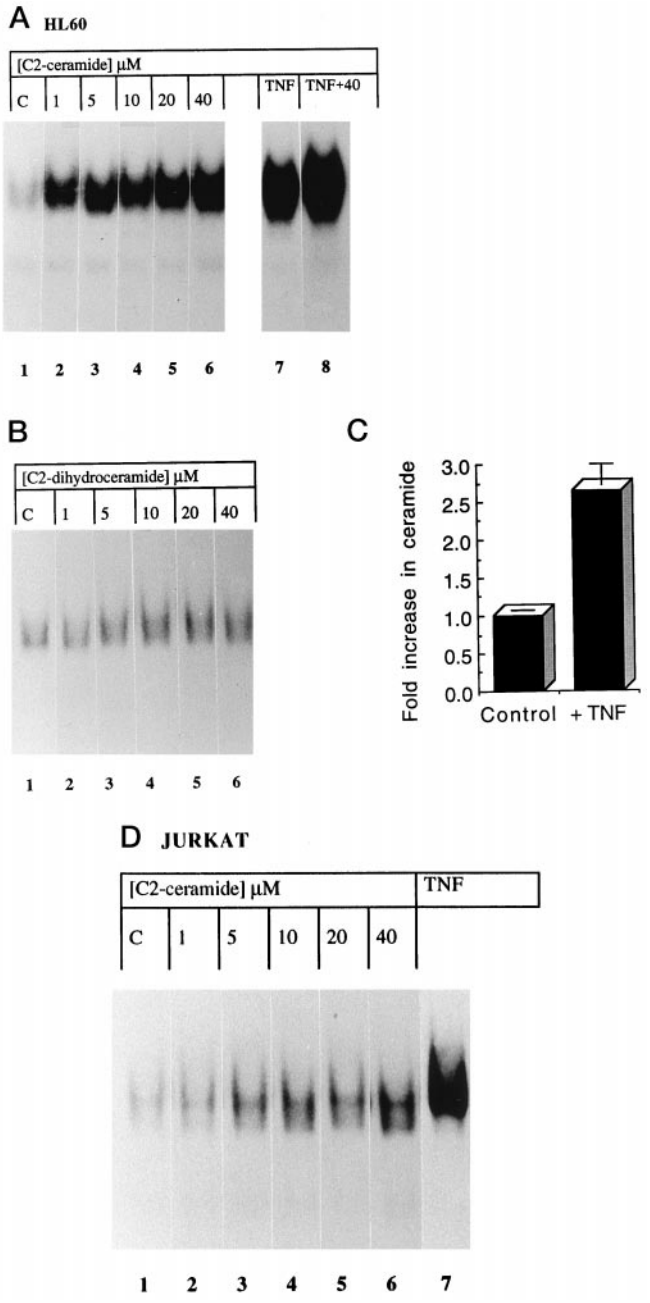

FIG. 2. **C_2-ceramide activates NFκB in HL60 promyelocytic leukaemia and Jurkat T lymphoma cells.** *A*, HL60 cells (5×10^6/ml) were treated with either C_2-ceramide (concentration indicated) (*lanes 2–6*) or an equivalent volume of vehicle control (ethanol) (*lane 1*) for 1 h. Alternatively, cells were treated with either vehicle control (*lane 7*) or C_2-ceramide (40 μM) (*lane 8*) for 30 min, prior to the addition of TNF (2 ng/ml for 1 h) *B*, HL60 cells (5×10^6/ml) were stimulated with dihydro C_2-ceramide (concentration indicated) (*lanes 2–6*) or an equivalent volume of vehicle control (ethanol) (*lane 1*) for 1 h. *C*, HL60 cells (1×10^6/ml) were stimulated with TNF (100 ng/ml) or vehicle control (medium) for 1 h. Extracts were prepared, and ceramide levels were measured by the diacylglycerol kinase procedure as described under "Experimental Procedures." Labeled ceramide was quantified by InstantImager™ (Packard Instrument Co., Meriden, CT) following TLC, where data are representative of two or three separate experiments (means ± S.E.). Base-line ceramide levels in nonstimulated cells were determined to be 89.9 ± 15.5 pmol/10^6 cells. *D*, Jurkat T cells (5×10^6/ml) were treated with either C_2-ceramide (concentration indicated) (*lanes 2–6*) or an equivalent volume of vehicle control (ethanol) (*lanes 1*) for 1 h. In the experiments shown as *A*, *B*, and *D*, nuclear extracts were prepared following stimulation and analyzed for NFκB binding activity as described under "Experimental Procedures." NFκB-DNA complexes are shown. Results are representative of at least three separate experiments.

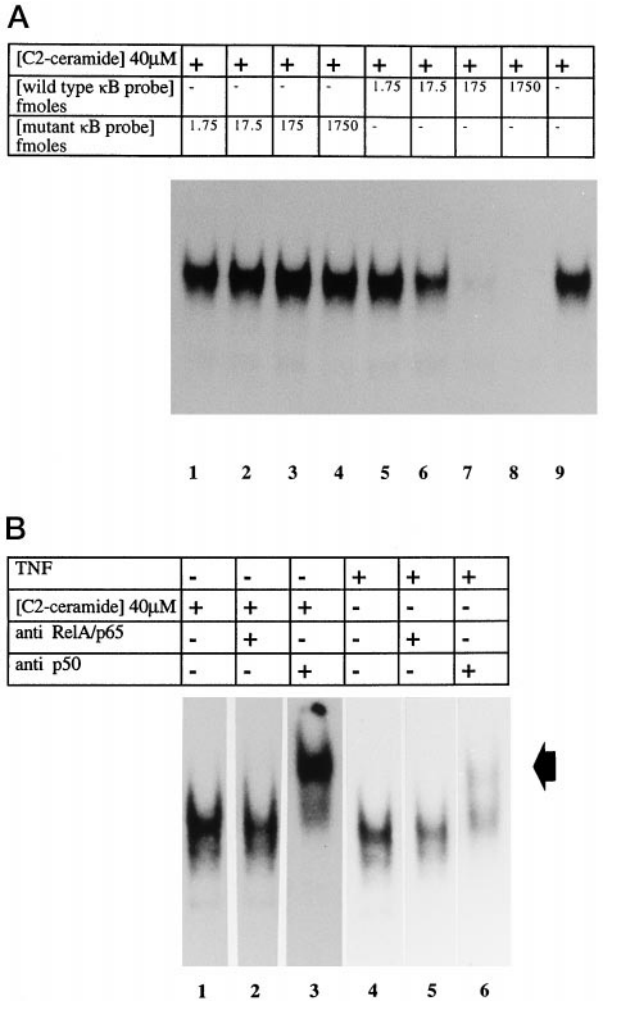

FIG. 3. **Activated NFκB complexes from C_2-ceramide-treated HL60 cells are specific for binding to the κB motif and predominantly contain the p50 subunit.** *A*, nuclear extracts from C_2-ceramide (40 μM)-treated HL60 cells were incubated with nonradioactive wild type (*lanes 5–8*) and mutant (*lanes 1–4*) probes for the κB consensus motif (concentrations indicated) at room temperature for 30 min prior to the addition of labeled probe. NFκB-DNA complexes are shown. *B*, nuclear extracts from C_2-ceramide (40 μM) and TNF-treated HL60 were left untreated (*lanes 1* and *4*) or incubated with antibodies to p65/RelA (*lanes 2* and *5*) or p50 (*lanes 3* and *6*) subunit components for 30 min on ice prior to the addition of labeled NFκB probe. Complexes were resolved as described under "Experimental Procedures." The positions of supershifted complexes are indicated. Results are representative of three experiments.

was also shown to be concentration-dependent, with both 40 and 100 μM showing an increase in p50 (Fig. 4*D*). The degradation of p105 was most evident at 100 μM C_2-ceramide.

Taken together, these data suggest that in HL60 cells, the lack of effect of C_2-ceramide on transactivation by NFκB, despite the presence of NFκB complexes, can be explained by p105 processing in excess of IκBα degradation. This would shift the balance of Rel-dependent transcription in the direction of nontransactivating (p50/p50) DNA binding forms of NFκB.

DISCUSSION

Ceramide has emerged as a second messenger implicated in multiple cellular responses, such as apoptosis, cell cycle arrest, and differentiation (reviewed in Ref. 30). Although its role is an issue of some debate, ceramide may function as a selective mediator of the cell killing effects of TNF, in addition to other cellular events in response to this cytokine. Because of the controversy that surrounds the role of this neutral lipid in

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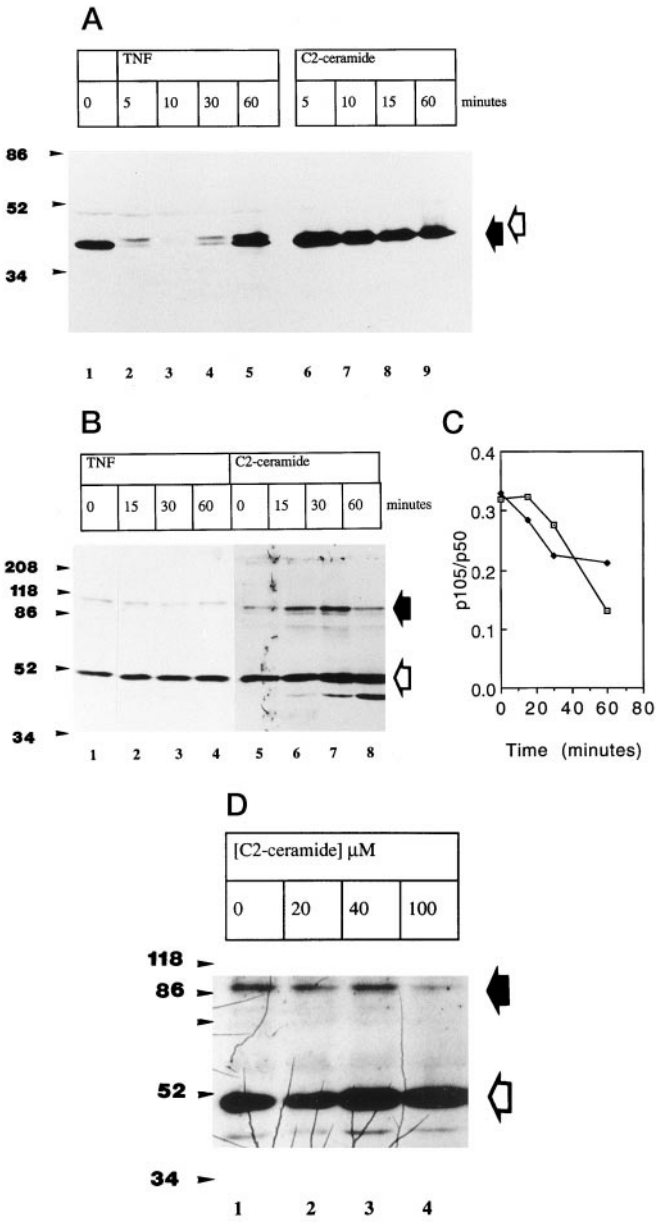

FIG. 4. **C_2-ceramide has a marginal effect on IκB degradation but promotes p105 processing in HL60 cells.** *A*, HL60 cells (2.5×10^6/ml) were treated with either C_2-ceramide (40 μM) (*lanes 6–9*) or an equivalent volume of vehicle control (ethanol) (*lane 1*) or TNF (2 ng/ml) (*lanes 2–5*) for various times indicated. Cell lysates were prepared following stimulation and analyzed for IκB degradation as described under "Experimental Procedures." *B*, HL60 cells (2.5×10^6/ml) were treated with either C_2-ceramide (40 μM) (*lanes 6–8*) or an equivalent volume of vehicle control (ethanol) (*lane 5*) or TNF (2 ng/ml) (*lanes 2* and *3*) for the various times indicated. Cell lysates were prepared following stimulation and analyzed for p105 processing to the p50 subunit as described under "Experimental Procedures." *Closed* and *open arrows* indicate the position of the IκB inhibitor protein and its phosphorylated form in *A* and the p105 protein and its p50 product in *B*, respectively. Molecular mass markers are shown in kilodaltons. Results are representative of three separate experiments. *C*, data from *B* were analyzed and quantitated by UVP transillumination densitometry scanning and Gelworks 1D Advanced™ software and is represented as the ratio of p105 precursor to p50 product, where (♦) and (□) correspond to data sets for TNF and C_2-ceramide-treated HL60 cells respectively. *D*, HL60 cells (2.5×10^6/ml) were treated with either C_2-ceramide (concentration indicated) (*lanes 2–4*) or an equivalent volume of vehicle control (ethanol) (*lane 1*) for 1 h. Cell lysates were prepared following stimulation and analyzed for p105 processing to the p50 subunit as described under "Experimental Procedures." *Closed* and *open arrows* indicate the positions of p105 and its p50 product, respectively. Molecular mass markers are in kilodaltons.

TNF-mediated signaling, we decided to compare the signaling events between TNF and ceramide that might culminate in the activation of the transcription factor, NFκB. In our study, we investigated the ability of C_2-ceramide to stimulate κB-linked gene expression. C_2-ceramide has previously been reported to potentiate and mimic TNF action in this response (13–15). We were unable to stimulate κB-linked CAT activity with this compound, however. This was in agreement with an earlier report where 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (a closely related analogue) failed to induce transactivation in a reporter gene assay in Jurkat T cells either alone or in combination with TNF (50). The absence of CAT stimulation can now be explained by our observation that selective activation of p50 occurs in response to ceramide. Specifically, C_2-ceramide and not its inactive congener caused a dose-responsive activation of NFκB in the hematopoetic cell line, HL60. On closer examination it was revealed that the pattern of Rel subunits in ceramide-activated complexes was distinct from that seen in TNF-activated complexes, with p50 predominating. The p50 antiserum employed was partially selective for p50 homodimeric forms of NFκB,[2] and the data obtained indicated that this subunit might be selectively activated in response to ceramide. We addressed p50 generation from the proteolytic processing of its precursor form, p105, as a possible target of ceramide, using an antibody that recognized both the parent protein and its cleaved product, the p50 subunit, and furthermore, compared our data with that obtained with TNF. We found a marked preference for p105 processing over IκBα degradation by ceramide, in contrast to TNF, which although capable of increasing p105 processing also caused a rapid degradation of IκBα (preceded by an apparent phosphorylation), in line with the classical pathway of NFκB activation. Our data therefore indicated that although ceramide activates NFκB, the subunit composition differs from that observed with TNF.

A number of other studies have examined whether ceramide can activate NFκB and probed the possible mechanism involved. Ceramide analogues have been found to activate NFκB (13–15), to potentiate the activation in response to TNF (47), or to have no effect (29, 47, 50, 51). The reasons for such marked discrepancies are unclear. Two studies that did not observe any effect were carried out in Jurkat (29, 50), which we found to be less responsive than HL60 cells. We also found that relatively high concentrations were needed to observe an effect and that treatment times of 1 h were required. These conditions may not be possible in some cell lines, where ceramide may induce apoptosis. Under the conditions used in our studies, no apoptosis was observed (not shown). Most recently, Gamard *et al.* (29) demonstrated that C_2-ceramide did not induce IκBα degradation in Jurkat T cells. This study also found no effect of this compound on NFκB activation, as assessed by gel shift analysis, although the cells were exposed for only 20 min at a C_2-ceramide concentration of 40 μM. We found that 40 μM C_2-ceramide activated NFκB in Jurkat T cells but that a 1-h contact time was required. A similar result was obtained in ML-1a leukemia cells (51), where a minimum contact time of 60 min was required to observe NFκB activation. The observation that a 1-h contact time was required may be significant, because TNF was found to increase ceramide levels in cells after a 1-h stimulation. As reported (29, 51), NFκB activation by TNF occurs much more rapidly than this, indicating that immediate activation of NFκB by TNF is unlikely to involve ceramide. It is possible that p105 processing by TNF, which we found to occur at a later time compared with IκBα degradation, may be mediated by ceramide. Different rates of IκBα and p105

[2] J. Imbert, personal communication.

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processing in response to TNF stimulation have also been observed by Melitts *et al.* (45). p105 processing as a mechanism of NFκB activation has received much less attention than the pathway involving IκBα degradation. Phosphorylation of p105 is required for its processing (42), similar to IκB-α. However, unlike IκBα, a novel E3 component of the proteosome is involved in p105 proteolysis (55). Phosphorylation occurs on multiple serines in the C-terminal PEST domain (42). The kinase or phosphatase (which would be inhibited) responsible for this modification is not known. Because ceramide has been shown to activate an as yet unidentified ceramide-activated protein kinase (16) as well as protein kinase C ζ (18), either may be involved. Our results would argue that the component of NFκB activation by TNF, which involves p105 processing rather than IκBα degradation, could be mediated by ceramide.

The enzyme(s) responsible for ceramide generation by TNF is still unresolved. Both acidic and neutral sphingomyelinase activities have been detected in extracts from TNF-treated cells (56). Evidence suggesting a role for acidic sphingomyelinase in NFκB activation has been presented (56). This has been disputed in two studies, however, demonstrating that TNF is able to activate NFκB in fibroblasts from Niemann Pick patients that lack acidic sphingomyelinase and in acid sphingomyelinase-deficient knockout mice (48, 52). In addition, another study has shown that an inhibitor of acidic sphingomyelinase, SR33557, did not inhibit NFκB activation by TNF (51). We found no effect of TNF on either neutral or acidic sphingomyelinase activities in HL60 cells (data not shown). An alternative mechanism must therefore be occurring that gives rise to the detected increase in ceramide, which could involve inhibition of ceramide metabolism or its increased biosynthesis (30).

The ability of ceramide to increase p50 homodimers in the relative absence of p50/p65 heterodimers may have consequences for gene expression. p65 is a potent transcription activation subunit, whereas p50 is not, because of the absence of a trans-activator domain (31, 32). In fact, evidence exists where p50 homodimers might function as negative regulators of κB-dependent transcription *in vivo* (44). Thus the ratio of transcriptionally active to inactive dimeric complexes is paramount in determining gene expression, in addition to the relative affinity of distinct NFκB binding sites for these transcription factors (57). Furthermore, p105 processing may also contribute indirectly to κB binding by generating dimers that associate with newly synthesized IκB, as has been previously suggested (42, 58). We found no inhibitory effect on the expression of the NFκB-linked reporter gene, indicating that the ability of TNF to rapidly increase p50/p65 heterodimers would overcome any inhibitory effect of p50 homodimers. Much higher levels of p50 homodimers may be required to see inhibition, as has been shown in studies on the interleukin-2 promoter in T cells (44).

The ability of C_2-ceramide to increase p50 homodimers may, however, account for some of the anti-proliferative properties reported for ceramide and analogues, such as down-regulation of c-*myc* gene transcription (27). In support of this proposal, a decrease in the rate of c-*myc* gene transcription has been directly related to a significant increase in the binding of p50 homodimers, which fail to transactivate the c-*myc* promoter (59). Down-regulation of c-*myc* expression has previously been shown to reflect perturbations in regulatory processes contributing to growth arrest and apoptosis (reviewed in Ref. 60), and ceramide has been shown to induce down-regulation of c-*myc* mRNA levels (by an unknown mechanism), acting possibly via ceramide-activated protein phosphatase (27). C_2-ceramide has also been shown to suppress the expression of the cytochrome P-450 2C11 (CYP2C11) gene (61). The protein product of this gene is a member of a family of enzymes whose content has been found to decrease in hepatic cells during infection and inflammation. Ceramide is thought to mediate this down-regulation, although a mechanism is not suggested. Because the gene for CYP2C11 is NFκB regulated, we would hypothesize that its down-regulation may involve increases in p50 homodimers.

The pro-apoptotic effects of ceramide could also involve the generation of p50 homodimers. NFκB activation has been shown to be anti-apoptotic (62), presumably through the induction of anti-apoptotic genes. An increase in p50 homodimers by ceramide could block the expression of such genes and thereby promote apoptosis.

In conclusion, our study indicates that the activation of NFκB by ceramide involves p105 processing in preference to IκBα degradation. This phenomenon may be involved in that aspect of NFκB activation by TNF that comprises the generation of p50 homodimers rather than IκBα degradation followed by release of the p50/p65 heterodimer.

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