Ceramide has been implicated as an intermediate in the signal transduction of several cytokines including tumor necrosis factor (TNF). Both ceramide and TNF activate a wide variety of cellular responses, including NF-κB, AP-1, JNK, and apoptosis. Whether ceramide transduces these signals through the same mechanism as TNF is not known. In the present study we investigated the role of the T cell-specific tyrosine kinase p56\textsuperscript{lck} in ceramide- and TNF-mediated cellular responses by comparing the responses of Jurkat T cells with JCaM1 cells, isogeneic Lck-deficient T cells. Treatment with ceramide activated NF-κB, degraded IκBα, and induced NF-κB-dependent reporter gene expression in a time-dependent manner in Jurkat cells but not in JCaM1 cells, suggesting the critical role of p56\textsuperscript{lck} kinase. These effects were specific to ceramide, as activation of NF-κB by phorbol 12-myristate 13-acetate, lipopolysaccharide, H\textsubscript{2}O\textsubscript{2}, and TNF was minimally affected. p56\textsuperscript{lck} was also found to be required for ceramide-induced but not TNF-induced AP-1 activation. Similarly, ceramide activated the protein kinases JNK and mitogen-activated protein kinase kinase in Jurkat cells but not in JCaM1 cells. Ceramide also induced cytotoxicity and activated caspases and reactive oxygen intermediates in Jurkat cells but not in JCaM1 cells. Ceramide activated p56\textsuperscript{lck} activity in Jurkat cells. Moreover, the reconstitution of JCaM1 cells with p56\textsuperscript{lck}-tyrosine kinase reversed the ceramide-induced NF-κB activation and cytotoxicity. Overall our results demonstrate that p56\textsuperscript{lck} plays a critical role in the activation of NF-κB, AP-1, JNK, and apoptosis by ceramide but has minimal or no role in activation of these responses by TNF.

Ceramide is synthesized as a signaling intermediate on activation of various enzymes including acidic, basic, and neutral sphingomyelinase and ceramide synthetase (1). These enzymes are activated on treatment of cells with a wide variety of stress stimuli, including tumor necrosis factor (TNF). Both TNF and ceramide activate similar cellular responses, including NF-κB, AP-1, JNK, and apoptosis (1). Although initially several groups reported that several TNF-mediated effects require generation of ceramide (2–11), the precise role of ceramide in TNF signaling has been highly controversial. Whereas several reports indicate that ceramide mediates TNF-induced cellular responses (2–11), others suggest that ceramide is not a critical intermediate in TNF signaling (12–23). That ceramide may be involved in TNF-induced apoptosis but not in NF-κB activation or other cellular responses has also been demonstrated (24–27). We have reported evidence that ceramide is needed but not sufficient for TNF-mediated apoptosis (25). The kinetics of activation of most of these cellular responses by ceramide is usually slower than that by TNF, thus providing one argument that ceramide is not needed for TNF signaling (1).

To explore this question, we used the JCaM1 cell line, a genetic variant of Jurkat deficient in p56\textsuperscript{lck} protein because of the deletion of exon 7 in p56\textsuperscript{lck} mRNA (28). p56\textsuperscript{lck} is a cytoplasmic tyrosine kinase, has a molecular size of 56 kDa, is a member of the Src family that is expressed highly in T cells, and binds to the cytoplasmic domain of the CD4 receptor (29), all of which make it a candidate for the ceramide and TNF pathways. It is required for T cell signaling in the human Jurkat T cell leukemia line (30). This protein-tyrosine kinase mediates NF-κB activation upon interaction of the human immunodeficiency virus type 1 envelope glycoprotein gp120 with the CD4 receptor (31). The precise role p56\textsuperscript{lck} plays in the ceramide or TNF-induced signaling is not known, however. We compared the cellular responses induced by ceramide with those induced by TNF in this cell line. We also used JCaM1 cells that had been reconstituted by transfection with the p56\textsuperscript{lck} gene (30). The studies indicated that ceramide can activate p56\textsuperscript{lck} and is required for ceramide-induced activation of NF-κB, AP-1, JNK, MAPK kinase, and apoptosis. Although several cellular responses of ceramide mimicked those of TNF, p56\textsuperscript{lck} was not found to be essential for TNF-induced cellular responses.

**EXPERIMENTAL PROCEDURES**

**Materials**—C<sub>2</sub> ceramide (N-acetyl-D-sphingosine), NaCl, and bovine serum albumin were obtained from Sigma. The phospho-specific anti-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibody was obtained from New England Biolabs, Inc. Anti-bodies-anti-mycotics (penicillin, streptomycin, and amphotericin B), RPMI 1640 medium, and fetal bovine serum were obtained from Life Technologies, Inc. Bacteria-derived recombinant human transcription factor κB; EMSA, electrophoretic mobility shift assay; JNK, c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; SEAP, secretory alkaline phosphatase; MTT, modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide; FMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; PV, pvernadate; MAPK, mitogen-activated protein kinase; MEF, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

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man TNF, purified to homogeneity with a specific activity of 5×10^6 units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Antibody against IκBα, p50, p65, JNK1, c-Jun, c-Fos, Cyclin D1, c-Raf, Lck, and double-stranded oligonucleotide having the AP-1 consensus sequence were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). IκBα, NF-κB, and AP-1 were previously reported (30). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1× antibiotics-antimycotics.

**NF-κB Activation Assay**—To assay NF-κB activation, we prepared nuclear extracts and performed electrophoretic mobility shift assays (EMSA) as described (32).

**AP-1 Activation Assay**—The activation of AP-1 was determined as described (32).

**Western Blot for IκBα**—To assay IκBα, postnuclear (cytoplasmic) extracts were prepared (32) from treated cells and resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose filter paper with rabbit polyclonal antibodies against either phospho-IκBα or IκBα, and detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

**c-Jun Kinase Assay**—The c-Jun kinase assay was performed by a modified method as described earlier (32). Briefly, after treatment of cells (3×10^6/ml) with TNF or ceramide for 15 min, cell extracts were prepared by lysing cells in buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, and 1 mM dithiothreitol. Cell extracts (150–250 μg/sample) were immunoprecipitated with 0.03 μg of anti-JNK antibody for 60 min at 4°C. Immune complexes were collected by incubation with protein A/G-Sepharose beads for 45 min at 4°C. The beads were washed with lysis buffer (4× 400 μl) and kinase buffer (2×400 μl), and then cell extracts were preincubated by incubating the cells for 30 min on ice in 0.05 ml of buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, and 1 mM dithiothreitol for 30 min. The lysate was centrifuged, and the supernatant was collected. Cell extract protein (50 μg) was resolved on 7.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with anti-PARP antibody, and then detected by chemiluminescence (ECL, Amersham Pharmacia Biotech). Apoptosis was represented by the cleavage of 116-kDa PARP into a 85-kDa peptide product.

**p56^lck Kinase Assay**—The p56^lck immunocomplex kinase assay was performed by a modified method (35). Briefly, after treatment of cells (5×10^6/ml) with either TNF or ceramide for 15 min, cell extracts were prepared by lysing cells in buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, and 1 mM dithiothreitol. Cell extracts (800 μg/sample) were immunoprecipitated with 0.5 μg of anti-p56^lck antibody for 12 h at 4°C. Immune complexes were collected by incubation with protein A/G-Sepharose beads for 1 h at 4°C. The beads were washed with lysis buffer (4× 400 μl) and kinase buffer (2× 400 μl), and then cell extracts were preincubated by incubating the cells for 30 min on ice in 0.05 ml of buffer containing 20 mM HEPES, pH 7.4, 1 μM dithiothreitol, 25 mM NaCl). Kinase assays were performed for 30 min at 37°C in 20 mM HEPES, pH 7.4, 10 mM MgCl_2, 1 μM dithiothreitol, and 10 μCi of [γ-^32P]ATP. Reactions were stopped with the addition of 15 μl of 2× SDS sample buffer, boiled for 5 min, and subjected to SDS-PAGE (9%). The p56^lck autophosphorylation band was analyzed by a PhosphorImager (Molecular Dynamics).

**MAPK Kinase Assay**—Cells were treated with different concentrations of TNF or ceramide for 30 min at 37°C. The cells were washed with phosphate-buffered saline and extracted with lysis buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, 1 mM dithiothreitol, and 1 mM sodium orthovanadate. A 50-μg aliquot of protein was resolved on each lane on 10% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and probed with the phosphospecific anti-p44/p42 MAPK (Thr202/Tyr204) antibody (New England Biolabs) raised in rabbits (1:3000 dilution). The membrane was then incubated with peroxidase-conjugated anti-rabbit IgG (1:3000 dilution), and bands were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

**NF-κB-dependent Reporter Gene Transcription**—Ceramide-induced, NF-κB-dependent reporter gene transcription was measured as described previously (33). Briefly, cells (0.1×10^6 cells/well) were plated in 6-well plates and then transfected with plasmid DNA (0.5 μg) for NF-κB promoter DNA that had been linked to heat-stable secretory alkaline phosphatase (SEAP) by the calcium phosphate method. After 10 h, cells were treated with different concentrations of TNF or ceramide. Twelve hours later, cell culture-conditioned medium was harvested, and 25 μl was analyzed for alkaline phosphatase activity essentially as described by the protocol of CLONTECH Inc. (Palo Alto, CA). The activity of SEAP was assayed on a 96-well fluorescent plate reader (Fluoroskan II, Lab Systems) with excitation set at 360 nm and emission at 460 nm. This reporter system was specific, because TNF-induced NF-κB-SEAP activity was inhibited by overexpression of IκBα mutants lacking either Ser^27 or Ser^32 (33).

**Cytotoxicity Assay**—The ceramide-induced cytotoxicity was measured by the modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (34). Briefly, cells (10,000 cells/well) were incubated in the presence or absence of the indicated test sample in a final volume of 0.1 ml for 72 h at 37°C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well. After a 2-h incubation at 37°C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethyl formamide) was added. After 15 min, cell extracts were measured using a 96-well multispecimen autoreader (Dynatech MR 5000), with the extraction buffer as a blank.

**Immunoblot Analysis of PARP Degradation**—Ceramide- and TNF-induced apoptosis was examined by proteolytic cleavage of PARP (32). Briefly, Jurkat and JCaM1 cells (2×10^5/ml) were activated with different concentrations of TNF and ceramide, for 15 min, cell extracts were prepared by incubating the cells for 30 min on ice in 0.05 ml of buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, and 1 mM dithiothreitol for 30 min. The lysate was centrifuged, and the supernatant was collected. Cell extract protein (50 μg) was resolved on 7.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with anti-PARP antibody, and then detected by chemiluminescence (ECL, Amersham Pharmacia Biotech). Apoptosis was represented by the cleavage of 116-kDa PARP into a 85-kDa peptide product.

**RESULTS**

To study the role of p56^lck in cellular signaling activated by ceramide and TNF, we used JCaM1 cells, which are known to be p56^lck-deficient. Jurkat cells were used as a p56^lck-positive control. Most ceramide-induced cellular responses are similar to those of TNF. To determine how ceramide signaling differs from that of TNF, the two agents were compared throughout this study.

**Ceramide Activates NF-κB in Jurkat Cells but Not in JCaM1 Cells**—Although both TNF and ceramide activate NF-κB, whether activation requires p56^lck is not known. Jurkat and JCaM1 cells were treated with various concentrations of either TNF or ceramide for 30 min, and nuclear extracts were prepared and examined for NF-κB activation by EMSA (Fig. 1A). NF-κB (upper panels) activated NF-κB in both Jurkat and JCaM1 cells in a dose-dependent manner, with optimum activation (5-fold) at around 100 pM. Ceramide (lower panels) activated NF-κB in a dose-dependent manner in Jurkat cells optimally at 5 μM (4-fold), but no activation was found in JCaM1 cells. These results suggest that p56^lck-dependent NF-κB activation is required for ceramide-induced but not for TNF-induced activation. To determine if this effect was time-dependent, Jurkat and JCaM1 cells were treated with either TNF (100 pM) or ceramide (10 μM) for different times and then examined for NF-κB activation (Fig. 1B). TNF activated NF-κB in both cell types with comparable kinetics (upper panels). Ceramide (lower panels) activated NF-κB in a time-dependent manner in Jurkat cells with optimum activation occurring at 15 min, but no significant activation of NF-κB was observed in p56^lck-deficient JCaM1 cells, even after 60 min.

**NF-κB Activation Induced by PMA, LPS, H_2O_2, Ceramide, and TNF**—NF-κB is activated by a wide variety of stimul
through a pathway involving overlapping and nonoverlapping steps (36). Thus we sought to examine if p56\textsuperscript{Lck} is required for NF-\textkappaB activation induced by PMA, LPS, and H\textsubscript{2}O\textsubscript{2}. Jurkat and JCaM1 cells were stimulated with PMA (25 ng/ml), serum activated LPS (1 \textmu g/ml), H\textsubscript{2}O\textsubscript{2} (250 \textmu M), TNF (0.1 nM), and ceramide (10 \textmu M) for 30 min at 37 °C. After these treatments, nuclear extracts were prepared and then assayed for NF-\textkappaB and then assayed for NF-\textkappaB by EMSA (Fig. 1C). PMA, LPS, TNF, and H\textsubscript{2}O\textsubscript{2} activated NF-\textkappaB

**Fig. 1. Effect of TNF and ceramide on NF-\textkappaB activation.**

A, Jurkat and JCaM1 cells (2 × 10\textsuperscript{6/ml}) were stimulated with different concentrations of TNF or ceramide for 30 min. After these treatments, nuclear extracts were prepared and then assayed for NF-\textkappaB as described under “Experimental Procedures.” B, cells were incubated at 37 °C with 0.1 nM TNF and 10 \textmu M ceramide for the indicated times. After these treatments nuclear extracts were prepared and then assayed for NF-\textkappaB. C, effect of PMA, LPS, H\textsubscript{2}O\textsubscript{2}, TNF, and ceramide on NF-\textkappaB activation. Jurkat and JCaM1 cells were stimulated with PMA (25 ng/ml), serum activated LPS (1 \textmu g/ml), H\textsubscript{2}O\textsubscript{2} (250 \textmu M), TNF (0.1 nM), and ceramide (10 \textmu M) for 30 min at 37 °C. After these treatments nuclear extracts were prepared and then assayed for NF-\textkappaB. D, supershift and specificity of NF-\textkappaB. Nuclear extracts were prepared from untreated or ceramide-treated (10 \textmu M) Jurkat cells (2 × 10\textsuperscript{6/ml}), incubated for 15 min with different antibodies and unlabeled wild type and mutant NF-\textkappaB, and then assayed for NF-\textkappaB as described under “Experimental Procedures.”
in both cell types, but again ceramide activated the transcription factor only in Jurkat cells. These results suggest that the mechanism of activation of NF-κB by ceramide differs from that of other inducers. Components of Ceramide-induced NF-κB and Its Specificity—Activated NF-κB typically consists of p50 and p65 homodimers or heterodimers (37). To determine the composition of the ceramide-induced NF-κB complex, we prepared nuclear extracts from untreated or ceramide-treated (10 μM) Jurkat cells (2 × 10⁶/ml), incubated then for 15 min with different antibodies and unlabeled NF-κB probe, and then assayed them for NF-κB by EMSA (Fig. 1D). Both anti-p50 and anti-p65 antibodies supershifted the NF-κB complex, whereas irrelevant anti-cyclin D1, anti-c-fos, or preimmune serum had no effect on the complex. The NF-κB band disappeared by competition with wild-type oligo but not with mutant oligo.

Ceramide Did Not Induce IκBα Degradation in JCaM1 Cells—NF-κB activation by most inducers requires IκBα degradation (37). Previously it has been shown that NF-κB activation induced by UV, pervanadate (PV), or reoxygenation does not coincide with IκBα degradation (38). Whether p56⁶⁶k is required for ceramide-induced IκBα degradation was also examined (Fig. 2A). As expected, TNF-induced IκBα degradation reached maximum at 15 min in both Jurkat and JCaM1 cells. The resynthesis of IκBα occurred at 30 min in Jurkat cells but at 60 min in JCaM1 cells, suggesting that Lck may decrease the rate of resynthesis of TNF-induced IκBα (Fig. 2A, upper panels). Like TNF, ceramide induced IκBα degradation, reaching the maximum at 15 min in Jurkat cells (Fig. 2A, lower left panel). In p56⁶⁶k-deficient JCaM1 cells, however, no ceramide-induced IκBα degradation was observed (Fig. 2A, lower right panel).

Ceramide Did Not Induce IκBα Phosphorylation in JCaM1 Cells—That PV activates NF-κB without IκBα phosphorylation in p56⁶⁶k-deficient JCaM1 cells has been reported (38). Here we examined what effect p56⁶⁶k had on ceramide-induced IκBα phosphorylation. To detect the phosphorylated form of IκBα on the Western blot, we used antibodies specific to the serine 32 phosphorylated form of IκBα. Cytoplasmic extracts treated with either 0.1 nM TNF or 10 nM and then assayed by Western blot for IκBα (A) and phosphorylated IκBα (B) in cytosolic fractions.

with ceramide or TNF for different times were probed with antibodies against the phosphorylated IκBα and examined by chemiluminescence. TNF induced IκBα phosphorylation as early as 5 min in both cell types (Fig. 2B, upper panels), whereas ceramide induced IκBα phosphorylation in Jurkat cells but not in JCaM1 cells (Fig. 2B, lower panels). Because p56⁶⁶k is a protein-tyrosine kinase and IκBα phosphorylation detected is on serine, p56⁶⁶k must regulate an IκBα kinase that phosphorylates IκBα directly.

NF-κB-dependent Reporter Gene Expression—NF-κB binding to the DNA and IκBα degradation is not sufficient to suggest that p56⁶⁶k is required for NF-κB-dependent reporter gene expression (39). Therefore, the effect of p56⁶⁶k on ceramide-induced reporter gene expression was examined. As shown in Fig. 3, TNF induced reporter gene expression in both Jurkat and JCaM1 cells in a dose-dependent manner. Ceramide induced expression in a dose-dependent manner in Jurkat cells but not in JCaM1 cells, suggesting that p56⁶⁶k was also required for ceramide-induced NF-κB-mediated reporter gene expression.

AP-1 Activation—Most agents that activate NF-κB also activate AP-1. Whether AP-1 activation by either TNF or ceramide requires p56⁶⁶k was investigated. To determine the role of p56⁶⁶k in AP-1 activation, Jurkat and JCaM1 cells were treated with various concentrations of either TNF or ceramide for 30 min, and the nuclear extracts were prepared and examined for AP-1 activation by EMSA (Fig. 4A). TNF activated AP-1 in both Jurkat and JCaM1 cells in a dose-dependent manner, with optimum activation at around 100 pM (upper panels). Ceramide activated AP-1 in a dose-dependent manner in Jurkat cells, but no activation was found in JCaM1 cells (lower panel). These results suggest that p56⁶⁶k kinase is also not required for TNF-induced AP-1 activation but is required for ceramide-induced
activation. To determine if this effect is time-dependent, we treated Jurkat and JCaM1 cells with either TNF (100 pM) or ceramide (10 μM) for different times and then examined the cells for AP-1 activation (Fig. 4B). TNF activated NF-κB in both cell types with similar kinetics. Ceramide activated NF-κB in Jurkat cells in a time-dependent manner, but again no significant activation was observed in p56lk-deficient JCaM1 cells.

Like NF-κB, AP-1 can be activated by a wide variety of stimuli (40). To examine if p56lk is required for AP-1 activation induced by PMA, LPS, and H2O2, Jurkat and JCaM1 cells were stimulated with PMA (25 ng/ml), serum activated LPS (1 μg/ml), H2O2 (250 μM), TNF (0.1 nM), and ceramide (10 μM) for 30 min at 37 °C. After these treatments, nuclear extracts were prepared and then assayed for AP-1 as described under “Experimental Procedures.”
LPS, TNF, and \( \text{H}_2\text{O}_2 \) activated AP-1 in both cell types, but ceramide activated it only in Jurkat cells. These results suggest that the mechanism of activation of AP-1 by ceramide differs from that of other inducers.

To determine the composition of ceramide-induced AP-1 complex, nuclear extracts were prepared from untreated or ceramide-treated (10 \( \mu \text{M} \)) Jurkat cells (2 \( \times 10^3/\text{ml} \), incubated for 15 min with different antibodies and unlabeled AP-1 oligo, and then assayed for AP-1 by EMSA (Fig. 4D). Both anti-c-Fos and anti-c-Jun antibodies supershifted the AP-1 complex, whereas irrelevant anti-cyclinD1, anti-p50, or preimmune serum had no effect on the complex. The AP-1 band disappeared by competition with wild-type oligo.

**JNK Activation**—The activation of AP-1 requires the activation of a stress-activated protein kinase, JNK (40). To determine the role of p56\(^{lck} \) in JNK activation, Jurkat and JCaM1 cells were treated with various concentrations of either TNF or ceramide for 15 min, and the cell extracts were prepared and examined for JNK activation by immune complex kinase assays (Fig. 5). TNF activated JNK in both Jurkat and JCaM1 cells in a dose-dependent manner, with optimum activation (6-fold) at around 1000 pM concentration (upper panels). Ceramide activated JNK in a dose-dependent manner in Jurkat cells, but no activation was found in JCaM1 cells (lower panel). These results suggest that p56\(^{lck} \) kinase plays no role in TNF-induced JNK activation but it does for ceramide-induced activation.

**MAPK Kinase Activation**—The activation of JNK and NF-\( \kappa \)B is regulated by an upstream kinase MAPK kinase or MEK (40, 41). To determine the role of p56\(^{lck} \) in MEK activation, we treated Jurkat and JCaM1 cells with various concentrations of either TNF or ceramide for 30 min, prepared the cell extracts, and examined them for MEK activation by Western blot using an antibody that detects the phosphorylated form of MAPK (Fig. 6). TNF activated MEK in both Jurkat and JCaM1 cells in a dose-dependent manner, with optimum activation at around 100 pM concentration (upper panels). Ceramide activated MEK in a dose-dependent manner in Jurkat cells, but no significant activation was found in JCaM1 cells (lower panel). These results suggest that p56\(^{lck} \) kinase plays no significant role in TNF-induced MEK activation, but it does play an important role in ceramide-induced activation.

**Apoptosis Induction**—Several reports indicate that ceramide can induce apoptosis in various cell types (see Ref. 1). Whether p56\(^{lck} \) is required for the cytotoxic effects of ceramide is not known. To determine the role of p56\(^{lck} \) in cytotoxicity, Jurkat and JCaM1 cells were treated with various concentrations of either TNF or ceramide for 72 h and then examined for cell viability by MTT dye uptake assay (Fig. 7A). TNF induced cytotoxicity in both Jurkat and JCaM1 cells in a dose-dependent manner, with optimum effect occurring around 1 nM concentration (upper panels). Ceramide induced cytotoxicity in a dose-dependent manner in Jurkat cells, but no significant cytotoxicity was found in JCaM1 cells (lower panel). These results suggest that p56\(^{lck} \) kinase plays no significant role in TNF-induced cytotoxicity, but it does play an important role for ceramide-induced cytotoxic effects.

The cytotoxic effects of TNF are mediated through the activation of cascade of caspases (see Ref. 42). Caspase-2, -3, -7, and -9 are known to cleave PARP protein substrate. Whether ceramide activates these caspases and whether the activation of these caspases requires p56\(^{lck} \) are not known. To determine
the role of p56\textsuperscript{lck} in caspase activation, we treated Jurkat and JCaM1 cells with various concentrations of either TNF or ceramide for 24 h and then prepared the cellular extracts and examined them for PARP cleavage by Western blot analysis. TNF induced PARP cleavage in both Jurkat and JCaM1 cells in a dose-dependent manner, with optimum effect at around 1 nM concentration (upper panels). TNF-induced PARP cleavage was somewhat enhanced in p56\textsuperscript{lck}-deficient JCaM1 cells. Ceramide induced PARP cleavage in a dose-dependent manner in Jurkat cells, but no significant caspase activation was found in JCaM1 cells (lower panel). These results suggest that p56\textsuperscript{lck} kinase plays an important role in ceramide-induced activation of apoptosis but perhaps plays little role in TNF-induced apoptosis.

Ceramide-induced p56\textsuperscript{lck} Activation—From the studies indicated above it is clear that p56\textsuperscript{lck}-deficient cells are unable to activate NF-\kappaB, AP-1, JNK, MEK, and apoptosis induced by ceramide. This implies that ceramide must mediate its effects through activation of p56\textsuperscript{lck} kinase. We have previously shown that TNF can activate p56\textsuperscript{lck} kinase (35). Whether ceramide can activate p56\textsuperscript{lck} is, however, not known. To determine the activation of p56\textsuperscript{lck}, we first assayed p56\textsuperscript{lck} protein in Jurkat...
cells and its absence in JCaM1 cells by Western blot analysis using p56\(^{lck}\) antibodies. As shown in Fig. 8A, p56\(^{lck}\) protein was present in Jurkat cells but not in JCaM1 cells. Then activation of p56\(^{lck}\) was examined by treating cells with different concentrations of either TNF or ceramide for 15 min and then testing them for autophosphorylation of p56\(^{lck}\) (upper panels). Ceramide also activated p56\(^{lck}\) in Jurkat cells but not in JCaM1 cells (lower panels). These results suggest that p56\(^{lck}\) is activated by both TNF and ceramide but is required only for ceramide-mediated cellular responses.

Ceramide-induced Cellular Responses Can Be Reversed by Transfection of p56\(^{lck}\) Gene in JCaM1 Cells—To further confirm the role of p56\(^{lck}\) in ceramide signaling, we used JCaM1 cells that had been reconstituted by transfection of the p56\(^{lck}\) gene (30). The reconstituted cells expressed p56\(^{lck}\) protein (Fig. 8A) and this protein could be activated by TNF and ceramide in a dose-dependent manner (Fig. 8A). We further examined these cells for ceramide-induced NF-\(\kappa B\) activation (Fig. 9B), JNK activation (Fig. 9C), and cytotoxicity (Fig. 9D). The presence of p56\(^{lck}\) reversed the ceramide-induced activation of NF-\(\kappa B\), JNK, and cytotoxicity in a dose-dependent manner, and it had no significant effect on TNF-induced activation.

**DISCUSSION**

Although ceramide activates several cellular responses similar to those activated by TNF, whether they are mediated through the same mechanism is not understood. By using p56\(^{lck}\)-deficient cells, in this report we demonstrate that Lck is required for ceramide-induced activation of NF-\(\kappa B\), AP-1, JNK, MEK, and apoptosis, but not for that induced by TNF. We found that in control Jurkat cells both TNF and ceramide induced p56\(^{lck}\) activity, but it was needed only for ceramide-induced cellular responses. The transfection of p56\(^{lck}\)-deficient cells with p56\(^{lck}\) gene reconstituted the ceramide-induced cellular responses.

To our knowledge this is the first report to indicate that p56\(^{lck}\) is required for ceramide-mediated activation of NF-\(\kappa B\), AP-1, JNK, MEK, and apoptosis. Three pieces of independent evidence in our studies suggest the role of p56\(^{lck}\) in ceramide signaling. First, ceramide signaling is interrupted in p56\(^{lck}\)-deficient cells. Second, ceramide can activate p56\(^{lck}\) kinase activity in cells where signaling is intact. Third, reconstitution of p56\(^{lck}\)-deficient cells with p56\(^{lck}\) gene reverses ceramide signaling. The activation of NF-\(\kappa B\) by human immunodeficiency virus type 1-derived envelope glycoprotein gp-120 has been shown to require p56\(^{lck}\) (31). The p56\(^{lck}\) has also been shown to be required for NF-\(\kappa B\) activation induced by reoxygenation and by PV (38). Based on p56\(^{lck}\)-deficient Jurkat variants, the role of p56\(^{lck}\) in PV-induced tyrosine phosphorylation of I\(\kappa B\)α and NF-\(\kappa B\) activation was suggested (38). Our laboratory and Imbert et al. (38) showed that PV induced phosphorylation of I\(\kappa B\)α at position 42 (43). How tyrosine phosphorylation activates NF-\(\kappa B\) is not understood. In the present studies we found that ceramide induces serine phosphorylation of I\(\kappa B\)α in Jurkat cells but not in p56\(^{lck}\)-deficient cells, thus suggesting that p56\(^{lck}\) indirectly affects serine phosphorylation of I\(\kappa B\)α. Thus p56\(^{lck}\) must modulate the function of I\(\kappa B\)α kinase, which phosphorylates I\(\kappa B\)α (37).

We showed that p56\(^{lck}\) is required for ceramide-induced activation of JNK and MEK. TNF-induced activation of JNK and MEK was found to be p56\(^{lck}\)-independent. Our results are consistent with a previous report that T-cell antigen receptor-mediated activation of the MAPK pathway requires p56\(^{lck}\) kinase (44). Like us, Denny et al. (44) employed p56\(^{lck}\)-deficient JCaM1 cells to demonstrate the requirement for p56\(^{lck}\). The activation of JNK by 1-selectin was also found to be mediated through p56\(^{lck}\) kinase (45).

How p56\(^{lck}\) mediates the activation of NF-\(\kappa B\), AP-1, JNK, MEK, and apoptosis by ceramide is not clear. The activation of these cellular responses requires the generation of reactive oxygen intermediates (37, 40, 46, 47). For instance, overexpression of the antioxidant enzymes superoxide dismutase and \(\gamma\)-glutamylcysteine synthetase has been shown to suppress the activation of NF-\(\kappa B\) induced by ceramide (32, 48). Thus it is possible that p56\(^{lck}\)-induced reactive oxygen intermediate generation mediates the activation of NF-\(\kappa B\) induced by ceramide. H\(_2\)O\(_2\) has been shown to activate p56\(^{lck}\) enzyme (49) but whether p56\(^{lck}\) can mediate reactive oxygen intermediate generation has not been reported. These observations also suggest that p56\(^{lck}\) is located upstream in the ceramide signaling pathway. Although we have previously reported that TNF activated p56\(^{lck}\) (35), this is the first report to indicate that ceramide can also activate this kinase. Recently, it was shown by Hanna et
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In our studies we found that although Lck deficiency interrupted the ceramide signaling, TNF-mediated cellular signaling was unaffected, thus suggesting that TNF does not mediate its effects through ceramide. This conclusion is in agreement with reports that show that TNF-induced NF-κB activation and apoptosis through activation of a tyrosine kinase: another group, however, showed that in nontransformed T lymphocytes the p56<sup>lck</sup> deficiency induces cell cycle arrest and hypersusceptibility to apoptosis. Overall our results demonstrate for the first time that ceramide can activates p56<sup>lck</sup> and the latter plays a major role in ceramide signaling but not in TNF signaling.

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Protein Tyrosine Kinase p56\textsuperscript{\textit{lck}} is Required for Ceramide-induced but Not Tumor Necrosis Factor-induced Activation of NF-\textit{kB}, AP-1, JNK, and Apoptosis

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